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Modulation of signal transduction pathways relevant to atherosclerosis by dietary chemopreventive agents

Abstract

Atherosclerosis, an inflammatory, proliferative disorder, is one of the leading causes of death and morbidity in the Western world. Epidemiological and experimental evidence show that consumption of certain dietary constituents lead to a decrease in the incidence of the disease.

The dietary constituents curcumin, resveratrol, epigallocatechin-3-gallate (EGCG), but not indole-3-carbinol (I3C) inhibited the activation of NF-κB in B lymphoblasts and HUVEC. EGCG inhibited NF-κB activation in human umbilical vein endothelial cells (HUVECs) by blocking the activity of IKKα, thus preventing subsequent phosphorylation and degradation of IκBα. In B lymphoblasts inhibition of NF-κB activation by EGCG, appeared to be independent of an effect on IκBα. EGCG also prevented the formation of inducible extracellular and intracellular ROS in B lymphoblasts.

EGCG inhibited the growth of B lymphoblasts and HUVEC, which was mediated through a cell cycle arrest and induction of apoptosis. Further analysis of this growth inhibitory effect, identified a number of important cell growth regulators that were a target of EGCG. In HUVEC, EGCG growth inhibition appeared to involve p53, CDK1, cyclin D1, and HMOX-1, but was independent of Pin1 and XIAP. In B lymphoblasts, EGCG growth inhibition appeared to be independent of any significant affect on p53, CDK1, cyclin D1, Pin1 and XIAP, but may involve the loss of NF-κB activity or increases in HMOX-1 protein.

Treatment with EGCG produced changes in the expression of a number of disease relevant genes including *HMOX-1*, as detected by microarray analysis. This increase in *HMOX-1* mRNA corresponded to an increase in HMOX-1 protein. In B lymphoblasts, further analysis revealed that the increase in HMOX-1 was dependent on the PI3K and p38 pathways.

The data obtained characterise some of the chemopreventive properties of curcumin, resveratrol, I3C and particularly EGCG, facilitating the design of preventive dietary intervention studies in healthy volunteers or groups of at risk patients.

Dedicated to My Family

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List of Abbreviations

AA	Arachidonic acid
AAPH	2, 2'-azobis.[2-amidinopropane] dihydrochloride
ACAT	Acyl-CoA:cholesterol acyltransferase
AGE	Advanced glycated end-product protein
AIBN	Azobisisobutyronitrile
AIF	Apoptotic inducing factor
ANOVA	Analysis of variance
APAF-1	Apoptotic protease activating factor-1
AP-1	Activating protein-1
ApoB	Apolipoprotein B-100
APS	Ammonium persulphate
ARE	Anti-oxidant response element
ATP	Adenosine 5' triphosphate
BIR	Baculovirus IAP repeat
BSS	Balanced salt solution
bZIP	Basic leucine zipper
CCL_4	Carbon tetrachloride
CDK	Cyclin dependent kinase
CDKI	Cyclin dependent kinase inhibitor
cGMP	Cyclic guanosine monophosphate
CHK	Checkpoint kinase
CHOAS	Cambridge heart antioxidant study
COX2	Cyclo-oxygenase 2
CTD	COOH terminal domain
CVD	Cardiovascular disease
DASH	Dietary approaches to stop hypertension
DD	Death domain
DISCs	Death-inducing signalling complexes
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide 5'triphosphate

DTT	Dithiothreitol
EBV	Epstein Barr virus
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EC	Epicatechin
ECg	Epicatechin gallate
EDTA	Ethylene diaminetetraacetic acid
EGC	Epigallocatechin
EGCG	Epigallocatechin-3-gallate
EGM	Endothelial growth medium
EGTA	ethyleneglycol-bis(b-aminoethyl)-N,N,N',N'-tetraacetic acid
ELAM-1	Endothelial leukocyte adhesion molecule-1
ERK	Extracellular signal regulated kinase
EST	Expressed sequence tags
FADD	Fas-associated death domain
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FOX	Ferrous oxidation in xylenol orange
GADD45	DNA-damage protein 45
GCK	Germinal centre kinase
GGT	Gamma-glutamyl transpeptidase
GOT	Glutamate-oxalacetate-transaminase
GPT	Glutamate-pyruvate-transaminase
GPx	Glutathione peroxidase
GSNO	s-nitrosoglutathione
HDL	High density lipoprotein
HEPES	N-[2-hydroxyethyl]piperazine-N'-[ethanesulfonic acid]
HHS	Helsinki heart study
HMOX-1	Hemeoxygenase-1
HPK1	Hematopoietic progenitor kinase 1
HRP	Horseradish peroxidase
HUVEC	Human umbilical vein endothelial cell
IAP	Inhibitor of apoptosis
ICAM-1	Intracellular cell adhesion molecule-1

IFN-γ	Interferon gamma
IκB	Inhibitor of κB
IKK	IκB kinase
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IRAK	Interleukin-1R-activated kinase
I3C	Indole-3-carbinol
JNK	c-Jun N terminal kinase
KAB	Kinase assay buffer
LDL	Low density lipoprotein
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
MCP-1	Monocytic chemotactic protein-1
MEKK	MAPK kinase kinase
MEK	MAPK kinase
MMP	Matrix metallo proteinase
mRNA	Messenger ribonucleic acid
NAK	NF-κB activating kinase
NCI	National Cancer Institute
NF-κB	Nuclear factor kappa B
NIK	NF-κB inducing kinase
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NTD	NH_2 terminal domain
Ox-LDL	Oxidised low-density lipoprotein
PA	Plasminogen activator
PAGE	Polyacrylamide gel electrophoresis
PAI-1	Plasminogen activator inhibitor-1
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
PIG	Proline oxidase
pRb	Phosphorylated retinoblastoma protein
RAGE	Receptor for advanced glycated end-product
RH	Rel homology

RIPK	Receptor interacting kinase
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
Poly DIDC	Poly deoxyinosinic-deoxycytidylic acid
RB	Retinoblastoma protein
RNA	Ribonucleic acid
RNase	Ribonuclease
ROS	Reactive oxygen species
RT	Room temperature
RT-PCR	Reverse transcriptase polymerase chain reaction
SAPK	Stress activated protein kinase
SDS	Sodium dodecyl sulphate
SMC	Smooth muscle cell
SNP	Sodium nitroprusside
TAK1	TGFβ-activated kinase
tBHQ	Tert-butylhydroquinone
TBS	Tris buffered saline
TBS-T	Tris buffered saline tween
TE	Tris EDTA buffer
TEMED	N, N, N', N'-tetramethylethylenediamine
TF	Transcription factor
TGFβ	Transforming growth factor β
TNFα	Tumour necrosis factor alpha
TRADD	TNF receptor-associated death domain protein
TRAF2	TNF receptor-associated factor 2
VCAM-1	Vascular cell adhesion molecule-1
VSMC	Vascular smooth muscle cell
VLDL	Very low density lipoprotein

Chapter One

Introduction

1.1 Synopsis

Cardiovascular disease remains one of the major killers in modern society, with up to 12 million deaths annually. In order to develop a viable and effective treatment strategy, the complex pathogenesis of the disease must first be understood. This thesis will describe one of the major types of cardiovascular disease termed atherosclerosis, and what is currently known about the disease process. Furthermore it will highlight a potential method of combating this disease through dietary intervention/prevention.

In this thesis introduction, a prelude to the pathogenesis behind the disease will be given, as well as introducing the concept of chemoprevention, the agents under investigation, and finally ascribing the relevance of studying the NF-κB signalling pathway and downstream consequences to atherosclerosis.

There were a number of aims initially defined at the inception of this investigation, which are resolved in ensuing chapters. One intention was to examine whether there were differences in pathways relevant to atherosclerosis, such as NF-κB, between two types of lymphoblastoid cells, one consisting of cells derived from subjects with hypertension (a predisposing condition to developing atherosclerosis) and one consisting of cells from normal or normotensive subjects. A further objective was to appraise whether a panel of these lymphoblastoid cell lines, would reflect any changes in the endothelial cell model HUVEC (human umbilical vein endothelial cell), thus making them suitable surrogate cells for the less readily available endothelial cells that line the arterial lumen.

1.2 Cardiovascular disease

Cardiovascular disease (CVD) is the main cause of death and morbidity in the developed world, with 1.5 million deaths per annum in the European Union alone. To emphasise the scale of the problem, the number of people who die of CVD is almost as great as the combined number from the next seven leading causes of death. One in eight male deaths before the age of 65 is the result of CVD, such as heart failure or stroke. The social and economic problems brought about by CVD are immense. The greatest single cause of adult disability and economic burden to the UK alone, consuming over 10 billion pounds sterling per annum, is the cardiovascular complication leading to strokes (British Heart Foundation, UK).

It was once believed that CVD and its underlying cause, atherosclerosis, were the inevitable consequences of aging alone. More recently the disease has been recognised as a problem in affluent societies. There is now an emerging global problem of obesity and type II diabetes, risk factors, which will culminate inevitably in an increased global burden of heart disease. Therefore, CVD such as atherosclerosis is now deemed to be a preventable disease.

1.2.1 Atherosclerosis

Atherosclerosis is a progressive multifactorial disease with numerous predisposing conditions, including smoking, diabetes, hyperlipidemia, hypertension, mechanical stress and inflammation. Typically the disease affects the large and medium sized muscular arteries, such as the coronary, carotid, and arteries of the lower extremities, as well as the elastic arteries such as the aorta and iliac vessels (As reviewed in Collins 1993). Current understanding of the mechanisms behind atherosclerosis suggest a complex interplay of inflammatory/immune and fibroproliferative mediators that act on a number of different cell types.

Several theories have been proposed which attempt to define the mechanisms behind atherogenesis, the processes which lead to atherosclerosis. The lipid hypothesis suggests that changes induced during the disease process are reactive responses to lipid infiltration. The influence of lipids on cardiovascular disease is clearly evidenced in patients with genetic hyperlipidemias, in which homozygous individuals rarely live beyond 26 years. The administration of anti-lipid therapies such as statins is one of the few strategies that have induced regression of atherosclerosis in randomised clinical trials (Brown *et al.* 1990). However nearly 20 % of those adequately treated patients had adverse cardiovascular events, emphasising the importance of nonlipid risk factors (Randomised trial of cholesterol lowering in 4444 patients with coronary vascular disease, The Scandinavian simvastatin survival study (4s) Pedersen, 1994). Other theories of atherogenesis include the thrombogenic hypothesis (abnormal blood elements acting as a nidus for fibrin deposition), the mesenchymal hypothesis (proliferation of smooth muscle cells and collagen deposition), and the monoclonal hypothesis (lesion development from a few mutated smooth muscle cells) (Smith *et al.* 1996; Kerenyi *et al.* 1985; Murry *et al.* 1997). It is likely that atherogenesis is a combination of a number of these propositions. In fact, Ross (1993; 1995) proposed a unifying hypothesis suggesting atherogenesis was

dependent on cellular interactions in the arterial vessel wall following endothelial injury (figure 1.1). This injury, which is one of the earliest events to occur during plaque formation, can be caused by direct physical trauma, oxidised lipoproteins, bacteria, viruses, mechanical stress, or oxidative stress induced by reactive oxygen intermediates. Endothelial dysfunction results in anomalous signalling mechanisms culminating in the release of various chemotactic factors (such as monocytic chemotactic protein 1, MCP-1) and the up-regulation of adhesion molecules (for example vascular cell adhesion molecule-1, VCAM-1 and endothelial leucocyte adhesion molecule-1, ELAM-1). These chemotactic factors, as well as oxidised lipoproteins, act on circulating monocytes, platelets, and T lymphocytes, which bind to the newly expressed adhesion molecules present on the surface of the endothelial cells lining the artery. The cells transmigrate through into the subendothelial space, where in the presence of oxidised lipoproteins and various chemokines, they release potent growth-regulatory molecules that can act in an autocrine and paracrine manner. The monocytes proliferate and differentiate into macrophages. These macrophages take up oxidised lipoproteins to form foam cells. In humans, such fatty streak lesions can usually be found in the aorta in the first decade of life, the coronary arteries in the second decade, and the cerebral arteries in the third and fourth decades. As well as monocytic proliferation vascular smooth muscle cells (VSMCs) begin to proliferate and migrate across the internal elastic lamina into the intimal layer. Further chemotactic factors are released by the numerous cells types, which act to enhance cellular infiltration and further proliferation of endothelial, smooth muscle and macrophage cells. Stimulated VSMCs allow for the deposition of extracellular matrix, thus converting the initial lesion to a fibrous plaque. As the disease progresses foam cells undergo apoptosis thus contributing to the lipid filled core of the plaque. The plaque continues to develop into the arterial lumen until sufficiently large enough to block blood flow. However, the most important clinical complication is an acute occlusion due to the formation of a thrombus or blood clot, resulting in myocardial infarction or stroke. Usually the thrombosis is associated with rupture or erosion of the lesion.

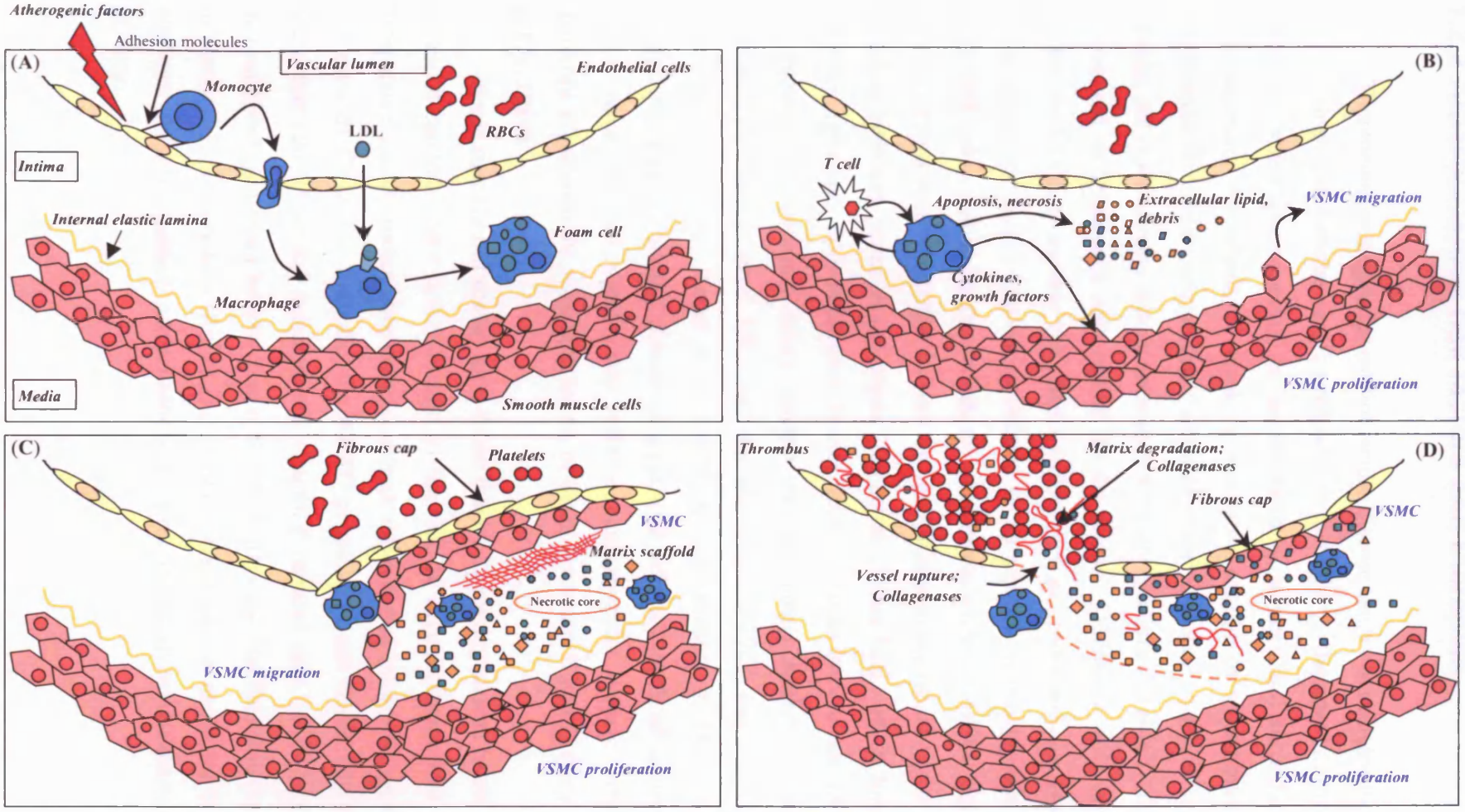

Figure 1.1 - The mechanisms behind atherogenesis. *Cardiovascular risk factors alter the vascular endothelium, which triggers a cascade of events including the recruitment and infiltration of leukocytes (A). Cytokines and growth factors are released by inflammatory cells and vascular cells, causing VSMCs to migrate and proliferate (B). Vascular cells continue to proliferate and synthesise extracellular matrix components on the luminal side of the vessel wall forming the fibrous cap of the atherosclerotic lesion (C).Inflammatory mediators lead to the expression of proteases, rendering the plaque weak and susceptible to rupture and thrombus formation (D).*

1.2.2 Atherosclerotic risk factors and therapies

Therapeutic strategies for cardiovascular disease can try to mitigate the influence of injury or intervene with the vessel's response to that injury. Attempts to limit or eliminate injury will affect atherogenesis, as demonstrated by primary and secondary prevention trials regarding the effects of smoking cessation, control of hypertension, and lipid modification on cardiovascular death rates. In order to reduce injury, it is important to consider the types of risks factors associated with atherosclerosis. Table 1.1 lists the major genetic and environmental risk factors for the disease. Obviously little can be done to combat the effects of some of the genetic factors such as gender and age. However, many of the other risk factors can be considered in an attempt to reduce atherogenesis. As mentioned before damage to the endothelium can be caused by oxidative stress induced by reactive oxygen species (ROS). As well as contributing to the initial site of injury, ROS including hydrogen peroxide and superoxide act to oxidise LDL, which further enhances disease progression. Epidemiological studies have shown an inverse relationship between the frequency of coronary artery disease and the dietary intake of fresh fruits and vegetables (Verlangieri *et al.* 1985; Acheson and Williams 1983) and antioxidant vitamins (Stampfer *et al.* 1993; Rimm *et al.* 1993). A large number of cross-cultural studies (comparing rates of coronary disease in areas with different levels of antioxidant vitamin intake) have reported significantly reduced cardiovascular disease incidence and/or mortality in populations with high levels of plasma vitamin E and C (Gey *et al.* 1987a, 1987b, 1989).

Some case control studies have found that by increasing plasma levels of vitamin E, C, and β-carotene there is a reduction in the risk of angina (Riemersma *et al.* 1991). Prospective cohort studies have correlated high self-reported intakes of antioxidants with low rates of CVD. For example, the 40,000 population male health professional study found that 100 IU per day supplemental vitamin E reduced rates of CVD, that 60 IU per day conferred significant benefit relative to 7.5 IU day, and that β-carotene was protective in current and past smokers (Rimm *et al.* 1993). Conversely, in one study involving 9000 high-risk patients vitamin E had no beneficial effect on cardiovascular outcomes (Yusuf *et al.* 2000).

Factors with a strong genetic component	
Increasing age	Association made between development of CVD and increasing age
Male gender	Men develop CVD at more than twice the rate of women
Heredity (family history of the disease)	Taking into account other known factors, family history remains a very significant risk factor
Systemic inflammation	Elevated levels of inflammatory molecules such as C-reactive protein are associated with CVD, as are inflammatory diseases such as rheumatoid arthritis
Hypertension (high blood pressure)	Associations observed with epidemiological studies.
High blood levels of cholesterol, especially LDL cholesterol	Associations demonstrated in epidemiological studies and supported by studies of genetic disorders and animal models
Other undesirable blood lipid levels, including low levels of HDL cholesterol and high levels of triglycerides	Associations demonstrated in epidemiological studies and supported by studies of genetic disorders and animal models
Diabetes and obesity	Associations observed in epidemiological studies and in animal models
Depression and behavioural traits	Associations observed in several population studies
Environmental factors	
Physical inactivity	Significant independent associations with CVD
Cigarette smoking	Strong associations observed in epidemiological studies
High fat diet	Population migration and epidemiological studies indicate strong association with lifestyle especially diet
Infectious agents	Epidemiological studies provide suggestive evidence for an association with various infectious agents, such as *Chlamydia pneumoniae*

Table 1.1 – Major genetic and environmental risk factors for atherosclerosis

It is likely that these conflicting findings are due to the fact that different doses of antioxidants were used. Also the treated patients suffered from advanced levels of

atheroslerosis and hence the therapy may not have been effectual, as the oxidative reactions tend to occur early on in atherosclerosis.

A number of randomised intervention studies have been performed on subjects having lesions or a past history of disease (secondary prevention trial). Williams *et al.* (1971), randomly assigned 33 patients with severe claudication due to atherosclerosis in the peripheral vessels to 9 months of vitamin E therapy or placebo. The improvement in symptom-free walking distance in vitamin E group was twice that of the placebo group, at the end of the treatment. This compares with the more recently reported Cambridge Heart Antioxidant Study (Stephens *et al.* (CHOAS), 1996). In this study a controlled trial in which 2002 heart disease patients with angiographically proven coronarography atherosclerosis, were given vitamin E or an inactive placebo. Among the subjects who received vitamin E, approximately half received a dose of 800 IU/day, whereas those who enrolled at a later date received 400 IU/day. Vitamin E treatment significantly reduced the combined risk of cardiovascular death and nonfatal myocardial infarction.

Another factor that can be considered when attempting to reduce 'injury' is the plasma level of lipids such as LDL. LDL cholesterol plays a particularly fundamental role because it is directly involved in the early damaging stages of atherosclerosis. In the Helsinki heart study (HHS), treatment with the fibrate gemfibrozil produced a 34 % reduction in incident coronary events with a 35 % reduction in plasma triglyceride levels, a 9 % increase in HDL cholesterol levels and an 11 % reduction in plasma LDL cholesterol concentration (Frick *et al.* 1987). In addition to using a fibrate, is a study using a statin known as pravastatin (Sacks *et al.* 1996). Results from the CARE study provided evidence of a pronounced benefit of treatment with pravastatin. This statin brought about an 18 % reduction in plasma total cholesterol, which was associated with a 24 % relative risk reduction in cardiovascular events.

Dietary modifications can have profound effects on total serum cholesterol levels without the requirement of cholesterol lowering drugs. One such example is the Oslo trial (Hjermann *et al.* 1981), where the effect of dietary modification and also the risk factor of smoking were examined in 1232 healthy men aged 40-49 years. These men had high serum cholesterol levels (7.5 to 9.8 mmol/L), were mostly smokers (80 %), had systolic blood pressures above 150 mm/Hg and were at very high risk for cardiovascular disease. They were randomised into 2 groups, where one intervention group received dietary and antismoking advice, and the control group did not receive any advice. The advised diet was low in saturated fat and high in fibre (fresh fruits and vegetables). Saturated fat was

decreased from 18 % to 8 % of total energy intake, and saturated fat was partly replaced by n-6 polyunsaturated fatty acids. This resulted in a 13 % difference in serum cholesterol between experimental and control groups. Besides the difference in diet, 25 % of smokers in the experimental group had stopped smoking, with only 17 % in the control group. These differences in diet and smoking were associated with a 47 % difference in the total of fatal and nonfatal myocardial infarction and sudden death between the two groups, thus emphasising the importance of changes in diet and smoking.

The risk factor of hypertension offers potentially another point open to therapy, to prevent the initial stages of endothelial injury. The disease of hypertension can take many forms (such as essential hypertension), and is caused by a number of different factors. In many subjects, a hypertensive state is brought about by salt sensitivity. For this form of hypertension the causative mutations all lead to increased reabsorption of sodium in the renal tubules. However, in most individuals diagnosed with high blood pressure, the basis behind their abnormal blood pressure remains unknown. This is because it is an interplay of genetic and environmental factors, ultimately resulting in sufficient abnormalities in gene expression to yield the pathological elevations of blood pressure. Whatever the cause, an elevation in blood pressure is associated with damage to endothelial cells lining the arterial lumen resulting in cytoskeletal rearrangement and altered morphology, but also changes in endothelial gene expression (as reviewed in Cines *et al.* 1998). This is evidenced by the prevalence of atherosclerosis at coronary branch points of vessels (points most exposed to changes in blood pressure and thus shear stress). In subjects with essential hypertension, numerous studies have shown a reduction in arterial stiffness thus blood pressure, associated with various pharmacologic classes of anti-hypertensive agents including β-blockers, diuretics, angiotensin converting enzyme inhibitors and calcium antagonists. In addition to these agents, similar decreases in blood pressure can be obtained ostensibly by altering dietary intake of certain foods. For example, the dietary approaches to stop hypertension (DASH) trial, resulted in a significant decrease in diastolic blood pressure by increasing consumption of fruits, vegetables and dairy products with low saturated fat content. Such a diet is low in saturated fat and high in potassium, calcium, and magnesium. The trial provided the first evidence that potassium, calcium and magnesium in addition to sodium, are important dietary determinants of blood pressure (Sacks *et al.* 1999; Kesteloot *et al.* 1984). The more recently published results of the second DASH trial, in which sodium intake was lowered and intake of potassium, calcium and magnesium was increased, showed that the combined effect of sodium reduction and the

DASH diet was greater than their single effects (Sacks *et al.* 2001). These results highlight the importance of diet towards the therapy of disease, thus a healthy diet should be an integral part in the treatment of hypertension.

In addition to therapies that attempt to prevent or limit damage to the endothelium, other strategies aimed at ameliorating the normal response of the endothelium in response to injury have been considered. Many of these therapies work to reverse endothelial dysfunction by restoring normal physiological gene expression. Therefore a possible target would be the transcription factor NF-κB. For example, suppression of NF-κB by dietary derived polyphenols could prevent the expression of genes germane to atherosclerosis (for an in-depth discussion see section 1.8.3). Chemopreventive agents such as EGCG, can work on both aspects of therapy; that is they mitigate the influence of injury and/or intervene in the vessel's pathological response to that injury (see below).

1.3 Synopsis of Chemoprevention

Chemoprevention can be defined as the reversion, suppression or prevention of disease by use of chemicals, notably natural chemicals of dietary origin. Because chronic diseases such as cardiovascular disease and cancer are the leading causes of death in modern society, anything that can substantially reduce the risk of these diseases can be of great benefit to public health. Ideally, a chemopreventive agent should encompass the following inherent qualities to make it suitable for human consumption; (1) high efficacy against multiple targets, (2) high bioavailability, (3) minimised or no adverse side effects, (4) inexpensive, (5) easy administrative routes such as oral consumption and (6) human acquiescence.

Rather than altering habitual dietary intake to prevent potential future chronic illness, the general public normally endure drug treatments or submit to surgical procedures that are commonly arduous and painful. However, it is recognised that prevention or chemoprevention of disease as opposed to remediation is the optimum approach to curative medicine.

The occurrence of many diseases, such as cardiovascular disease and cancer, varies widely throughout the world, and is often influenced by lifestyle and environmental factors. For many years, it has been acknowledged that the consumption of a balanced diet rich in fruits and vegetables can prevent or control the onset of several pathological conditions such as cardiovascular disease and cancer. For instance epidemiological

evidence has suggested an elevated risk of developing cardiovascular disease such as atherosclerosis, synonymous with a high intake of saturated fat. However diets that are low in saturated fat and cholesterol correlate with a reduced incidence of coronary events. In fact it is well accepted that an increased intake of vegetables and fresh fruits i.e. food sources high in vitamins and antioxidants, culminates in a decrease in the risk of developing heart disease. Thus chemoprevention offers an additional strategy to decrease cardiovascular pathologies.

It is envisaged that chemopreventive agents are to be consumed over prolonged periods of time. Any agent that is used on a long-term basis and is strong enough to reduce the risk of developing a chronic disease is also likely to have other effects in the body, some of which may be undesirable. Consequently, it is important to evaluate both the risks and benefits of the chemopreventive therapy for each individual subject. However in the majority of cases, especially using dietary agents, the benefits of chemoprevention far exceed the risks for subjects that are at high risk of disease (against which the chemopreventive agent protects).

Research in the subject of chemoprevention has identified a multitude of dietary constituents, which possess various protective properties. It is now apparent that these compounds, which comprise a diverse number of structures, can act though a range of similar or dissimilar mechanisms. For example, many agents act as reactive oxygen scavengers, mopping up excess reactive intermediates, and thereby increasing the overall antioxidant capacity of the target tissue. Other agents work through direct interaction with signal transduction intermediates, consequently impinging on the outcome of these signal pathways.

The chemopreventive agents under investigation were resveratrol, curcumin, I3C an EGCG, selected on the basis of existing epidemiological and experimental evidence.

1.4 Resveratrol

Resveratrol (3, 5, 4'-trihydroxystilbene) is part of the stilbene family of compounds occurring in a number of plant species and is particularly enriched in grape skins, berries and peanuts (Sanders *et al.* 2000). The stilbenes exist as low molecular weight phenolics, which function as anti-fungal phytoalexin compounds, enabling the plant to overcome pathogen attack. Resveratrol can exist as cis- and trans-isomeric forms. However, in this study only the effects of the trans-isomer (figure 1.2) were examined.

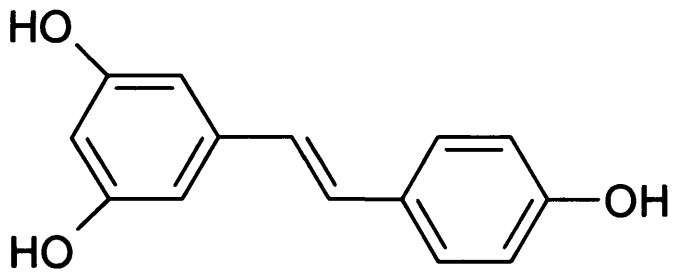

***Figure 1.2** - Chemical structure of trans-resveratrol (3, 5, 4'-trihydroxystilbene)*

Due to its high concentration in grape skins, significant amounts of resveratrol are present in wine. Epidemiological evidence indicates that consumption of red wine helps prevent cardiovascular disease (Rimm *et al.* 1991; St Leger *et al.* 1979). In fact data from the World Health Organisation indicate that consumption of wine (especially red wine) may reduce the risk of cardiovascular disease by 40 % (Renaud *et al.* 1992). The interest in compounds present in red wine was first stimulated by the "French paradox" theory. This theory comes from the observation that despite consumption of a high fat diet and smoking tendencies of the population of southern France, there was a surprisingly low incidence of coronary vascular disease (Renaud *et al.* 1992). This intriguing observation was attributed to the high consumption of red wine by the French subjects. Further work on this theory suggested that protection was partially due to the ethanol present in wine. Ethanol acts through a haemostatic mechanism and causes an increase in circulating HDL (as reviewed in Rimm *et al.* 1996). More recently however, numerous studies have provided evidence for the bioactivity of phenolic components present in wine including resveratrol (as reviewed in Soleas *et al.* 1997). Resveratrol was first considered as possessing biological activity in 1992 by Siemann *et al.* Since then its properties have been extensively investigated.

1.4.1 Resveratrol mechanisms of action

Until recently the proposed cardioprotective mechanisms of action have not been extensively investigated. Resveratrol has been associated with a reduction in general inflammatory mediators (Kimura *et al.* 1985; Subbaramaiah *et al.* 1998; Wadsworth *et al.* 1999). More specifically, resveratrol inhibits cytokine production, including interferon

(IFN)-γ, and IL-2 in splenic lymphocytes and TNFα and IL-12 in peritoneal macrophages, possibly due to inhibition of NF-κB activity (Gao *et al.* 2001).

One of the more investigated properties of resveratrol is the ability to act as a potent antioxidant. Oxygen derived free radicals and/or oxidative stress play a significant role in various stages of atherosclerosis. More specifically, reactive oxygen intermediates cause the initial 'injury' to the endothelial cells, but also oxidise lipoproteins such as LDL present in the lumen and medial layers of the artery, which contributes to the early and progressive stages of the disease. Wine was found to increase the antioxidant capacity of serum in human subjects (Whitehead *et al.* 1995). In this study, serum antioxidant capacity was evaluated in nine subjects, who ingested red wine, or white wine (on separate occasions). One hour after consumption, red wine was found to increase the antioxidant capacity by 18 %, and white wine by 7 %. The increase was attributed to the polyphenolics present in the wine, including resveratrol. Resveratrol has also been found to inhibit oxidative modification of LDL in a number of systems (Zou *et al.* 1999; Frankel *et al.* 1993; Belguendouz *et al.* 1997; Fremont *et al.* 1999). In one such study by Zou *et al.* (1999), human LDL was oxidised using Cu^{2+} or an azo compound AAPH (or 2, 2'-azobis.[2-amidinopropane] dihydrochloride, a free radical generator). Resveratrol was found to protect LDL against both Cu^{2+}- induced and azo compound-initiated oxidative modification *in vitro*, which may have been attributable to its free radical scavenging capacity. In a similar study, trans-resveratrol was found to be more efficient than cis-resveratrol at protecting porcine LDL against peroxidative degradation by Fe^{2+} and AAPH. The protective effect was due to both chelating and free radical scavenging abilities of resveratrol. Further to the LDL modulatory role, is the evidence that resveratrol is able to increase the HDL to LDL cholesterol ratio (Gaziano *et al.* 1993), which is associated with a decreased risk of atherosclerosis. Interestingly, in one study resveratrol had no effect on lipoprotein profile and did not prevent peroxidation of serum lipids in 'normal' rats (Turrens *et al.* 1997). These contrasting findings may be due to the use of different concentrations and incubation conditions of resveratrol. Also the rat study was performed *in vivo*, and many of the reported effects are *in vitro*, except for the report by Whitehead *et al.* (1995) who examined the effect of red and white wine as a whole, and not just resveratrol.

Resveratrol also inhibits the peroxidation of membrane lipids. In rat liver microsomes, Blond *et al.* (1995) showed that in non-enzymatic or in NADPH-dependent

peroxidation, the concentration required to produce 50 % inhibition was about three times lower with resveratrol than with quercitin (another polyphenol present in wine).

By inhibiting the deleterious effects of ROS, resveratrol may function to protect the cell from apoptosis. In fact a report by Sun *et al.* (1997) using ROS and heavy metal sensitive PC12 cells, showed that upon addition of iron, resveratrol protected cells from peroxidative stress and hence ROS-induced apoptosis. This anti-apoptotic effect has been confirmed by Manna *et al.* (2000) who demonstrated resveratrol abrogated TNFα-induced cytotoxicity and caspase activation. Conversely, resveratrol was found to suppress lymphoblast cell transformation and induce apoptosis via a p53-dependent pathway (Huang *et al.* 1999). The apoptotic property of resveratrol was confirmed by Gao *et al.* (2001), who disclose that resveratrol inhibited cell growth and also induced apoptosis in leukaemic cells, which in part was due to suppression of NF-κB activity.

Modulation of cell growth and apoptosis may also be due to suppression of other signalling pathways germane to cellular proliferation and apoptosis, such as mitogen activated protein kinase (MAPK) family. In porcine coronary arteries, short-term treatment with resveratrol substantially inhibited phosphorylation and hence activation of the extracellular signal regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase 1 (JNK1) and p38, MAPK pathways (El-Mowafy *et al.* 1999). Manna *et al.* (2000) confirmed JNK inhibition and possibly ERK 1/2 inhibition (as confirmed by inhibition of MEK, an upstream regulatory kinase of ERK 1/2) in other cell types.

The favourable effects of resveratrol on the cardiovascular risk factors are not limited to its antioxidant action. As well as acting to mop-up or scavenge ROS, resveratrol modulates the expression of various genes important to atherosclerosis. Tissue factor is a cell surface receptor for clotting/coagulation factor VII(a) (Rapaport *et al.* 1995). Inappropriate *in vivo* expression of tissue factor in vascular cells has been shown to be responsible for thrombotic disorders such as atherosclerosis (Taubman *et al.* 1997; Wilcox *et al.* 1989). Tissue factor may also play a role in cell adhesion and migration (Ott *et al.* 1998), which are also important factors in atherosclerosis. Resveratrol in a concentration-dependent manner inhibited the expression of tissue factor in endothelial cells stimulated with a variety of agonists, including IL-1β, TNFα and lipopolysaccharide (LPS). Using nuclear run-on analysis this inhibition in expression was at the level of transcription. Interestingly, resveratrol did not significantly alter the binding abilities of a number of important transcription factors, namely c-Fos/c-Jun and c-Rel/p65, which are required for the induction of tissue factor promoter in endothelial cells (Mackman *et al.* 1997).

However, a report by Manna *et al.* (2000) found that resveratrol blocked TNFα-induced phosphorylation, nuclear translocation of p65 and NF-κB reporter gene transcription, in myeloid, lymphoid, and epithelial cells. The suppression of NF-κB coincided with suppression of AP-1. This would suggest that the effects mediated by resveratrol are cell-specific. A more recent study by Pendurthi *et al.* (2002a, 2002c) found that resveratrol inhibited the expression of LPS-induced expression of tissue factor in human monocytes and a monocytic cell line, THP-1, in a concentration-dependent manner, by inhibiting NF-κB.

Other pro-atherogenic genes which are modulated by resveratrol include cell adhesion molecules. The adhesion of circulating monocytes to dysfunctional endothelium is one of the earliest steps in formation of the atherosclerotic lesion (Ross *et al.* 1993). Two reports demonstrate that resveratrol inhibited leukocyte adhesion by inducing a decrease in adhesion molecule expression in pathophysiologically challenged endothelial cells (Ferrero *et al.* 1998; Pendurthi *et al.* 2002b). One of the reports by Ferrero *et al.* (1998), found that resveratrol, at concentrations as low as 100 nmoles/L, significantly inhibited intracellular adhesion molecule 1 (ICAM-1) and VCAM-1 expression by TNFα- and LPS-stimulated endothelial cells, (thus demonstrating the efficacy of resveratrol). It is likely that inhibition of adhesion molecule expression is due to the suppression of NF-κB transcriptional activity, which has been shown in a number of independent reports (Collins *et al.* 1995; Manna *et al.* 2000; Tsai *et al.* 1999; Pendurthi *et al.* 2002b).

The effects of resveratrol on platelet aggregation and activity have been investigated. The initial step in thrombus formation in atherosclerosis is the adhesion of platelets onto vascular subendothelial connective tissue exposed upon endothelial injury. Platelet aggregation is linked to the synthesis of eicosanoids from arachidonic acid (AA). Resveratrol was found to reduce and/or inhibit platelet aggregation both *in vitro* (Wang ZR *et al.* 2002; Zbikowska *et al.* 1999; Pace-Asciak *et al.* 1995) and *in vivo* (Wang ZR *et al.* 2002). This reduction in platelet aggregation is due to the inhibition of pro-atherogenic eicosanoid synthesis, such as thromboxane (Pace-Asciak *et al.* 1995; Kimura *et al.* 1985; Mikhailidis *et al.* 1983) and prostaglandin family members (Pace-Asciak *et al.* 1995; Kimura *et al.* 1985; Zbikowska *et al.* 1999), which may be due to cyclo-oxygenase 2 (COX2) inhibition as reported by Subbaramaiah *et al.* (1998).

Resveratrol also functions to maintain normal physiological blood pressure. Chen *et al.* (1996) observed that resveratrol caused a nitric oxide (NO)-mediated relaxation of precontracted endothelium-intact rat aorta. Hsieh *et al.* (1999) confirmed that NO was

produced following resveratrol treatment of bovine pulmonary artery endothelial cells. However arterial vasodilation-induced by resveratrol was also found to be independent of NO as demonstrated by Demrow *et al.* (1995). In this report resveratrol treatment was associated with an increased vasodilatory prostacyclin synthesis.

1.5 Indole-3-carbinol

It has long been recognised that members of the *brassica* family, that is the cruciferous vegetables including Brussels sprouts, cabbage, broccoli and cauliflower, have many beneficial and potential cancer chemopreventive activities. This view is based on both experimental testing of crude extracts and epidemiological studies. In the majority of cases biological activities of these crude extracts can be linked to specific fractions or isolates obtained from species of this closely related family, for example indole-3-carbinol (I3C). I3C is a hydrolysis product of glucobrassicin (figure 1.3).

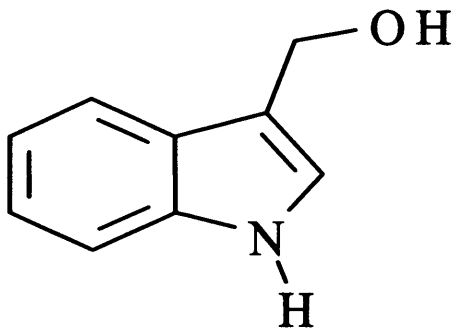

***Figure 1.3** - Chemical structure of indole-3-carbinol*

1.5.1 I3C mechanisms of action

To date much of the work concerning the chemopreventive properties of I3C has focussed on cancer rather than cardiovascular chemoprevention (as reviewed in McDanell 1988). However, some of the chemopreventive properties described for cancer are also applicable for cardiovascular disorders. The various reports into the chemopreventive properties of I3C have suggested that they may be associated with its ability to modulate the cell cycle or induce apoptosis (Cover *et al.* 1998, 1999; Telang *et al.* 1997). Work by Cover *et al.* (1998), investigated the effect of I3C on the cell cycle in MCF-7 breast carcinoma cells. In this report, I3C suppressed cell growth, which was associated with a

reversible G_1 cell cycle arrest. This cell cycle arrest was preceded by an I3C-induced decrease in CDK6 expression, independent of oestrogen signalling. Also, I3C inhibited endogenous phosphorylation of the retinoblastoma protein (pRb) and *in vitro* CDK6-mediated phosphorylation of pRb. Furthermore, the CDK inhibitors p21 and p27 levels were increased by 50 %, when maximal growth arrest was observed. These observations indicate that I3C can affect many regulators of the cell cycle leading to growth regulation. Cellular proliferation and apoptosis are significant pathological changes associated with various stages of atherosclerosis.

Telang *et al.* (1997) examined the effects of treating chemically transformed breast epithelial 184-B5/BP derived cells and mammary carcinoma–derived MDA-MD-231 cells with I3C. I3C (50 μM) was found to increase the Q/P ratio by 137-210 % (Q = G_0, P = S + M phase), which resulted in a two-fold increase in cellular apoptosis and a 54 -61 % inhibition of growth. This growth inhibitory effect was attributed in part to an increase in the formation of an antiproliferative estradiol metabolite.

Interestingly, cellular metabolism of estradiol has been reported to alter in response to carcinogenic insult and is related to the risk of developing breast cancer (Telang *et al.* 1991, 1992; Suto *et al.* 1992; Liehr *et al.* 1996). Levels of estradiol can also have profound effects on the cardiovascular system, reducing the risk of developing arterial disease (as reviewed in Mendelsohn *et al.* 1999). Oestrogens can have numerous direct effects on blood vessels, which can be divided into rapid or nongenomic and longer-term or genomic effects. Rapid effects include enhancement of vessel vasorelaxation by both endothelium-dependent and independent mechanisms. Oestrogen can modulate the influx of extracellular calcium into VSMCs by modulating L-type calcium channels present on cell membranes (Jiang *et al.* 1991; Freay *et al.* 1997). At physiologic concentrations, oestrogen stimulates the opening of calcium-activated potassium channels through nitric oxide- and cyclic guanosine monophosphate-dependent pathway, (White *et al.* 1995; Wellman *et al.* 1996), thus relaxing smooth muscle and promoting vasodilation. Normal endothelium secretes NO. Physiologic concentrations of oestrogen cause a rapid release of NO (without altering gene expression) (Yuhanna *et al.* 1999; Caulin-Glaser *et al.* 1997), which both relaxes vascular smooth muscle and inhibits platelet activation (Moncada *et al.* 1993). Longer-term effects can result from oestrogen-induced increases in genes important for vasorelaxation, such as inducible nitric oxide synthase, and prostacyclin synthase (Weiner *et al.* 1994; Binko *et al.* 1998). Other anti-atherogenic effects mediated by oestrogen include the *in vitro* inhibition of VSMC proliferation, migration (Kolodgie *et al.* 1996;

Bhalla *et al.* 1997), and also adhesion molecule expression (Caulin-Glaser *et al.* 1996). Therefore modulation of oestrogens by I3C could potentially protect against atherosclerosis through a number of different mechanisms.

Other chemopreventive qualities of I3C include the effect on cytochrome p450 enzymes and phase II drug metabolising enzymes. There are several reports in the literature that demonstrate the I3C-induced elevation of cytochrome p450 isoenzyme activity (Manson *et al.* 1998; Birt *et al.* 1986; Bailey *et al.* 1987; McDanell *et al.* 1987; Rijnkels *et al.* 1998) and phase II enzymes (Manson *et al.* 1997). In terms of cancer chemoprevention, phase II enzymes act to eliminate potential carcinogens from the body. While p450 enzymes can also detoxify potential carcinogens, they may also activate potential carcinogens.

Other p450 family members can affect vascular homeostasis (i.e. vascular tone, cellular proliferation) and/or the metabolism of cholesterol, hence the significance to cardiovascular disease (as reviewed in Fleming *et al.* 2001). One report by Rijnkels *et al.* (1998) has shown that I3C induced cytochrome p450-IA activity in Caco-2 human colon carcinoma cells. LeBlanc *et al.* (1994) investigated the effect of I3C on cytochrome p450 family members, namely cholesterol ester hydrolase and cholesterol 7α-hydroxylase, and hence cholesterol homeostasis in a murine model. Oral administration of 500 and 750 mg/kg/day I3C to mice for 1 week resulted in increased liver mass and microsomal protection content. Hepatic microsomal cholesterol levels were not significantly altered following treatment with 100 and 250 mg/kg/day I3C, but were significantly decreased following treatment with 500 and 750 mg/kg/day. Conversely, the lower doses of 100 and 250 mg/kg/day I3C decreased serum cholesterol levels, whereas the higher doses had no effect. These changes were not attributable to hepatic hypertrophy as phenobarbital (an agent that induces liver hypertrophy), had no effect on microsomal or serum cholesterol levels. I3C had no effect on either cholesterol ester hydrolase or cholesterol 7α-hydroxylase activity, but did elevate hepatic acyl-CoA:cholesterol acyltransferase (ACAT) activity, which is the enzyme responsible for the formation of hepatic cholesteryl esters.

As with other chemopreventive agents I3C possesses antioxidant properties. The cytoprotective effect of I3C on carbon tetrachloride (CCl_4) mediated hepatotoxicity in mice was examined (Shertzer *et al.* 1988). I3C pre-treatment by gavage, 1 hour prior to intraperitoneal injection of CCl_4 produced a 63 % decrease in CCl_4-mediated centrilobular necrosis and a related 60 % decrease in plasma alanine aminotransferase activity (a marker of liver necrosis). The toxicological effects of CCl_4 were mediated by radical species

generated during reductive metabolism by p450 enzymes. Three separate systems, which were based on lipid peroxidation, were used to evaluate the potential modulatory effect of I3C on ROS-induced damage. One system consisted of phospholipid dissolved in chlorobenzene, with peroxidation initiated by the thermal and photodecomposition of azobisisobutyronitrile (AIBN), another system consisting of phospholipid vesicles in phosphate buffer (pH 7.4), with peroxidation initiated by ferrous/ascorbate, and finally mouse liver microsomes containing an NADPH-regenerating system, with peroxidation initiated with CCl_4. I3C inhibited lipid peroxidation in all three systems in a concentration-dependent manner. In the AIBN and ferrous/ascorbate systems, greater inhibition by I3C was noted under conditions of low free radical generation. I3C was most effective in the microsomal CCl_4-initiated system. The inhibitory effect was attributed to the I3C radical scavenger ability.

1.6 Curcumin

Curcumin (figure 1.4) is the major polyphenolic component of turmeric powder derived from the rhizome of the plant *Curcuma longa*, found in south and southeast tropical Asia. As well as being used as a dietary spice, turmeric has been used for centuries as a traditional medicine to treat inflammatory disorders (Nadkarni KM, 1976). In addition to its anti-inflammatory actions, curcumin also has strong antioxidant properties as demonstrated in numerous studies (Toda *et al.* 1985; Jitoe *et al.* 1992).

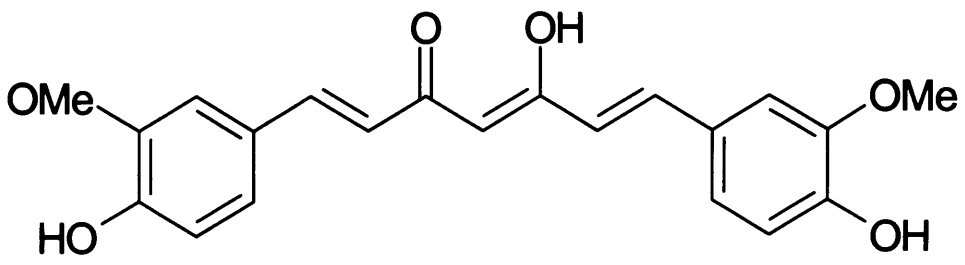

***Figure 1.4** - Chemical structure of curcumin*

1.6.1 Curcumin mechanisms of action

The precise cardioprotective mechanisms of action of curcumin have not been clearly defined. Curcumin has many effects depending on the cell type and concentration under study. One preventive action of curcumin on atherogenesis and related cardiovascular disease is through its effect on LDL modification. An investigation by Ramirez-Tortosa *et al.* (1998) examined the effects of an extract from *Curcuma longa*, ZCL4, on the *in vitro* peroxidation of human LDL. The turmeric extract increased the *in vitro* resistance of human LDL to oxidation. When LDL was subjected to $CuSO_4$ mediated oxidation, the addition of this extract to the medium increased the lag phase and decreased the formation of conjugate dienes, because it stopped the initial oxidation process. A more recent study by Ramirez-Tortosa *et al.* (1999) examined the effect of this same extract ZCL4, in an *in vivo* model of experimental atherosclerosis in rabbits fed with a high fat and cholesterol diet. Intake of ZCL4 significantly decreased oxidation of serum LDL-cholesterol. In addition, administration of ZCL4 resulted in higher serum levels of vitamin E, compared to those found in animals receiving a diet enriched with vitamin E, thus indicating a vitamin E-sparing effect. Furthermore, the thoracic aorta suffered a milder atheroma plaque formation in the animals treated with the extract than those receiving the control diet.

As well as *in vivo* animal studies, the effect of the turmeric extract on human blood lipid peroxide levels has been investigated. One study by Ramirez-Bosca *et al.* (1995) administered ZCL4, to men ranging in age from 27 to 67 years. At the end of treatment period of 45 days there was a significant decrease in blood serum lipid peroxides. There was no associated liver or kidney toxicity as established by measuring enzyme levels of glutamate-oxalacetate-transaminase (GOT), glutamate-pyruvate-transaminase (GPT), gamma-glutamyl transpeptidase (GGT) and alkaline phosphatase. Further examination also demonstrated that HDL-and LDL-peroxides were reduced by 25-50 % by the 60^{th} day of treatment. This lowering effect of the curcuma extracts was confirmed by the work of Babu *et al.* (1997). In this study, blood levels of LDL and VLDL cholesterol, triglycerides and phospholipids were lowered by curcumin in rats in which diabetes had been induced by streptozotocin.

Other favourable chemopreventive qualities of curcumin include the effects on pro-atherogenic gene expression, possibly mediated through suppression of important transcription factors like NF-κB. For example the effect of curcumin on the adhesion of

leukocytes has been investigated (Kumar *et al.* 1998; Jobin *et al.* 1999). Treatment of endothelial cells (EC) with TNFα for 6 hours augmented the adhesion of monocytes to EC, and this adhesion was due to increased expression of ICAM-1, VCAM-1, and ELAM-1. Pre-treatment of EC for 1 hour with curcumin completely blocked their adhesion to monocytes, as well as the cell surface expression of the aforementioned adhesion molecules. This inhibition of cell surface adhesion molecules was thought to be due to suppression of NF-κB activity (Kumar *et al.* 1998).

As with resveratrol, curcumin alters the expression of the pro-atherogenic gene encoding tissue factor. The study by Bierhaus *et al.* (1997) investigated the effect of curcumin on the TNFα-induced expression of tissue factor, the central mediator of coagulation involved in atherogenesis. When bovine aortic endothelial cells were pre-incubated in the presence of curcumin, TNFα-induced tissue factor gene transcription and expression were reduced. Transient transfection studies with tissue factor-promoter plasmids revealed that both NF-κB- and AP-1-dependent tissue factor expression were reduced following treatment with curcumin. These inhibitory responses were due to distinct mechanisms, as curcumin inhibited TNFα-induced IκBα degradation and NF-κB translocation, whereas inhibition of AP-1 was direct by interfering with DNA binding.

The effect of curcumin on pro-inflammatory gene expression, including *IL-8* (Jobin *et al.* 1999), *TNFα* and *IL-1β* (Chan *et al.* 1995) has been investigated. Jobin *et al.* (1999) found that curcumin inhibited IL-1β-mediated *IL-8* gene expression in IEC-6, HT-29, and Caco-2 epithelial cells. *ICAM-1* gene expression was also inhibited, confirming the work of Kumar *et al.* (1998). This inhibitory effect was again due to suppression of cytokine-induced NF-κB DNA binding activity, RelA nuclear translocation, IκBα degradation, IκB serine 32 phosphorylation and IKK activity. It was also concluded that gene expression may also in part be due to curcumin inhibition of the ERK pathway, as demonstrated by MEK1 inhibition. Suppression of LPS-induced synthesis of TNFα and IL-1β in a human monocytic macrophage cell line, by curcumin concentrations as low as 5 μM has been reported by Chan *et al.* (1995). Again this inhibition was attributed to suppression of NF-κB activity. Inhibition of NF-κB by curcumin protected the cells from TNFα-induced apoptosis as curcumin inhibited the TNFα-induced cytotoxicity of actinomycin-D-treated L929 fibroblasts.

A more recent report in *ex vivo* cultured BALB/c mouse peritoneal macrophages, by Chan *et al.* (1998) showed that curcumin (1-20 μM) reduced the production of

inducible-nitric oxide synthase (*iNOS*) mRNA in concentration dependent manner. Furthermore, this *ex vivo* effect was also replicated *in vivo*, as two oral doses of curcumin (92ng/g of body weight) reduced *iNOS* mRNA expression in the livers of LPS-injected mice. It is likely that inhibition was again due to suppression of NF-κB, as the group have formerly established that curcumin inhibits NF-κB activation in a monocytic cell line. Also Murphy *et al.* (1996) have shown that iNOS gene expression is dependent on NF-κB activity, in murine macrophages. Although iNOS may function to induce vasorelaxation, it also catalyses the production of NO free radical, which is deleterious to endothelial cell homeostasis and thus contribute to atherosclerosis.

Inhibition of NF-κB by curcumin can also lead to suppression of cell growth, via cell cycle or apoptotic responses (Chen HW *et al.* 1998; Anto *et al.* 2000; Kawamori *et al.* 1999; Jiang *et al.* 1996). Cellular proliferation and apoptosis are aetiological changes associated with certain cells during atherogenesis (Ross *et al.* 1993). In vascular smooth muscle cells, curcumin inhibited serum-stimulated [^{3}H]-thymidine incorporation indicating an inhibitory effect on cell-proliferation. Curcumin arrested cell cycle progression and also induced apoptosis (Chen HW *et al.* 1998). The apoptotic effect may partly be mediated through the inhibition of protein tyrosine kinase activity, protein kinase C activity, *c-myc* mRNA expression and *Bcl-2* mRNA expression, but independent of p53 expression. This observation is not cell specific as Jiang *et al.* (1996) reported that curcumin induced cell shrinkage, chromatin condensation, and DNA fragmentation (characteristics of apoptosis), in immortalised mouse embryo fibroblast NIH 3T3, erb B2 oncogene-transformed NIH 3T3, mouse sarcoma S180, human colon cancer cell HT-29, human kidney cancer cell 293, and human hepatocellular Hep G2 cells.

Interestingly, curcumin-induced cell death was prevented in L-929 mouse fibrosarcoma cells following stable transfection of *RelA* gene encoding RelA/p65 subunit of NF-κB, whereas the parental cells underwent apoptosis. This resistance to curcumin-induced apoptosis was abrogated following transfection of a super-repressor form of IκBα into the *RelA*-transfected cells. The *in vitro* apoptotic effects have also been demonstrated *in vivo*, following administration of a 0.2 % curcumin diet in male F344 rats (Kawamori *et al.* 1999).

As well as impinging on the NF-κB pathway directly, curcumin also affects the activity of other important cellular signalling pathways germane to cell growth and survival, including JNK (Squires *et al.* 2003; Chen YR *et al.* 1998), and ERK 1/2 (Squires *et al.* 2003). In the report by Chen YR *et al.* (1998), curcumin inhibited the activation of

Traditionally tea was drunk to improve blood flow, eliminate toxins, and to improve resistance to diseases (Balentine *et al.* 1997). Today, tea consumption is associated with lifestyle and social habits, although increased public awareness of the health benefits from drinking tea has changed this prospective somewhat.

The three main types of tea include black, green and oolong, which have different chemical compositions, dependent on the type of processing that occurs immediately after picking. Green tea, which constitutes about 20 % of the tea produced, is picked and immediately steamed, or pan fired, then rolled to inactivate enzymes that may oxidise the polyphenols present in the leaf. Finally, the rolled leaves are air-dried at a high temperature. Epicatechins are the main compounds in green tea, accounting for its characteristic colour and flavour.

Black tea, which constitutes about 78 % of the tea produced, is plucked, withered, macerated, and then dried. The withering step lowers the moisture content and renders the leaf more assessable to maceration. Maceration disrupts the cell, allowing polyphenol oxidase to catalyse the oxidation of the catechins present in the leaf. The oxidation step, which continues for about 45-90 minutes, gives rise to the colour and taste of black tea, and is the result of the conversion of catechins to theaflavins and thearubigins. Theaflavins and thearubigins have been associated with chemopreventive qualities.

The final 2 % of the tea produced is known as oolong tea. Oolong tea is processed in a similar manner to black tea, except the oxidation step is much shorter. Thus normal oolong tea is about half fermented and thus contains more catechins compared to black tea, but not as much as green tea.

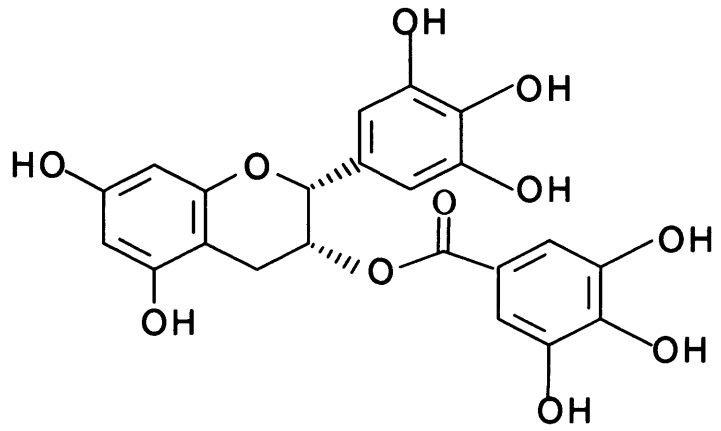

***Figure 1.5** - Chemical structure of epigallocatechin 3 gallate (EGCG)*

Most of the polyphenols present in green tea, accounting for 30 % of the dry weight, are flavonals commonly known as catechins. Epigallocatechin-3-gallate (EGCG) is

JNK in a T-lymphocyte cell line, following induction with PMA plus ionomycin, anisomycin, UV-C, γ radiation, TNFα or sodium orthovanadate. ERK activation by PMA plus ionomycin was also suppressed by curcumin. Curcumin moderately suppressed transfected MEKK1-induced JNK activation, but effectively blocked JNK activation caused by co-transfection of TGFβ-activated kinase (TAK1), germinal centre kinase (GCK), or hematopoietic progenitor kinase 1 (HPK1) or kinases that activate JNK. However curcumin did not directly inhibit JNK, SEK1/MKK7, MEKK1 or HPK1 activity. Taken together these data suggest that curcumin may affect the JNK pathway by interfering with the signalling molecule(s) at the same level or proximally upstream of the MAPKKK level. Also, it may reveal a possible mechanism of suppression of NF-κB signalling by curcumin, and hence the anti-inflammatory effect.

Another favourable chemopreventive quality of curcumin includes the effect on matrix metalloproteinases. A report by Thaloor *et al.* (1998) investigated the effect of curcumin on endothelial cell migration, attachment, and tube formation on Matrigel (a model for angiogenesis). Endothelial cell migration and attachment is also important in the early and progressive stages of atherosclerosis (Ross *et al.* 1993). In HUVEC curcumin had no effect on endothelial cell migration or attachment to either plastic or Matrigel. Zymographs of curcumin-treated culture supernatants showed a decrease in the gelatinolytic activities of secreted 53- and 72-kDa metalloproteinases. This decrease in gelatinolytic activity of the 72-kDa metalloproteinase, may have been due to curcumin mediating an effect at both the transcriptional and translational level of this gene as demonstrated by Western and Northern analysis.

1.7 Epigallocatechin 3 gallate (EGCG)

The consumption of tea is a very ancient habit and was first practiced in China and India about 5000 years ago. The tea plant, *Camellia sinensis*, is now cultivated in over 30 countries around the globe and currently consumed worldwide although at greatly varying levels. Not only is there variation between countries, but also variation in the population of a country. This variation ranges from the consumption of no cups, up to 20 or more cups per day (Graham *et al.* 1992). Excluding water, it is now generally accepted that tea is the most consumed beverage in the world, with per capita worldwide consumption of approximately 120 ml per day (Graham *et al.* 1992).

the major catechin constituent of green tea (figure 1.5), accounting for more than 10% on a dry weight basis.

1.7.1 Bioavailability and metabolism of EGCG

Two thirds of the U.K. population above 10 years of age (around 37 million people) are reported to drink tea daily, on average three cups, and 90 % of such tea is prepared from tea bags. The estimated U.K. dietary intakes of total tea catechins, calculated on the average of 3 cups per day (200 ml cup, 1 % tea leaves w/v), are 61.5, 92.7, and 405.5 mg/day from fruit teas, black teas, and green teas, respectively (Khokhar *et al.* 2002). These figures are estimates as they are dependent on brewing conditions, such as water temperature, the amount and time of stirring, as well as the initial dry weight of tea in the teabag.

For any of these catechins, including EGCG, to induce a biological effect they must first be absorbed into the body. Knowledge of their absorption and metabolism is essential to the understanding of their action *in vivo*. There have been many studies investigating the bioavailablity of various catechins both in animal models and also human subjects (Van het Hof *et al.* 1998; Van Amelsvoort *et al.* 2001; Miyazawa *et al.* 2000; Chen *et al.* 1997; Wiseman *et al.* 2001; Hollman *et al.* 1997; Nakagawa *et al.* 1997a, 1997b). Ingestion of both green and black tea results in rapid appearance of catechins into plasma. In rat and murine models the absorption of green tea extracts and of pure EGCG has been evaluated. Under conditions in which the anti-tumourigenesis activity of tea has been demonstrated in rats and mice (0.9 % decaffeinated or regular green tea, respectively, as the sole source of drinking fluid), the plasma EGCG, epigallocatechin (EGC), and epicatechin (EC) steady state levels were 37, 55, and 20 ng/ml for rats, compared to 124, 62, and 10 ng/ml for mice (as reviewed in Hollman *et al.* 1997). These disparities may be due to species differences and/or the use of dissimilar tea preparations. More recently, a number of more thorough studies have been performed in rats (Nakagawa *et al.* 1997a; Chen *et al.* 1997). One such study by Nakagawa *et al.* (1997a), have examined the absorption and tissue distribution of EGCG in the rat. Levels of EGCG were calculated 60 minutes after a single oral administration of EGCG (500mg/kg body weight). Levels of EGCG (in the free form) reached 12.3 nmol/ml in plasma, 48.4 nmol/g in liver, 0.5 nmol/g in brain, 565 nmol/g in small intestinal mucosa, and 68.6 nmol/g in the colonic mucosa. These results indicate that EGCG is absorbed (in the free form) from the digestive tract, with the intestinal mucosa

being the most enriched of the organs. This is suggestive of the intestines being the major absorption site of EGCG in mammals. Chen *et al.* (1997) confirmed the finding that EGCG can be readily absorbed (in free form) in the rat. Interestingly, data from this study indicate that EGCG was better absorbed when given through drinking fluid. In addition, it appears that EGCG was mainly excreted through the bile, whereas EGC and EC are excreted through the bile and urine.

A number of studies on the absorption and pharmacokinetics of tea catechins in humans have been reported (Van Amelsvoort *et al.* 2001; Van Het Hof *et al.* 1998; Das *et al.* 1971). One of the first of these studies was carried out by Das *et al.* (1971). Oral administration of (+)-catechin (83 mg/kg) to human subjects resulted in absorption, rapid metabolism, and excretion of the compound largely within 24 hours. Absorption of the (+)-catechin (in free form) in the gastrointestinal tract and presence in the blood was noted as early as 6 hours after oral administration. The presence of the free form of (+)-catechin plus 11 metabolites was detected in the urine, and free form (+)-catechin plus 2 metabolites were detected in the faeces. Importantly no serious side effects were observed from any of the human subjects, although 1 or two complained of having diarrhoea about 24 hours after oral intake of (+)-catechin.

In another study in humans, ten healthy human volunteers ingested 1.5 μM epicatechin gallate (ECg), EGC, or EGCG in a randomised crossover design (Van Amelsvoort *et al.* 2001). After deconjugation, catechins in plasma and 24 hour urine samples were determined by high performance liquid chromatography (HPLC). Also antioxidant activity was measured in plasma by determining ferric reducing activity (FRAP). The catechin levels in plasma after ingestion were significantly different, EGC rose quickly with a short elimination half-life (*t½ elimination* = 1.7 h), ECg was intermediate in rise but slowest in decline (*t½ elimination* = 6.9 h), with EGCG slowest in rise but intermediate in decline (*t½ elimination* = 3.9 h). At 24 hours, EGC and EGCG had returned to base levels, but ECg was still elevated. Peak maximum absorption was approximately 1.3 μM/l of blood for EGCG. Up to 13.6 % of the ingested EGC (partly methylated) was eliminated in the urine, however no ECg or EGCG were detected. Only EGC and ECg produced a significant increase in the antioxidant capacity of the plasma, but with EGCG no statistically significant effect was noted. In another study (Yang CS *et al.* 1998), 18 volunteers were given a beverage containing decaffeinated green solids (1.5, 3, or 4.5 g) with sucrose and coffee whitener after overnight fasting. The plasma concentrations of EGCG, EGC and EC reached peak levels between 1.5 to 2.5 h in almost

all subjects and declined to undetectable levels after 24 hours. When the dose of green tea was increased from 1.5 to 3.0 g, the maximum plasma concentrations of EGCG, EGC, and EC increased (2.5 to 5 fold), but did not significantly change further, even after an increase up to 4.5 g of green tea. The half lives of the terminal elimination phase of EGCG, EGC, and EC were from 3 to 5 hours. Most of the EGC and EC was eliminated in the urine. However, consistent with other findings no EGCG was detected in the urine. These levels were consistent with the study by Nakagawa *et al.* (1997b), who demonstrated that the concentration of EGCG in human plasma was in the low micromolar range (4 μM), which at these levels has been shown to induce biological effects *in vitro*.

In humans, there is currently little data on the tissue distribution of tea flavonoids after tea consumption. However one study by August *et al.* (1999) demonstrated significant levels of tea flavonoids detected in human colorectal tissue after ingestion of green tea extract (equivalent dose of 2-3 cups of tea) (August *et al.* 1999). Four hours after tea ingestion, increased levels of tissue catechin was noted, and was still evident after 24 hours. There was a decrease in prostaglandin E_2 levels in rectal tissue, demonstrating a cellular biological response at relevant tea consumption levels.

In a rat model, 8 days following consumption of green tea polyphenols, most catechins were detectable in the bladder, followed by the kidney, large intestine, oesophagus, lung, and prostrate. Comparatively low levels were detected in liver, spleen, heart, and thyroid gland (Yang CS *et al.* 2000). This compared to significant amounts of radiolabelled EGCG recovered in digestive tract tissues (14.5 %) (stomach, small intestine, and colon) and 2.1 % being distributed over lung, brain, heart, liver, kidney, spleen, pancreas, bladder, uterus, and ovary.

Taken together these studies clearly show that catechins including EGCG are absorbed into the human body and are detectable at low micromolar concentrations, doses at which a biological effect has been noted in *in vitro* systems.

An important aspect related to evaluating the absorption of catechins from a tea beverage, is the effect of milk. Polyphenols have a strong affinity for proline rich proteins such as milk. However, the consumption of milk and green or black tea does not affect the polyphenol concentration in blood, indicating that milk does not reduce tea polyphenol bioavailability (Van Het Hof *et al.* 1998).

In humans catechin biotransformation or metabolism tends to occur in the liver, although some colonic metabolism is also evidenced (Das *et al.* 1971). Studies in humans using radiolabelled catechin have shown that after oral administration, some 50 % of the

radioactivity was recovered in the urine, and only 0.5 to 3 % of this was in the form of catechin aglycone (Hackett *et al.* 1983). Regarding the metabolites, Lee MJ *et al.* (1995) reported after oral administration of EGCG and EC to human volunteers, the major conjugates in plasma were sulphates for EGCG and glucuronide for EC. Some 20 % of EGCG was also present as unconjugated compound. Also Van Amelsvoort *et al.* (2001) demonstrated the presence of the *O*-methylated form of EGC in humans after oral administration of 1.5 μM EGC. It is thought that the phenolic hydroxyl group of the catechin is the site of substitution for the glucuronide, sulphate and methyl group.

1.7.2 EGCG mechanisms of action

Although there has been much work investigating the health effects of tea constituents, the precise cardioprotective mechanisms of action of EGCG have yet to be clearly defined. Like many other polyphenolic compounds, EGCG functions as a potent antioxidant and/or anti-inflammatory agent. EGCG has been shown to have numerous *in vitro* and *in vivo* effects, impinging on various signalling pathways such as NF-κB (Ahmad *et al.* 2000), ERK, p38 (Chung *et al.* 1999) and JNK pathways (Dong *et al.* 1997).

One of the more characterised effects of EGCG is the ability to act as an antioxidant. ROS have been implicated in the initiation (by inducing endothelial injury), and progressive phases (by oxidising lipids) during atherosclerosis (Ross *et al.* 1993). Many studies have demonstrated the antioxidant capacity of flavonoids including EGCG (Kondo *et al.* 1999; Ohshima *et al.* 1998; Feng *et al.* 2001; Alvarez *et al.* 2002). Rice-Evans *et al.* (1996) found a relationship between antioxidant activity and structure in flavonoids and their homologues. This study has pointed out the importance of the phenolic group as an electron acceptor. Alvarez *et al.* (2002) demonstrated EGCG at concentrations of 1 – 100 μM, blocked the production of oxygen radicals by resident rat peritoneal macrophages stimulated with protein kinase C activator phorbol 12 myristate 13-acetate. This study demonstrates the desired biological effect was obtainable at a low physiologically relevant concentration.

As well as scavenging ROS, thus protecting against endothelial injury, EGCG and other related catechins may also act to prevent lipid peroxidation induced by ROS. Oxidation of LDL is recognised as an important step leading to atherosclerosis and there are several reports indicating that tea inhibits the oxidation of LDL *in vitro* (Wiseman *et al.* 1997; Miura *et al.* 1994; Salah *et al.* 1995). Studies testing the antioxidant effect of tea

polyphenols on LDL and very low-density lipoprotein (VLDL) oxidation indicate that EGCG is very effective and has a lipoprotein bound antioxidant activity greater than tocopherol (Vinson *et al.* 1995). In human aortic endothelial cells, tea extracts have been found to inhibit the formation of early lipid peroxidation products and late lipid peroxide decomposition products in a concentration-dependent manner (Pearson *et al.* 1998).

In addition to suppressing lipid peroxidation, green tea extracts also modify the plasma levels of circulating lipids. Yang *et al.* (1997) has found that green tea significantly reduces serum and liver cholesterol, and liver weight by lowering lipid deposition in hypercholesterolemic diet-induced rats. However in this study, HDL-cholesterol and triglyceride levels remained unchanged. Conversely, rats fed with 2.5 % green tea leaves in the diet showed a reduction in blood triglycerides and total cholesterol contents. Enhancement of the superoxide dismutase and phase II enzyme activities in the liver without any liver or kidney damage was also reported (Lin *et al.* 1998).

Data showing the effects of catechins on cholesterol modification in humans is slightly more equivocal. Serum lipid concentrations, resistance of LDL to oxidation, and oxidative damage to lipids remain unchanged in some human studies (McAnlis *et al.* 1998; Van Het Hof *et al.* 1997). However, a study by Yee *et al.* (2002) has indicated in human hepatoma cell line, HepG2, that an 8 hour pre-treatment with EC and EGCG, at concentrations of 10, 25 and 50 μM, inhibited apolipoprotein B-100 (apoB) secretion in a dose-dependent manner. Importantly total protein and albumin synthesis and secretion was unaffected, indicating a apoB-specific effect. Although a significant effect on apoB was observed, cellular lipids including free cholesterol, cholesterol ester, or triglyceride were unaffected. Another study in humans has shown that oral consumption of green tea can lower serum cholesterol levels (Kono *et al.* 1996). This concurs with a major study by Tokunaga *et al.* (2002), who examined the relationship between green tea consumption and serum lipids and lipoproteins in human subjects. The subjects consisted of 13,916 workers (8476 men and 5440 women) aged 40-69 years in central Japan. Health screening revealed that the subject cohort did not have morbid conditions affecting serum cholesterol levels. Serum concentrations of total cholesterol, LDL and triglyceride were measured at screening, and consumption of green tea and lifestyle characteristics were ascertained by a questionnaire. Daily consumption of green tea was reported by 87 % of subjects, and consumption was associated with statistically significant lower levels of serum total cholesterol in both men and women. Serum triglycerides and HDL cholesterol were not significantly changed. The inverse relationship of serum total cholesterol with green tea,

levelled off at the consumption of more than 10 cups per day. Interestingly, regression analysis (excluding the outliers) adjusting for age, bodymass index, ethanol intake, smoking habit, coffee intake and type of work, showed a daily consumption of one cup of green tea was associated with a reduction in serum total cholesterol by 0.015 mmol/l in men and women. This association was evident even after adjusting for selected dietary factors. In confirmation of these reports, Imai *et al.* (1995) have shown that green tea consumption was associated with decreased serum total cholesterol, but additionally a decrease in triglyceride levels and atherogenic index.

Other favourable chemopreventive qualities of EGCG include the effects on the maintenance of blood pressure homeostasis. The pathogenesis of hypertension represents and important risk factor for cardiovascular complications (Kitiyakara *et al.* 1998). An epidemiological study has shown an inverse relationship between consumption of black tea and systolic blood pressure (Tijburg *et al.* 1997). Also consumption of 1 mg of EGC and EGCG significantly lowered intraocular pressure in normotensive rabbits (Hodges *et al.* 1999). Furthermore, green tea catechins reduce renal hypertension, thus improving kidney function in rats (Yokozawa *et al.* 1994). Taken together these data are suggestive that green tea extracts are beneficial in the maintenance of normal blood pressure. However, Hodgson *et al.* found that drinking black or green tea at a dose equivalent to four standard cups, did not improve blood pressure in 20 normotensive humans subjects (Hodgson *et al.* 1999). In a second study, a cohort consisting of 13 subjects with high-normal systolic blood pressure and mild systolic hypertension (systolic blood pressure in the range of 130-150 mmHg), consumed either five cups of green tea or black tea, over 7 days. There was no significant change in systolic or diastolic blood pressure following consumption of green or black tea. A possible explanation to the variation between some of these findings may ostensibly be due to heterogeneity of individuals. This is because the pathogenesis of hypertension possesses a strong genetic component, with potentially many different altered gene products contributing to the disease (Hornstra *et al.* 1998).

Another potential benefit of tea consumption is the ability to act as an anti-inflammatory agent. Atherosclerosis is a disease with a strong inflammatory component (Ross *et al.* 1993). Studies in animal models show that green tea polyphenols decrease inflammation. Yang FJ *et al.* (1998) reported that mice fed an extract of green tea polyphenols had decreased TNFα production in response to an injection of LPS. Haqqi *et al.* (1999) reported that the ingestion of green tea polyphenol extract reduced joint disease in mice with adjuvant-induced arthritis. Crouvezier *et al.* (2001) investigated the effect of

EGCG, ECg, EGC and black tea extract on the production of pro- and anti-inflammatory cytokines in human leukocytes *in vitro*. ECg, EGC, and EGCG decreased the production of IL-1β, and enhanced the production of IL-10, but did not alter production of IL-6 or TNFα. Overall these data suggest an anti-inflammatory effect induced by tea-derived catechins. However, EGCG, *in vitro*, can also stimulate the release of IL-1 from monocytes (Tijburg *et al.* 1997) indicating a cell specific effect. Furthermore EGCG inhibits leukocyte transmigration through endothelial cell monolayers (Hofbauer *et al.* 1999), which is associated with the inflammation process in atherogenesis.

Several studies that have focussed on elucidating the anti-inflammatory mechanisms of tea catechins, have suggested that it is due to the inhibition of NF-κB. This is consistent with various studies, which have demonstrated that tea polyphenols inhibit NF-κB activation (Ahmad *et al.* 2000; Yang *et al.* 2001; Yang FJ *et al.* 1998; Kim *et al.* 2001). For example, EGCG (40-80 μM) lowered constitutive expression and activation of NF-κB in NHEK (normal human epidermal keratinocyte) cells, but at even lower levels (10 μM) effectively inhibited NF-κB expression in A431 cancer cells (Ahmad *et al.* 2000). In addition to effecting NF-κB expression, EGCG can act on up-stream regulators of the NF-κB pathway. Yang *et al.* (2001) have shown that EGCG blocks NF-κB activation by inhibiting IκB kinase activity in intestinal epithelial cell line IEC-6.

As well as impinging on the NF-κB pathway, tea flavonoids can also act on other important signalling pathways including the MAPK family of proteins. There have been many reports that demonstrate EGCG and related catechins can modulate ERK 1/2 activation (Ahn *et al.* 1999; Kovalenko *et al.* 1997; Heldin *et al.* 1999; Locher *et al.* 2002). Tea flavonoids can also modulate the JNK and p38 MAPK pathways. (Dong *et al.* 1997; Barthelman *et al.* 1998; Chen WX *et al.* 1999; Lu *et al.* 1998). Inhibition of NF-κB and/or MAPK pathways by tea flavonoids can influence vital cellular responses such as proliferation and/or apoptosis. Aberrant cellular proliferation and apoptosis are significant pathological changes associated with various stages of atherogenesis. A study by Locher *et al.* (2002) reported the effect of EGCG on the proliferation of VSMCs stimulated by native low-density lipoprotein (LDL). The group disclose that cellular proliferation was concentration-dependently inhibited by EGCG and a number of other polyphenolic EGCG derivatives. This effect may in part be due to inhibition of the ERK 1/2 pathway, which is typically recognised to be involved in aspects of cellular proliferation. Interestingly, Lu *et al.* (1998) describe the concentration-dependent inhibitory effect of EGCG (and other constituent polyphenols of tea), on the proliferation of a range of vascular smooth muscle

cells derived from dissimilar sources and also on human lymphoid type cells. This inhibitory effect was partly attributed to a decrease in protein tyrosine kinase activity, as well as a reduction in c-jun mRNA expression and inhibition of JNK1, but not ERK 1/2 or PKC activation.

EGCG can also affect other important mediators of cell growth and apoptosis. For example, EGCG has been shown to affect $p21^{waf1}$ (Liberto *et al.* 2000; Liang *et al.* 1999; Gupta *et al.* 2000; Bhatia *et al.* 2001), $p27^{kip1}$, cyclin dependent kinases 2 and 4, cyclins D and E (Liang *et al.* 1999; Bhatia *et al.* 2001), p53 (Liang *et al.* 1999, Gupta *et al.* 2000), and pRB (Liberto *et al.* 2000). Liang *et al.* (1999), have shown that EGCG (30 μM) induced a G_1 cell cycle arrest in asynchronous and synchronous MCF-7 breast carcinoma cells. This cell cycle arrest was accompanied with a change from hyper- to a hypophosphorylated pRB protein, a decrease in protein expression of cyclin D1 and E, suppression of CDK 2 and 4 activities, but an increase in $p21^{waf1}$, $p27^{kip1}$ and p53 expression. Apoptosis induced by EGCG has also been shown in a number of human carcinoma cells (Gupta *et al.* 2000; Ahmad *et al.* 1997; Bhatia *et al.* 2001). Induction of apoptosis may also be due to regulation of caspases, as black tea polyphenols induced apoptosis through cytochrome c release and activation of caspase-9 and 3 in human U937 cells (Pan *et al.* 2000a).

Both NF-κB and AP-1 (a down-stream target of the MAPK signalling pathways) have been found to regulate the expression of certain members of the matrix metalloproteinase family (MMP), (Chase *et al.* 2002; Bond *et al.* 2001). Tea flavonoids have been shown to inhibit the activity and expression of a number of MMPs. Some members of the MMP family have been implicated in atherogenesis. (Galis *et al.* 2002; Borden *et al.* 1997). Theaflavin and EGCG (25 μM) inhibited collagenases (probably MMP-2 and MMP-9) from mouse lung carcinoma cells (Sazuka *et al.* 1997). At significantly lower concentrations, which could be expected in plasma after tea consumption (IC_{50} < 0.1 μM), EGCG was also effective in reducing tumour invasion mediated by MMP activity (Demeule *et al.* 2000). It is likely that the gallate moiety was essential for MMP inhibition, as EGCG was the most potent inhibitor of MMP-2, -9 and –12 compared to EGC and EC.

1.8 NF-κB overview

NF-κB was first described 17 years ago by Sen and Baltimore (1986) as a B cell nuclear factor that bound a site in the immunoglobulin κ enhancer and was believed to be specifically expressed in B cells. It became apparent that NF-κB is in fact expressed in many cell types and thus not exclusive to B cells (as reviewed in Baldwin 1996). This pleiotropic transcription factor (TF) belongs to the Rel family of TFs and is involved in the activation of an exceptionally large number of genes in response to inflammation, viral and bacterial infections and other stressful situations requiring rapid reprogramming of gene expression. As well as their recognised role in inflammatory and immune type responses, NF-κB TFs also regulate several other important physiological processes, including developmental processes, cell growth, apoptosis, differentiation, redox metabolism and cell adhesion (as reviewed in Ghosh *et al.* 1998; Pahl *et al.* 1999). As NF-κB is activated by many external stimuli, it has not been clearly defined how these signals activate or indeed at what point their signalling pathways impinge on the NF-κB signalling pathway. However, several common features do exist for all of the major activation pathways studied (figure 1.6). In most normal quiescent or non-stimulated cells NF-κB is retained in the cytoplasm as an inactive form in close association with a sequestering complex consisting of any of several IκB proteins (IκBα, -β, -ε, -γ, p105, p100 and Bcl-3; as reviewed in Whiteside *et al.* 1997b). In response to a wide variety of stimuli, IκBα is phosphorylated by an upstream kinase complex known as IκB kinase (consisting of IKKα, IKKβ, IKKγ). NH_2-terminal phosphorylation (on serine 32 and 36 of IκBα) leads to the immediate recognition of the NH_2-terminus of IκBα by the recently identified β-TrCP-like F box-containing component of Skp-Cullin-F-Box (SCF)-type E3 ubiquitin-protein ligase complex (Yaron *et al.* 1998; Chen ZJ *et al.* 1995; Baldi *et al.* 1996; Maniatis *et al.* 1999). This consequently results in the polyubiquitination on lysines 21 and 22 (DiDonato *et al.* 1996) targeting IκBα for proteosomal degradation by the 26S proteosome. The degradation of IκBα liberates the NF-κB dimer (exposing the nuclear localisation sequence or NLS) allowing it to translocate to the nucleus. Translocation is thought to occur through interactions with proteins responsible for nucleocytoplasmic transport within a cell, known as karyopherins (Moroianu *et al.* 1998; Pemberton *et al.* 1998). Once in the nucleus NF-κB binds to consensus sequences on DNA, thus modulating the expression of specific genes. One of the target genes activated by NF-κB is that encoding IκBα. Newly

synthesised IκBα can enter the nucleus, remove NF-κB from the DNA, and export the complex back to the cytoplasm to restore the original latent state (Arenzana-Seisdedos *et al.* 1997).

There are, however, alternative pathways for activation of NF-κB that have been reported. For example UV radiation-induced degradation of IκBα (hence activation of NF-κB) appears to be independent of IKK activity, as serine 32, 36 or tyrosine 42 are not phosphorylated (Li *et al.* 1998). The mechanism of IκBα degradation in response to UV radiation is unknown. The second atypical activation pathway is a result of hypoxia or pervanadate treatment and is believed to require phosphorylation of IκBα at tyrosine 42 instead of serine 32 and 36 (Imbert *et al.* 1996). Phosphorylation of tyrosine 42 does not result in IκBα degradation. Again the exact mechanisms of activation behind this pathway are not known, although certain members of the Src family have been implicated.

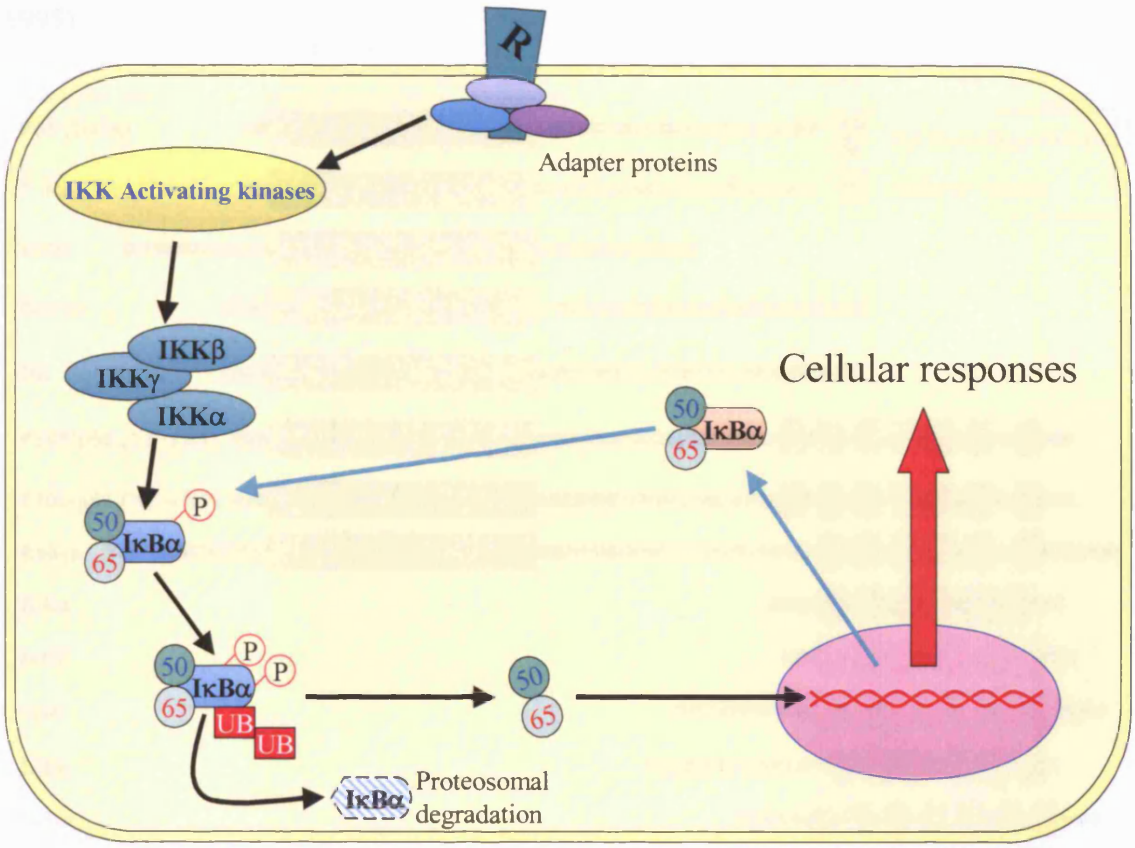

Figure 1.6 - Rel/NF-κB signal transduction. *Various signals converge on activation of the IκB kinase (IKK) complex. In many examples this occurs through receptor activation. IKK then phosphorylates IκB on serine residues 32 and 36, which targets it for ubiquitination and proteolysis. Liberated NF-κB (in this case p65 and p50) enters the nucleus and activates gene expression. One gene regulated by NF-κB is IκB. Newly synthesised IκB is thought to bind nuclear NF-κB, cause it to dissociate from the DNA and export it back out into the cytoplasm in an inactive state.*

1.8.1 The NF-κB, IκB and IKK proteins

There are presently five known members of the mammalian NF-κB/Rel family consisting of c-Rel, NF-κB1 (p50 and its precursor p105), NF-κB2 (p52 and its precursor p100), Rel A (p65) and Rel B (Baeuerle *et al.* 1994; Siebenlist *et al.* 1994; Miyamoto *et al.* 1995; Dejardin 1999). Each of these protein subunits contains the Rel homology domain (RHD; see figure 1.7) which functions in DNA binding, dimerisation, and interactions with IκB sequestering complex. Both NF-κB1 and NF-κB2 contain multiple copies of ankyrin repeats at their C-termini, which typically characterise the IκB forms. Processing of these subunits culminates in the formation of active p50 and p52 subunits. The p100 and p105 proteins do not function exclusively as precursor forms of p50 or p52, but also serve to regulate the activity of the other NF-κB subunits (as reviewed in Baldwin 1996; Dejardin 1995).

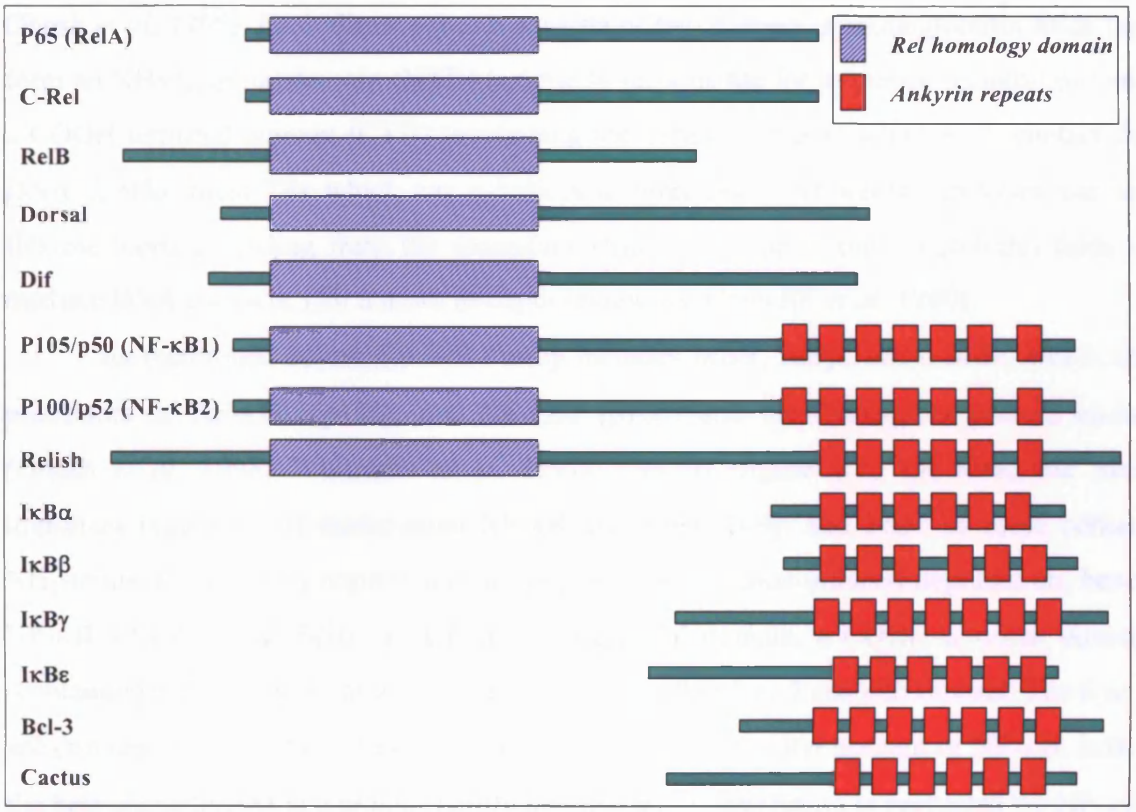

Figure 1.7 - The NF-κB and IκB families of proteins. *The NF-κB/Rel family is characterised by the presence of the Rel homology domain. The IκB proteins have multiple copies of the ankyrin repeat. NF-κB1 and 2 are proteins that contain both the Rel homology domain and ankyrin repeats. Dorsal, Dif, Cactus and Relish are Drosophila proteins demonstrating interspecies homology.*

Active or DNA-binding NF-κB, consists of a dimer. Classically this dimer is made up of p65 and p50 subunits. Other homo and/or hetero dimers are able to form, although combinations of certain dimers have not been demonstrated, for example RelB will only dimerise with p50 or p52. Each of the distinct dimers is able to bind to DNA with differing specificities and with different affinities. Consequently, this increases the ability of NF-κB to differentially regulate the expression of a plethora of genes. Classic NF-κB preferentially binds the sequence 5' GGGRNNYYCC 3', whereas the RelA/c-Rel dimer binds to a sequence 5' HGGARNYYCC 3' (H indicates A, C or T; R is purine; Y is pyrimidine) (Parry *et al.* 1994). Other factors contributing to NF-κB target gene specificity include differences in cell type expression of the various NF-κB subunits, differential subcellular localisation, and differential interactions with inhibitory IκB subunit complexes (Baeuerle *et al.* 1994).

The butterfly-like x-ray crystal structures of various forms of NF-κB bound to DNA have been revealed (Chen FE *et al.* 1998, Chen YQ *et al.* 1998; Cramer *et al.* 1997; Ghosh *et al.* 1995). Each dimer subunit consists of two β-sheet immunoglobulin folds that form an NH_2 terminal domain (NTD) (primarily responsible for sequence recognition) and a COOH terminal domain (CTD) (containing the dimer interface) which both contact the DNA. Unlike most TFs which use α-helices to bind DNA, NF-κB/Rel proteins use ten flexible loops extending from the secondary structure of these immunoglobulin folds to mediate DNA contacts. (for a more in-depth review see Chen FE *et al.* 1999).

As mentioned before the IκB family includes IκBα, IκBβ, IκBγ, IκBε, Bcl-3, the precursors of NF-κB1 (p105), and NF-κB2 (p100) and the Drosophila protein cactus (Ghosh *et al.* 1998; Whiteside *et al.* 1997a, 1997b) (figure 1.7). Of these the most important regulators of mammalian NF-κB are IκBα, IκBβ and IκBε as these contain NH_2-terminal regulatory regions that are required for stimulus-induced degradation, hence NF-κB activation. All IκBs contain an NH_2-terminal domain, a COOH-terminal domain (containing a PEST motif) and a core consisting of either 6 or 7 ankyrin repeats. The 6 or 7 ankyrin repeats form stacked helical domains that bind to the RH domain of NF-κB. IκBα, the best characterised is a unique family member as its expression is regulated by NF-κB. Newly synthesised IκBα enters the nucleus and binds to the COOH-terminal of the RH domains of NF-κB. This binding appears to disrupt the NF-κB/DNA association by masking the nuclear localisation signal of NF-κB. A nuclear export sequence on IκBα is

exposed on binding, and causes re-exportation of the NF-κB/IκB complex into the cytoplasm in an inactive state (Arenzana-Seisdedos *et al.* 1997).

The IκB-kinase (IKK) family of proteins are commonly accepted as the main kinases responsible for the phosphorylation of IκB. Typically this kinase signalsome consists of 3 proteins, namely IKK-1 (IKKα), IKK-2 (IKKβ) and IKKγ (NEMO, or IKKAP), although under certain conditions a fourth molecule, IKAP (IKK-complex associated protein) has been associated with the complex (DiDonato *et al.* 1997; Mercurio F. 1997). The actual IκB kinase activity resides in two subunits of the complex namely IKKα and IKKβ. IKKγ is an associated modulator of the other two subunits and is thought to confer a structural or stabilising function (DiDonato *et al.* 1997; Mercurio 1997). The function of IKAP has not been clearly elucidated. The IKKs share much structural similarity and can form homo- or heterodimers. Each subunit of IKK is phosphorylated in the activation domain on specific serine residues (and threonine for IKKγ) by upstream kinases including NF-κB inducing kinase (NIK), MEKK-1, receptor interacting kinase (RIP) and Interleukin-1R-activated kinase (IRAK) (as reviewed see Karin *et al.* 1999a, 1999b) and NF-κB activating kinase (NAK) (Tojima *et al.* 2000).

Some form of receptor-ligand binding activates these various kinases such as NIK. For example, TNFα on binding to the TNF receptor, induces receptor dimerisation and recruitment of TNF receptor-associated proteins like TNF receptor-associated death domain protein (TRADD), Fas-associated death domain protein (FADD), TNF receptor-associated factor 2 (TRAF2) (Rothe *et al.* 1995a), to its intracytoplasmic domain to generate intracellular signalling cascades such as activation of NIK, IKK, IκB and NF-κB.

1.8.2 NF-κB and Atherosclerosis

As mentioned before, the pleiotropic family of transcription factors NF-κB play an important role in the inducible regulation of a variety of genes involved in the inflammatory/immune and also proliferative responses of cells. There are several lines of evidence that suggest an involvement of NF-κB in atherogenesis. The NF-κB signal transduction pathway in murine aortic endothelial cells is primed for activation in regions predisposed to atherosclerotic lesion formation (Hajra *et al.* 2000). In humans activated NF-κB has been detected in the fibrotic-thickened intima/media and atheromatous areas of the atherosclerotic lesion (Brand *et al.* 1996; Wilson *et al.* 2002). NF-κB was found

activated in the SMCs, macrophages (Brand *et al.* 1996; Wilson *et al.* 2002) and foam cells (Wilson *et al.* 2002) of the atherosclerotic plaque, but also in endothelial cells in lesions with less advanced atherosclerosis (Brand *et al.* 1996). Little or no activated NF-κB was detected in vessels lacking atherosclerosis (Brand *et al.* 1996).

There are multiple mechanisms through which NF-κB may contribute to atherogenesis (figure 1.8).

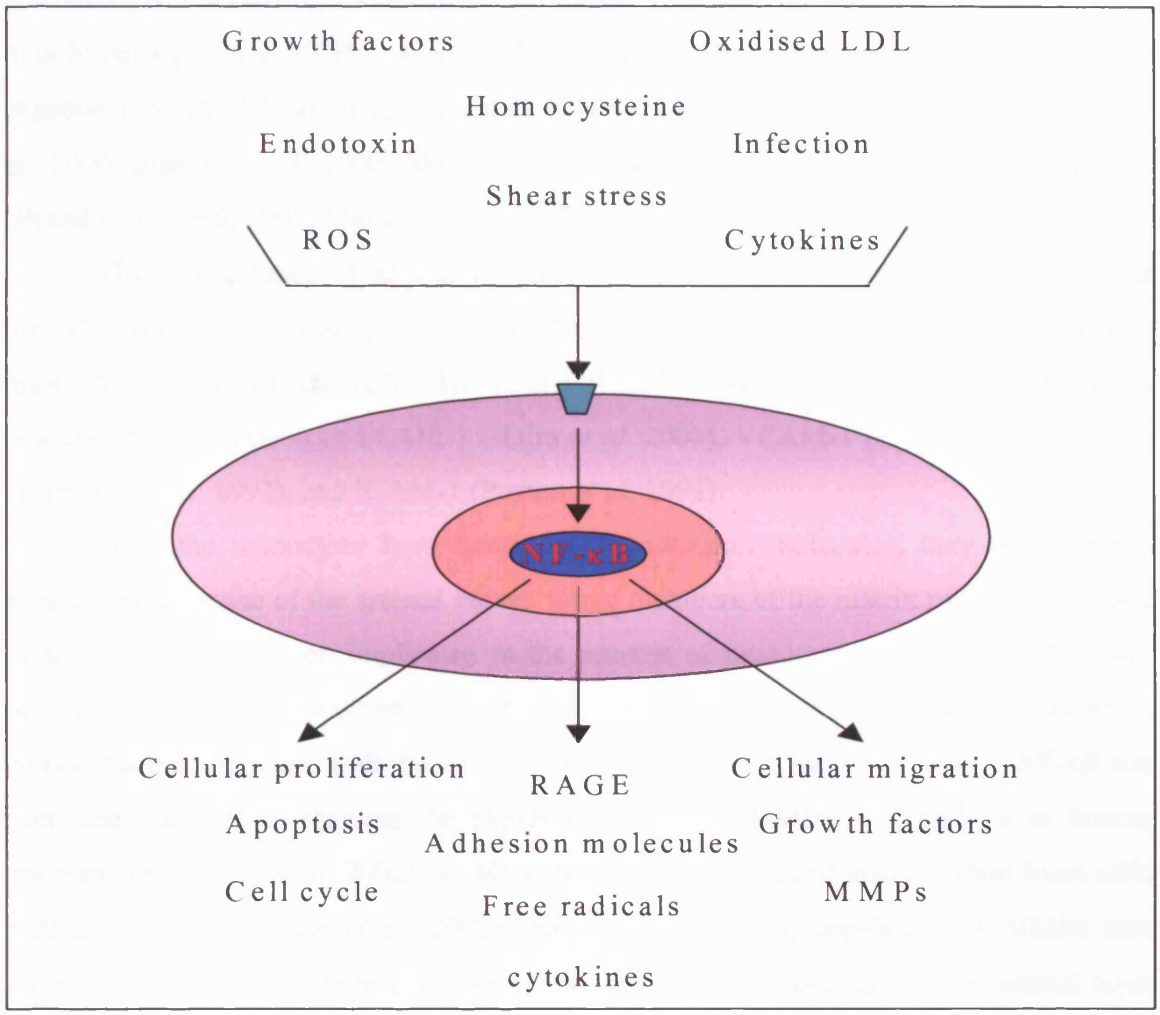

Figure 1.8 - NF-κB and atherogenesis. *This intracellular transcription factor activated by proatherogenic stimuli, is present in vascular cells, and is a central mediator regulating multiple atherogenic events.*

For example NF-κB may contribute to the initiation phases of atherosclerosis. Since it is activated by various proatherogenic factors including shear stress (Bhullar *et al.* 1998), homocysteine (Wang *et al.* 2000), vascular endothelial growth factor (Marumo *et al.* 1999), platelet derived growth factor (Marumo *et al.* 1997), fibroblast growth factor, tumour necrosis factor (Baeuerle *et al.* 1994), oxidised LDL (Parhami *et al.* 1993),

bacterial lipopolysaccharide (Muller *et al.* 1993), interleukin 1 (Ishikawa *et al.* 1999), viruses, and ROS. Activation of NF-κB by these proatherogenic factors culminates in the expression of genes demonstrated to play a role in atherogenesis. For example one of the early stages involved in plaque formation is the recruitment and adhesion of circulating monocytes and T and B lymphocytes. Monocyte chemoattractant protein (MCP-1) functions to recruit macrophages at the foci of endothelial damage. A wide variety of activated cells, including monocytes, fibroblasts, vascular endothelial cells, and smooth muscle cells produce MCP-1 *in vitro* (Rollins *et al.* 1996; Oppenheim *et al.* 1991). The expression of *MCP-1* can be up-regulated by NF-κB in a number of cell systems (Wang *et al.* 2000; Inadera *et al.* 2000; Marumo *et al.* 1999), and also in atherosclerotic lesions (Brand *et al.* 1996, 1997; Marumo *et al.* 1997).

The up-regulation of adhesion molecules is pivotal to the disease process allowing the adherence of circulating monocytes to injured endothelial cells. These adhesion molecules include ELAM-1, VCAM-1, and ICAM-1. Again NF-κB has been found to modulate the expression of ELAM-1 (Hajra *et al.* 2000), VCAM-1 (Cybulsky *et al.* 1991; Iademarco *et al.* 1992), and ICAM-1 (Poston *et al.* 1992).

Once the monocytes have bound to the adhesion molecules, they infiltrate the subendothelial space of the arterial vessel. Some members of the matrix metalloproteinase family (MMP) have been implicated in the process of monocyte infiltration. MMPs are secreted by cells involved in disease progression, which include VSMCs, monocytes/macrophages, endothelial cells and lymphocytes (Galis *et al.* 2002). NF-κB has been demonstrated to regulate the expression of genes *MMP-1*, *-3*, and *–9* in human macrophages (Chase *et al.* 2002), VSMCs (Bond *et al.* 2001), and also in rabbit foam cells produced *in vivo* (Chase *et al.* 2002). NF-κB-dependent up-regulation of MMPs also allows VSMCs and ECs to migrate across the internal elastic lamina into the intimal layer of the arterial vessel. Furthermore, proteinases participate in the proliferation of certain cell types including VSMCs (Dollery *et al.* 1995), a key part of the later stages of atherosclerosis.

In addition to monocyte adhesion, platelet aggregation at the foci of endothelial injury has also been associated with the early stages of atherogenesis. Evidence is accumulating to show that platelet activation exacerbates localised vascular inflammation, through the release of platelet proinflammatory mediators. Platelet activation modulates the activity of NF-κB in macrophages, consequently increasing production of IL-1β, IL-8 and MCP-1 (Neumann *et al.* 1997) thus enhancing the disease process.

As the plaque progresses up-regulation of various chemokines, including tissue factor (Wilcox *et al.* 1989), granulocyte macrophage colony stimulating factor (GM-CSF), TNFα (Baeuerle *et al.* 1994; Ross *et al.* 1993), IL-1 (Ross *et al.* 1993; Hiscott *et al.* 1993) and macrophage colony stimulating factor (M-CSF) (Baeuerle *et al.* 1994; Rosenfeld *et al.* 1992) is evident. Increased expression of these chemokines has been shown to be dependent on NF-κB, and is thought to enhance many of the processes involved in the intermediate stages of plaque formation. Also up-regulation of the receptor for advanced glycated end products (RAGE) has been shown to be dependent on NF-κB activation (Li JF *et al.* 1997). Advanced glycated end products are formed via the Maillard reaction and are the result of the non-enzymatic reaction between reducing sugars (such as glucose) with amino groups of proteins, lipids, and nucleic acids. AGE proteins are thought to promote atherogenesis by binding to their receptor, and bringing about a number of cellular responses. These range from induction of NF-κB (thus exacerbating the disease process), the generation of ROS and the up-regulation of VCAM-1 in endothelial cells (Schmidt AM *et al.* 1995). Subjects with elevated AGE proteins such as diabetics are thus predisposed to developing atherosclerosis.

Interestingly NF-κB has also been implicated in the later stages of the disease process. Many of the factors present in the atheroma, including TNFα, increased oxidative stress or oxidised LDL, activate NF-κB. NF-κB therefore serves to amplify and perpetuate the ongoing inflammation and matrix degradation within the plaque, with an increase in the release of cytokines, IL-1α and TNFα occurring. These cytokines lead to the persistent activation of NF-κB and/or the production of MMPs by VSMCs (Bond *et al.* 2001). These MMPs may result in fibrous cap weakening in the atherosclerotic plaque (Lee RT *et al.* 1997), resulting in various cardiovascular events such as vessel haemorrhaging, thrombosis and stroke. In conclusion activated NF-κB is present in numerous cell types in atheromatous plaques and offers an attractive chemopreventive and therapeutic target to provide an effectual strategy in combating and or alleviating the symptoms of disease.

1.9 Apoptosis

Apoptosis or programmed cell death is an important physiological process that plays an essential role in regulating cell number in tissue development and homeostasis. Moreover, apoptosis is also involved in a wide variety of pathological conditions ranging from neurodegenerative conditions, cancer, immunological diseases and also

cardiovascular disease. Apoptosis, an active process, was first described by Kerr *et al.* (1972), and is characterised by a number of typical morphological changes that occur in the dying cell. These range from membrane blebbing, nuclear fragmentation, chromatin condensation, loss of adhesion and rounding (in adherent cells), and cell shrinkage. Biochemical features associated with apoptosis can range from internucleosomal cleavage of DNA that culminates in oligonucleosomal 'laddering' (Cohen *et al.* 1994), phosphatidylserine externalisation (Martin *et al.* 1995a), and proteolytic cleavage of a number of intracellular substrates (Martin *et al.* 1995b).

These morphological and biochemical changes are elicited by a broad range of physiological or experimentally applied death stimuli and are observed in cells from diverse tissue types and species. This is consistent with the idea that independent signalling pathways converge on communal death-effector machinery that is evolutionarily conserved. This death-effector machinery typically consists of a family of cysteine proteases known as caspases. Cysteine protease activity can be detected in all cells undergoing apoptosis, regardless of their origin or the death stimulus, thus demonstrating the significance of caspases. Common to many of these death-signalling pathways is the involvement of the NF-κB signalling pathway. Section 1.9.1 describes how NF-κB may impinge on cell-death signalling pathways. However it is important to realise that apoptosis can occur independently of NF-κB.

1.9.1 NF-κB and Apoptosis

NF-κB can be pro-apoptotic, or also anti-apoptotic. The decision as to whether NF-κB is pro- or anti- apoptotic is dependent upon the specific cell type in which it is expressed and also the type of external stimulus. The first indication that NF-κB was involved in suppression of cell death came from studies on transgenic mice, which were deficient for the p65 (RelA) subunit of NF-κB. These mice died at embryonic day 15 and showed a massive degeneration of liver cells as a result of extensive apoptosis (Beg *et al.* 1995). *IKK-β* gene knockout mice and *IKK-β/IKK-α* double knockout mice die as embryos due to widespread hepatocyte apoptosis (Li *et al.* 1999; Tanaka *et al.* 1999). Male mice with an inactivated X-linked gene encoding IKK-γ/NEMO, die in mid gestation because of massive apoptosis of cortical and medulla lymphocytes in the thymus, and also degeneration of the liver (Makris *et al.* 2000; Rosenfeld *et al.* 2000). Interestingly cross breeding of RelA or IKK-β gene knockout mice with TNF-R1 or TNFα gene knockout

mice revealed partial rescue from embryonic lethality, suggesting a role for TNFα induction of cell death in the absence of NF-κB (Rosenfeld *et al.* 2000; Doi *et al.* 1999).

EBV infection induces B-lymphocyte proliferation and growth transformation (Grossman *et al.* 1994). Studies have shown that NF-κB inhibition causes spontaneous apoptosis in Epstein-Barr virus (EBV) transformed lymphoblastoid cells (Cahir-McFarland *et al.* 2000), thus demonstrating an important anti-apoptotic effect. In addition, constitutive NF-κB activation has been observed in certain proliferative-associated diseases such as Hodgkin's disease tumour cells, breast tumour cells (Sovak *et al.* 1997; Bargou *et al.* 1997) and in particular cells involved in atherogenesis, such as VSMCs (Dollery *et al.* 1995).

In support of a pro-apoptotic effect for NF-κB, is a report by Marianneau *et al.* (1997). In this report, Dengue-virus-infected HepG2 hepatocytes were rescued from apoptosis following the expression of NF-κB decoy oligonucleotides (double stranded NF-κB binding sites) implicating the role of NF-κB in virally induced apoptosis. Also several studies have shown that NF-κB promotes apoptosis in a variety of *in vitro* neuronal cell injury models (Clemens *et al.* 1997, 1998; Grilli *et al.* 1996; Grimm *et al.* 1996; Marinovich *et al.* 1996). Recently, two reports demonstrating NF-κB as a pro-apoptotic inducer in animal models have been reported. In one study, Qin *et al.* (1999) examined the effect of NF-κB in N-methyl-D-aspartate (NMDA) receptor-induced apoptosis of rat striatal mediumspiny neurons (which is an excitotoxic Huntington's disease model). Administration of the excitotoxin quinolic acid, which activates the NMDA receptor and induces apoptosis in the rat striatum, caused increased nuclear translocation of an NF-κB complex that contained the RelA and c-Rel subunits. This induction of apoptosis was attributed to the NF-κB dependent up-regulation of two death-promoting genes, namely *c-myc* and *p53*. The second study by Schneider *et al.* (1999) used a transgenic mouse containing a chimeric β-globulin gene that was under direct control by NF-κB (this acted as a model for strokes). Following induction of focal cerebral ischemia in these mice, there was an increase in NF-κB translocation and DNA binding (p50/RelA and p50/p50) and hence reporter gene transcription. Also, p50 knockout mice had significantly reduced ischaemic damage as compared to wild-type mice, suggesting that the absence of NF-κB provided protection from neuronal cell death.

The mechanisms behind apoptosis have been extensively studied (as reviewed in Hengartner *et al.* 2000; Chen *et al.* 2001). It is now believed that there are two main pathways leading to apoptosis, namely the intrinsic or mitochondrial mediated and

extrinsic or receptor-mediated pathways (figure 1.9). NF-κB is central to both the intrinsic and extrinsic pathways and regulates the activity of many of the apoptotic mediators. In addition, both pathways rely on the activation of a family of cysteine proteases, known as caspases, which cleave target proteins at aspartic acid residues. Caspases exist as proenzymes in an inactive state, and are dependent on proteolytic cleavage and subsequent molecular reorganisation to become active (Stennicke and Salveson, 1999).

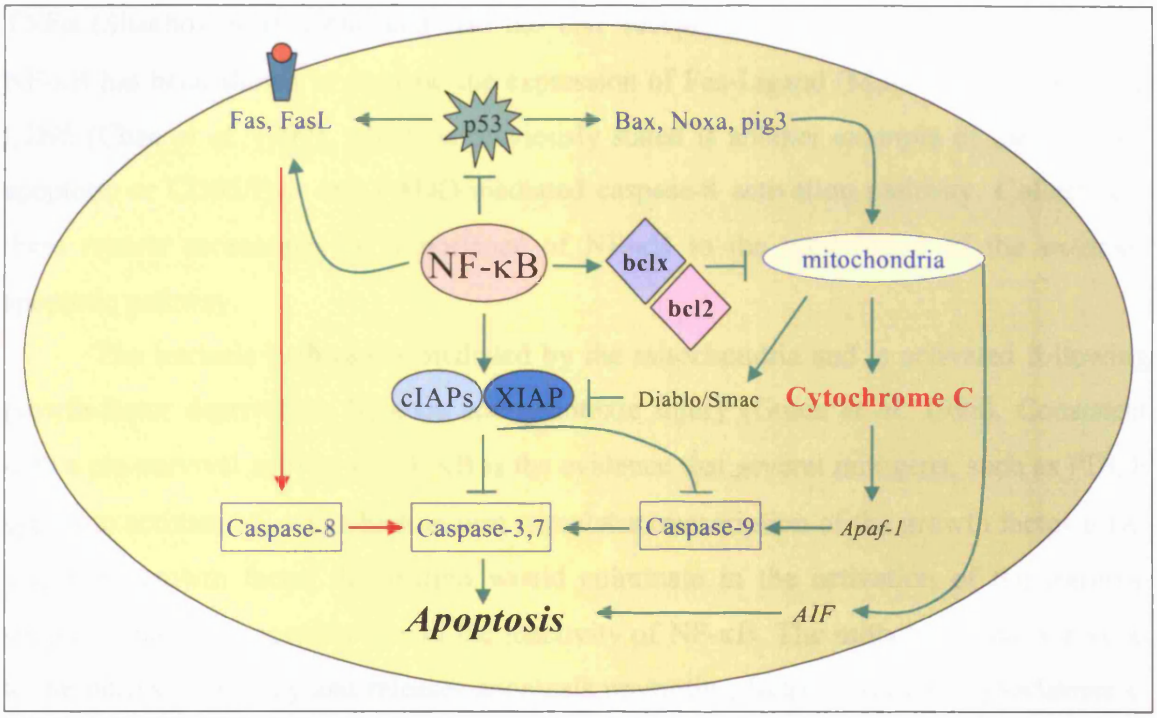

Figure 1.9- NF-κB is a central mediator of both extrinsic (red arrows) and intrinsic (green arrows) pathways leading to apoptosis. *Initiator caspases (8 and 9) can be activated by either ligand binding to the death receptor complex or by cytochrome c released from the mitochondria. Initiator caspases act on downstream effecter caspases (3 and 7), which commit the cell to apoptosis. NF-κB can function to block apoptosis via up-regulation of IAPs and/or Bcl-Xl, and/or inhibition of p53 function. (adapted from Chen et al. 2001).*

The extrinsic pathway is triggered as a result of ligand binding to its respective death domain associated receptors (TNF receptor 1, FAS-Ligand receptor/CD95). A typical example of a death domain-associated receptor is the TNFα receptor. On binding TNFα, aggregation of TNFα receptors and hence cytoplasmic death domains (DD) takes place (Hsu *et al.* 1995). Receptor oligomerisation culminates in the recruitment of adaptor molecules including TNF receptor associated death domain (TRADD) and Fas-associated death domain (FADD), to the DD of the receptor (Hsu *et al.* 1995). The recruitment of these adaptor proteins allows for the binding of initiator caspases, such as caspase 8 and 10

(Boldin *et al.* 1996; Fernandes-Alnemri *et al.* 1996). Aggregation in these crowded death-inducing signalling complexes (DISCs), and the low intrinsic protease activity of pro-caspase 8, may allow sufficient intramolecular processing so as to release active caspase (Muzio *et al.* 1998). Active caspase 8 then functions to cleave and thus activate downstream pro-caspases to produce executioner caspases such as 3, 6 and 7 (Grutter *et al.* 2000), which go on to bring about the morphological changes associated with apoptosis (as reviewed in Hengartner *et al.* 2000). NF-κB has been shown to regulate the expression of TNFα (Shakhov *et al.* 1990) and also the TNF receptor (Santee *et al.* 1996). Moreover, NF-κB has been shown to regulate the expression of Fas-Ligand (Matsui *et al.*1998), and CD95 (Chan *et al.* 1999), which as previously stated is another example of the extrinsic apoptotic or CD95/Fas- and FADD-mediated caspase-8 activation pathway. Collectively these reports accentuate the importance of NF-κB to the modulation of the extrinsic apoptotic pathway.

The intrinsic pathway is mediated by the mitochondria and is activated following growth-factor deprivation, hypoxia and genotoxic injury (Green *et al.* 1998). Consistent with a pro-survival activity of NF-κB is the evidence that several mitogens, such as PDGF appear to activate NF-κB, which in turn stimulates transcription of the growth factor gene. Therefore growth factor deprivation would culminate in the activation of the intrinsic apoptotic pathway, possibly due to the inactivity of NF-κB. The mitochondrion is pivotal to the intrinsic pathway and releases apoptosis promoting factors including cytochrome c, apoptosis inducing factor (AIF) and Diablo/Smac (De Laurenzi *et al.* 2000; Verhagen *et al.* 2000; Susin *et al.* 1999). Cytochrome c binds to the caspase activating protein, apoptotic protease activating factor-1 (Apaf-1), which then activates caspase-9 (Susin *et al.* 1999), and forms the complex known as the apoptosome (Zimmermann *et al.* 2001). Caspase-9 as part of the apoptosome, exerts an effect through activation of executioner caspases, such as caspase 3. AIF appears to bring about apoptosis in a caspase-independent manner (Lorenzo *et al.* 1999).

Important to the mitochondrial mediated apoptotic pathway is the Bcl-2 family of proteins. Subcellular localisation studies have shown that Bcl-2 and Bcl-Xl reside on the mitochondrial outer membrane and act in anti-apoptotic function (other anti-apoptotic members include Bcl-w, Bfl-1and Bcl-B). These anti-apoptotic members work to prevent cytochrome c release from the mitochondria, and thereby preserve cell survival (Kluck *et al.*1997). The pro-apoptotic members of the family including Bax, Bid, Bim, Bak, and Bad translocate to the mitochondria and bring about the release of cytochrome c (as reviewed in

Zimmermann *et al.* 2001). Some of the Bcl-2 family members such as Bfl1, Bcl-Xl, Nr13 and Bcl-2, have been revealed to be targets of NF-κB (Grumont *et al.* 1999; Chen FE *et al.* 1999; Lee HH *et al.* 1999; Lee FYJ *et al.* 1999; Mattson *et al.* 2001). Bfl1 overexpression can rescue cells deficient in c-Rel (Grumont *et al.* 1998) or IκBα super-repressor-expressing cells (Zong *et al.* 1999) from apoptosis induced by TNFα or antigen receptor ligation. Overexpression of Bcl-Xl also rescues IκBα super-repressor-expressing cells from TNF-α-induced apoptosis (Chen CL *et al.* 2000). The regulation of Bcl-2 family members by NF-κB also extends to pro-apoptotic member Bax (Bentires-Aij *et al.* 2001). Bax expression is enhanced in certain cells that express the IκBα-super-repressor and overexpression of NF-κB inhibits p53 stimulated *Bax* promoter activity (Bentires-Aij *et al.* 2001). Collectively these reports thus emphasise the significance of NF-κB in the intrinsic apoptotic pathway (also extrinsic through TNFα signal-mediated pathway).

The inhibitor of apoptosis (IAP) family, consist of a group of structurally related proteins that regulate apoptosis induced by a range of stimuli. Initially identified in baculovirus, IAPs are highly conserved through different species and contain a number of characteristic regions or motifs of homology. Typically IAP proteins possess two conserved motifs, the baculovirus IAP repeat (BIR) and the RING domain. The BIR domain consisting of a 70 amino-acid residue zinc-binding region is essential for the anti-apoptotic function of the IAPs and has been linked to the binding of and inhibition of caspases. At present eight human IAPs have been identified consisting of survivin (TIAP), apollon (Bruce), c-IAP1 (HIAP2, MIHB), c-IAP2 (HIAP1, MIHC), ML-IAP (Livin, KIAP) ILP-IAP (Ts-IAP), NAIP and XIAP. In the intrinsic pathway, Smac/Diablo released from the mitochondria binds to IAP family members and neutralises their anti-apoptotic activity, allowing initiation of caspase-dependent apoptosis (Du *et al.* 2000).

As well as Smac/Diablo interaction with IAPs, NF-κB also interacts with IAP family members through transcriptional regulation. There has been much work involved in elucidating the mechanisms behind IAP regulation. It has been acknowledged that there are at least three forms of regulatory control, transcriptional/post-transcriptional, regulation of stability and control of IAP activity by further regulatory proteins. Transcriptional control or expression of IAPs is thought to be under tight control. For example, the expression of survivin (an IAP family member) in HeLa cells is up-regulated during the G2/M phase of the cell cycle via NF-κB (Ghosh *et al.* 1998). In Jurkat cells, TNFα has been shown to up-regulate c-IAP2 where c-IAP2 protection from TNFα-induced apoptosis was dependent on

the presence of NF-κB (Chu *et al.* 1997). A more recent study on endothelial cells revealed the up-regulation of a number of IAPs following TNFα exposure (Stehlik *et al.* 1998). In this report, the authors provide evidence to suggest that NF-κB modulates IAP induction. This was concluded as overexpression of the endogenous cytoplasmic NF-κB inhibitor, IκB, totally suppressed IAP induction and sensitised the cells to apoptosis. This effect was reversed following the overexpression of XIAP, which restored the cytoprotective effect of the endogenous IAPs. Further to this study, a report by You *et al.* (1997) using a temperature activated v-Rel protein, demonstrated the ability to control the induction of endogenous ch-IAP1, and hence protect the cells from apoptosis. Therefore IAP expression by NF-κB or NF-κB activating agents accentuates a positive feedback mechanism whereby the c-IAPs (through their interaction with TRAF1 and 2 or through the reported interaction with RIP2) or XIAP (through its interaction with TAK1; Hofer-Warbinek *et al.* 2000) can potentiate the activation of NF-κB, which sequentially leads to increased levels of c-IAP2 or XIAP (Hofer-Warbinek *et al.* 2000) expression, reinforcing the anti-apoptotic effects of IAPs. In contrast, the zinc finger inhibitor of apoptosis A20 that is induced by NF-κB, acts as a negative regulator of NF-κB activation (Song *et al.* 1996).

The 57-kDa protein XIAP is most potent at inhibiting apoptosis triggered by a range of apoptotic stimuli. As previously mentioned its protective effects have been largely attributed to the direct inhibition of caspase function. XIAP binds to caspase 3, 7 and 9 through the interaction with its BIR motifs. Once bound to these different effector caspases, steric occlusion of the normal substrates of these caspases occurs, which prevents the propagation of the apoptotic signal. However, as stated before, IAPs inhibit apoptosis by non-caspase interactions mainly by modulating NF-κB and also to a lesser extent by JNK1. In actual fact c-IAP1 and 2 were first identified by virtue of their interaction with TRAF2 (Rothe *et al.* 1995b). Although this study was unable to demonstrate any cytoprotective role from this interaction, it suggested that IAP apoptotic suppression might be mediated in part through modulation of TNFα signalling cascade. With this in mind, the TNFα signalling cascade can be both pro- and anti-apoptotic, with the latter being mediated mainly through NF-κB modulation. Collectively these reports demonstrate the importance of NF-κB in the regulation of IAP gene expression. Given that IAPs modulate the activity of a number of caspases, it is clear that IAPs impinge on both the extrinsic and intrinsic apoptotic pathways.

The tumour suppressor, p53, has been considered one of the major contributors to apoptosis in response to a variety of stress inducers. The protein p53 is able to control the expression of many genes involved in reactive oxygen species synthesis or metabolism which contribute to the apoptotic process, including quinone oxidoreductase homologue (Pig 3), proline oxidase (Pig 6) homologue and glutathione peroxidase (GPx) (Polyak *et al.* 1997). In addition, p53 also regulates the expression of genes that directly control the process of apoptosis. These include eif24 (Pig 8) (Gu *et al.* 2000), Noxa (Oda *et al.* 2000), Fas, FasL and, as mentioned previously, Bax (Amundson *et al.* 1998). Positive regulation of Fas and FasL leads to the activation of the extrinsic pathway culminating in apoptosis, via caspase dependent mechanisms. The up-regulation of Bax facilitates the release of cytochrome c and triggers the cascade of events of the intrinsic apoptotic pathway. In addition p53 functions to induce growth arrest in part by enhancing the expression of $p21^{wafl}$, a cyclin dependent kinase inhibitor. Increased expression of $p21^{wafl}$ by p53 leads to either a G1 or G2/M cell cycle arrest or apoptosis (El-Diery *et al.* 1994; Bunz *et al.* 1998). Studies have demonstrated that p53 is a transcriptional target of NF-κB (Wu *et al.* 1994) and that expression is partially dependent on NF-κB activity (Kirch *et al.* 1999). This again emphasises the importance of NF-κB in the regulation of apoptosis to both the intrinsic and extrinsic pathways.

1.10 Cell Cycle

The control of cellular proliferation is achieved through regulation of the cell cycle, of which there are typically four phases. These phases are made up of an S (synthesis), M (mitotic), G_1 (gap1) and a G_2 (gap 2) phase, that can be differentiated on the basis of biochemical and morphological features. The biochemical characteristic of the S phase is the replication of nuclear DNA and thus doubling of the genetic information. In the M phase, division of the chromosomes between daughter cells is carried out. The G_1 phase occurs between the M through to S phase, whereas G_2 covers the period between S through to M phase. From the G_1 phase, there is also an out of cycle phase, in which the cell remains in a quiescent state known as G_0 phase (figure 1.10). Appropriate signals such as presence of various growth factors, can induce the cell to return from G_0 into G_1 phase and thus proceed through the cell cycle.

The various cell cycle events are highly coordinated to occur in a defined order with exact timing, requiring precise control mechanisms. Control of the cell cycle is due to

extrinsic and also intrinsic mechanisms. Extrinsic control mechanisms are determined by growth conditions such as nutrient supply. A cell may undergo arrest of the cell cycle if external ambient physiological conditions are not favourable. Intrinsic control mechanisms ensure that the cell cycle is executed completely, to allow both daughter cells to have a complete and identical genetic composition. For example mechanisms exist that check completion of S phase, consequently then allowing mitosis to take place i.e. entry into M phase. Another control mechanism ensures that a cell only enters S phase if preceded by mitosis. A further control mechanism exists that monitors whether the cells in G1 phase are large enough and have reached a critical size, to initiate another round of cell division. A final mechanism monitors DNA integrity or damage which can occur to the cellular genome via internal or external signals. If the DNA is damaged the cell cycle is arrested allowing for restoration to take place via various repair enzymes. If the damage is rectified then the cell can re-enter the cell cycle and division can take place. However if the damage remains then a cell may undergo apoptosis.

Both intrinsic and extrinsic control mechanisms are dependent on the coordinated synthesis, activation, and degradation of a family of proteins known as the cyclins that act as catalytic subunits for their respective kinases, known as cyclin dependent kinases (CDKs). Activity is further modulated by kinases and phosphatases that act to phosphorylate and dephosphorylate CDK, respectively. Furthermore, CDK activity is also modulated by association with one of a number of specific inhibitors (CDKI) or cell cycle checkpoint proteins, including $p21^{waf1}$, $p27^{kip1,85}$, and growth arrest and DNA-damage protein 45 (GADD45). Section 1.10.1 draws attention to the involvement of NF-κB in the regulation of some of these cell cycle regulatory proteins (section 1.10.1), and hence how NF-κB may regulate progression of the cell cycle.

1.10.1 NF-κB and proliferation

Evidence that NF-κB is involved in cell cycle progression has come from many independent studies. One such study showed that NF-κB activity was elevated during the G0 to G1 cell cycle transition in mouse fibroblasts (Baldwin *et al.* 1991). NF-κB activation was also required for cell cycling in other cell types, including regenerating liver cells, and oestrogen receptor-negative breast cancer cells (Baldwin *et al.* 1991; Cressman *et al.* 1994; Duckett *et al.* 1995; Biswas *et al.* 2000). NF-κB has been found to directly interact with cell cycle regulatory proteins. For example, the INK4 CDK inhibitors, which cause cell

cycle arrest by inhibiting CDK4 and 6, can also bind to and inhibit NF-κB through their ankyrin repeats. Conversely, overexpression of the unrelated CDK inhibitor p27 increases transactivation by NF-κB (Wolff *et al.* 1999).

As mentioned previously the cell is subject to a number of external controls. Information about the external environment is translated through to the nucleus via signalling pathways. Mitogenic signals in the form of growth factors bind to specific receptors and initiate a cascade of events that influences cell cycle progression. These external control mechanisms monitor growth factor/nutrient availability and can stop or promote cell division in G_1 (represented by 'R' on figure 1.10), depending on physiological conditions.

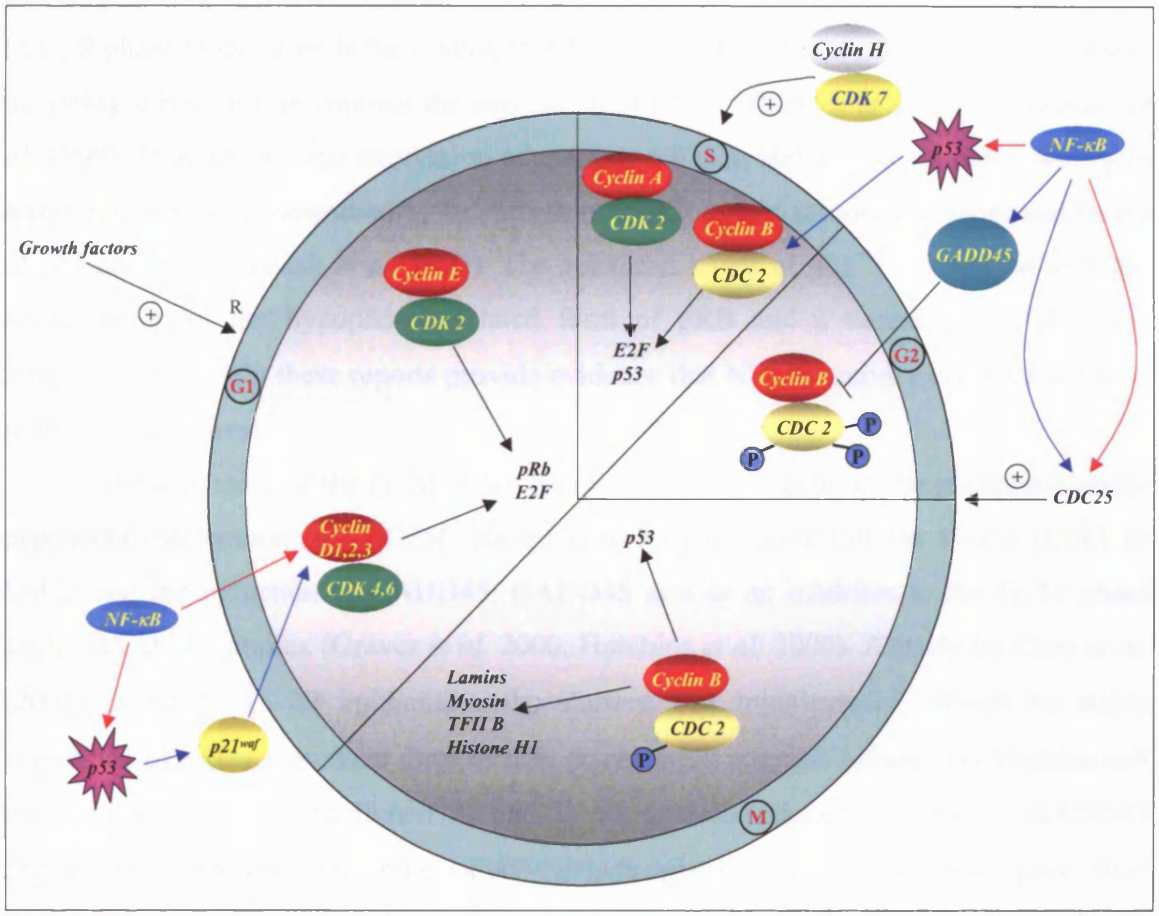

Figure 1.10 - An overview of the four phases of the eukaryotic cell cycle, indicating the main CDKs and respective cyclin-substrates. *The black arrows represent activation and phosphorylation, and the red or blue arrows represent positive or negative transcriptional regulation, respectively. R represents a restriction point. (Adapted from Biochemistry of signal transduction and regulation, second edition, by Gerhard Krauss, published by Wiley-VCH, 2001).*

In favourable conditions, cell progression from G1 to S phase is dependent on p53 activity, which governs the expression of the CDKI $p21^{wafl}$ (Sandor *et al.* 2000; Jimenez *et al.* 1999). Down-regulation of $p21^{wafl}$ allows the association of D-type cyclins with CDK4 and 6, which bring about G_1/S phase transition through phosphorylation of pRB. Phosphorylation of pRB results in the release of E2F, which is required for activation of S phase-specific genes (Nevins *et al.* 1992; Weinberg *et al.* 1995).

Two NF-κB binding sites have been found in the human cyclin D1 promoter sequence, providing direct evidence for the involvement of NF-κB in cell cycle transition (Hinz *et al.* 1999; Guttridge *et al.* 1999). In both studies, inhibition of NF-κB by over-expression of an IκBα super-repressor led to delayed cell cycle progression, and this effect could be rescued by ectopic expression of cyclin D1. Further to the involvement of NF-κB in G_1/S phase progression is the finding that NF-κB regulates the expression of p53 (Wu *et al.* 1994), which in turn controls the expression of $p21^{wafl}$ (Sandor *et al.* 2000; Jimenez *et al.* 1999). In addition, over expression of chicken c-Rel in HeLa cells caused a cell cycle arrest, which was accompanied by a decrease in CDK2 kinase activity and increased levels of p53 and $p21^{wafl}$ (Bash *et al.* 1997). The enhanced levels of $p21^{wafl}$ correlated with the accumulation of the hypophosphorylated form of pRB and a decrease in E2F DNA binding. Collectively these reports provide evidence that NF-κB activity can be associated with cell cycle arrest.

The activation of the G_2/M phase checkpoint is dependent on the phosphorylation-dependent inactivation of CDC25C phosphatase by checkpoint kinases 1 or 2 (Chk1 or Chk2) and the induction of GADD45. GADD45 acts as an inhibitor to the G_2/M phase cyclin B/CDC2 complex (Graves *et al.* 2000; Hutchins *et al.* 2000). A study by Chen *et al.* (2001), using BEAS-2B epithelial cells, showed that inhibition of NF-κB by stable expression of a kinase mutated form of IKK-β, enhanced arsenite-induced G_2/M phase cell cycle arrest. This was attributed in part to an arsenite-induced increase in GADD45 protein, which was due to the relief of NF-κB-induced suppression of GADD45 gene. Also this G_2/M phase arrest may have been due to regulation of CDC25 family members. Arsenite was found to induce *de novo* CDC25A expression, but markedly reduced the levels of CDC25B and CDC25C proteins, two phosphatases that activate CDC2/cyclin B complex.

The status of NF-κB activity was also a determining factor for vanadate-induced cell cycle arrest. In normal epithelial cells vanadate had less effect on cell cycle transition. However, in the cells where NF-κB activation was blocked, vanadate displayed a

significant G2/M phase cell cycle arresting effect (as reviewed in Chen *et al.* 2001). Indirectly, NF-κB can potentially affect the expression and activity of the CDK1. This is due to the finding that NF-κB can modulate the expression of p53 (Wu *et al.* 1994), which in turn has been shown to reduce the both tyrosine (Taylor *et al.* 1999, Azzam *et al.*1997, Passalaris *et al.* 1999) and threonine (Schneider *et al.* 1998) phosphorylation of CDK1. Furthermore there have been several reports in the literature that demonstrate p53-mediated repression of the CDK1 gene (Taylor *et al.* 1999; Yun *et al.* 1999). Reported by a number of groups is the involvement of $p21^{waf1}$ in the p53 mediated repression of CDK1 gene transcription, hence demonstrating another potential point at which NF-κB may indirectly affect cell cycle transition (Chang *et al.* 2000, Taylor *et al.* 2001).

Another protein important to the regulation of the cell cycle is Pin1. Although there is no evidence to suggest a direct interaction with NF-κB, Pin1 can regulate the expression, activity and stability of various pivotal cell cycle regulatory proteins, that interact with NF-κB including CDC25C (Crenshaw *et al.* 1998), cyclin D1 (Wulf *et al.* 2001), and p53 (Zheng *et al.* 2002; Zacchi *et al.* 2002).

1.11 Microarray

Cells have to respond appropriately to ambient factors in their surroundings to allow for the preservation of a homeostatic environment. This regulation is achieved by modulating the active working components of the cellular machinery, which occur through specific changes in the activity of their constituent proteins. These changes are achieved through either post-translational protein modifications or through stoichiometric changes in protein levels. The latter changes occur through selective modification in the rate of gene transcription and/or translation. It is in identifying these changes in gene transcription that the technique of microarray can be employed.

Since the recent advent of microarray the applications of this technique have been shown to be immense. It permits the study of temporal and spatial changes in expression of a plethora of genes in response to various treatments, time points and concentrations, instead of attempting to examine putative gene targets one by one. This can be useful in assigning genes or gene clusters to specific biological events and for analysing their interactions in a biological system, which may be useful in disease investigation. The development of this technique promises to enhance our understanding, and provide an insight into the complex cellular functions that subsist inside cells.

The methodology behind microarray is represented as in figure 1.11. In theory, microarrays work on the reverse principle to Northern blotting. A cDNA array is comprised of many micron sized gene specific polynucleotides that are spotted or arrayed on to a glass slide. Total cellular RNA is purified from a test and control cell line and converted to fluorescently labelled cDNA by RT-PCR. The control cDNA is labelled with one fluorophore (usually Cyanine3-dUTP, a red colour) and the test cDNA is labelled with another (usually Cyanine5-dUTP, a green colour). The control and test cDNA are then mixed and allowed to hybridise to the array slide overnight. The array slide is then washed before being scanned with a dual laser scanner. The fluorescent signals from the two fluorophores are detected and integrated to give an overall image representing the gene expression profile of the experiment. A gene that is equally expressed in both control and test shows up as a yellow colour (an equal mixture of the two fluorophores). Conversely, a gene that is either elevated or inhibited by the test compound, take on the colour of the test or control fluorophore whichever is in excess.

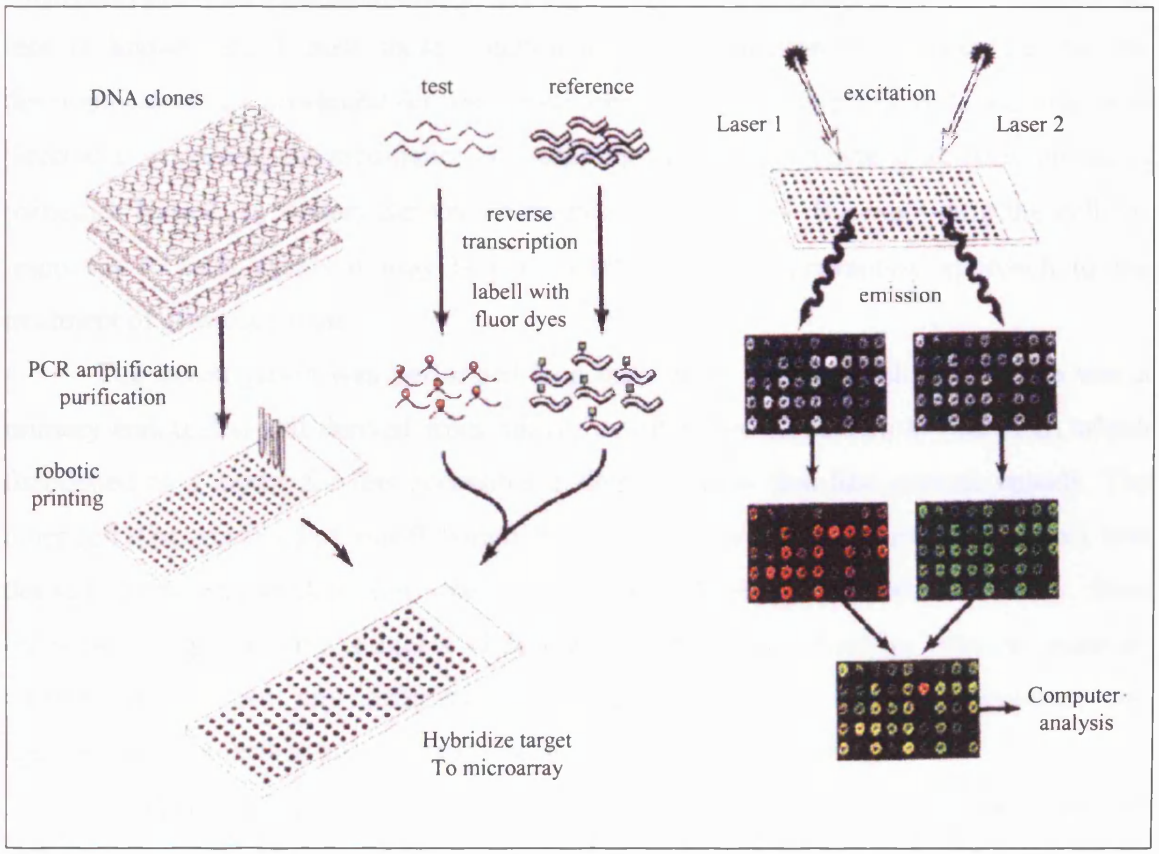

Figure 1.11 - Scheme of cDNA microarray method *(adapted from Duggan et al. 1999)*

When considering heart disease, this technique may be beneficial for a number of reasons. Firstly, it may allow for the development of a more global comprehension of the gene expression abnormalities that predispose to the development of atherosclerosis. This may help to identify potential novel molecular targets for therapeutic intervention. Also this technique would permit a better understanding of the molecular mode of action of potential cardiovascular chemopreventive agents. Moreover, it may allow for the prediction of potential side effects during preclinical and toxicological development of chemopreventive agents. Finally it may allow for the characterisation of patients most likely to benefit from the chemopreventive/therapeutic agent, thus optimising treatment strategies for future patients.

1.12 Thesis Aims

Epidemiological and experimental studies have provided some understanding of the chemopreventive actions/properties of dietary compounds in relation to cancer. However, less is known about how these compounds might function to protect against the development of cardiovascular disease. Therefore, the overall objective of this project was directed at examining the modulation of signalling pathways germane to atherosclerosis, in particular NF-κB, by dietary derived chemopreventive agents. By examining the cellular responses to such agents it may help to identify a novel preventive approach to the treatment of atherosclerosis.

The investigation was performed in a number of different cell types. One was a primary endothelial cell derived from human umbilical veins, known as HUVEC, which functioned as a model for less accessible endothelial cells that line arterial vessels. The other cell type consisted of four B-lymphoblastoid cell lines (immortalised with EBV), two derived from independent normotensive subjects (C143 and C149) and two from independent hypertensive subjects (H308 and H242). The intention was to examine whether there were any differences in response, between the normotensive and hypertensive (subjects at an increased risk to atherosclerosis) cell lines.

Another aim was to evaluate whether these more easily obtainable lymphoblastoid cell lines, would make a suitable surrogate for aortic endothelial cells. This would be ascertained by comparing their responses with the aortic endothelial cell model, HUVEC. Consequently this may help when evaluating future chemopreventive efficacy.

To accomplish the overall aims, a number of individual objectives were carried out.

1.12.1 Objectives

- The first objective was to evaluate the effect of four chemopreventive agents namely resveratrol, curcumin, indole-3-carbinol and epigallocatechin 3-gallate on the NF-κB signalling pathway. The NF-κB signalling pathway was investigated, as it is germane to atherosclerosis, because it is pivotal to inflammatory and immune type responses. There is also evidence to suggest that NF-κB is up regulated in animal and human atherosclerotic areas of the lesion. From this series of experiments it was resolved to focus on EGCG mechanisms of action, because it gave consistent results on efficacy. Also from reading the literature it appeared that the EGCG is more bioavailable than the other chemopreventive agents.

- The second objective was to examine the oxidant/antioxidant nature of the chemopreventive agent EGCG, which was important for two reasons. Firstly, if EGCG functioned as an antioxidant then it may prevent the damage caused by ROS during plaque formation. Secondly, it has been shown that the addition of EGCG and other tea polyphenols to cell culture medium, generated significant levels of H_2O_2, with the corollary that this 'artifactual' effect may account for some or all of the reported effects of EGCG in cell culture studies.

- The third objective was to evaluate the downstream consequences of EGCG-induced modulation of NF-κB. These downstream responses that were relevant to atherosclerosis included examining the effect of EGCG on cell growth regulation, by determining the effect of EGCG on the cell cycle and induction of apoptosis.

- The fourth objective was to further characterise the effect of the EGCG-induced cell growth inhibition and induction of apoptosis, particularly examining cell cycle and apoptotic regulatory proteins, of which several are NF-κB regulated.

- The final objective was to examine any potential differences in gene expression profiles between normotensive and hypertensive lymphoblastoid cell lines using cDNA microarray analysis. This may highlight any genes that give rise to the hypertensive disease status of the cells. Also the effect of EGCG on the gene expression profile in HUVEC and in the normotensive and hypertensive cell lines

was scrutinised. Again this may potentially identify novel genes modulated by EGCG that may be beneficial in the prevention of atherosclerosis.

It was hoped that this investigation might provide information on how these chemopreventive agents, especially EGCG, impinge on the NF-κB pathway to control aberrant cell proliferation and thus may be beneficial when attempting to design preventive dietary intervention studies in healthy volunteers or groups of at risk patients.

Chapter Two

Materials and methods

2.1 Materials

2.1.1 General chemicals and reagents

All general chemicals were purchased from Sigma-Aldrich (Poole, UK), unless otherwise stated. Indole-3 carbinol, curcumin, resveratrol and epigallocatechin-3-gallate were obtained from Sigma-Aldrich. The protease inhibitor cocktail consisted of AEBSF (104 nM), aprotinin (0.08 mM), leupeptin (2 mM), bestatin (4 mM), pepstatin A (1.5mM), E-64 (1.4 mM), was purchased from Sigma-Aldrich. PMA was purchased from Calbiochem, Merck Biosciences (Nottingham, UK). RPMI and endothelial growth media were purchased from Gibco-BRL (UK) and TCS cell works (UK) respectively. Acrylamide (30 % acrylamide: bis acrylamide) and ECL film for western analysis were obtained from Anachem and Amersham respectively. The inhibitors LY294002, U0126, SB203580, SP600125 were purchased from Calbiochem. The GST-IκBα was purchased from Santa-Cruz Biotechnology (Santa Cruz, USA)

2.2 Buffers

Buffers were prepared using autoclaved 18Ω ultrapure water. Where necessary, the pH of solutions was altered using 12N HCl or 5M NaOH as required.

Ammonium Persulfate (Initiator)

10 % (w/v) ammonium persulfate

Annexin V-binding buffer

10 mM Hepes (pH 7.4)
150 mM NaCl
5 mM KCl
1 mM $MgCl_2$
1.8 mM $CaCl_2$

Balanced salt solution (BSS) (pH 7.3)

140 mM NaCl
5 mM KCl
2.8 mM NaCO
1mM $MgCl_2$
1.5 mM $CaCl_2$
15 mM HEPES
0.06 mM $MgSO_2$

Cytosolic wash buffer

20 mM Tris base
150 mM NaCl
5 mM glucose
1X Protease inhibitor cocktail (Boehringer Manheim)

Di-sodium phosphate EDTA (SSPE) (20 x) pH 7.4

3 M NaCl
1 mM NaH_2PO_4
20 mM EDTA

DNTP Mixture (Pharmacia Biotech)

20 mM dGTP
20 mM dCTP
20 mM dATP
8 mM dTTP

EMSA binding buffer (2 X)

8% (w/v) ficoll

40 mM Hepes (pH 7.5)

EMSA loading Buffer

10 % (w/v) bromophenol blue

FOX reagent

2.5 mM ammonium ferrous sulphate

1 mM xylenol orange (dissolved in 250 mM sulphuric acid)

H8 buffer

20 mM Tris HCl (pH 7.5)

2 mM EDTA

2 mM EGTA

6 mM β-mercaptoethanol

1X protease inhibitor cocktail

Hybridisation buffer

71 % (v/v) deionised formamide

3.6 x Denhardts

0.7 % (w/v) SDS

Kinase assay buffer (KAB)

25 mM HEPES (pH 7.4)

25 mM β-glycerophosphate

24 mM $MgCl_2$

0.5 mM EDTA

Kinase lysis buffer

20 mM Tris (pH 7.5)

150 mM NaCl

1 mM EDTA

1 mM EGTA

1 % (w/v) triton X-100

2.5 mM sodium pyrophosphate

1 mM β-glycerolphosphate

1 mM Na_3VO_4

1 X protease cocktail inhibitor

Microarray buffers and equipment

RLT Buffer (Qiagen UK)

RW1 Buffer (Qiagen UK)

RPE Buffer (Qiagen UK)

RNase-free water

0.1 M DTT

RNAsin

Superscript II reverse transcriptase (Gibco-BRL, UK)

1 mM Cy3-Fluor-dUTP (Amersham, UK)

1 mM Cy5-Fluor-dUTP (Amersham, UK)

5 x First strand buffer (Gibco-BRL, UK)

8 μg/μl Oligo dT_{25}

1 μg/μl Oligo dA_{80}

Microcon YM-30 (Princetown separations, UK)

4 mg/ml tRNA (Gibco-BRL, UK)

Nuclear lysis buffer A

10 mM HEPES (pH 7.8)

10 mM KCl

2 mM $MgCl_2$

1 mM dithiothreitol (DTT)

0.1 mM EDTA

0.2 mM NaF

0.2 mM Na_3VO_4

1X protease inhibitor cocktail

Nuclear lysis buffer B

10 % (v/v) Nonidet P40

Nuclear lysis buffer C

50 mM HEPES (pH 7.8)

50 mM KCl

300 mM NaCl

0.1 mM DTT

10 % (v/v) glycerol

0.2 mM NaF

0.2 mM Na_3VO_4

1X protease inhibitor cocktail

SDS solution

10 % (w/v) SDS

Stripping buffer

2 % (w/v) SDS

62.5 mM Tris-base

0.8 % (v/v) β-mercaptoethanol

Tris-borate EDTA (TBE) (5 X)

1.78 M Tris-Borate
40 mM EDTA

Tris-buffered saline with tween-20 (TBST) (10 X)

0.05 M Tris (pH 7.5)
0.15 M NaCl
1 % (v/v) tween-20

Tris-EDTA (TE) buffer

1 M Tris base (pH 8)
0.1 M EDTA

Triton lysis buffer

20 mM Tris (pH 7.4)
137 mM NaCl
25 mM β-glycerol phosphate
2 mM NaPPi
2 mM EDTA
10 % (v/v) Triton-X 100
0.5 mM DTT
1 X protease inhibitor cocktail

Western BSA blocking buffer

5 % (w/v) electrophoresis grade bovine serum albumin (BSA) in TBST

Western blocking buffer (non-fat milk)

5 % (w/v) non-fat milk (Marvel) in TBST

Western electrophoresis buffer (10 X)

0.025 M Tris (pH 8.3)

0.192 M Glycine

0.1 % (w/v) SDS

Western running Gel Buffer (4 X)

1.5 M Tris-HCl (pH 8.8)

Western sample buffer (3 X)

50 mM Tris-base (pH 6.8)

12 % (w/v) SDS

36 % (v/v) glycerol

2 % (w/v) DTT

0.02 % (w/v) bromophenol blue

Western stacking Gel Buffer (4 X)

0.5 M Tris (pH 6.8)

Western transfer buffer (1 X)

48 mM Tris

39 mM glycine

20 % (v/v) MeOH

0.037 % (w/v) SDS

Western sample buffer (5 X)

2.4 % (w/v) SDS

60 % (v/v) glycerol

250 mM Tris base

500 mM DTT

0.1 % (w/v) bromphenol blue

2.2.2 Antibodies

Antibody against	Details
NF-κB p65	Rabbit polyclonal (C20) Santa-Cruz Biotechnology Inc (Santa Cruz, USA)
NF-κB p50	Rabbit polyclonal Kind gift from Dr Nancy Rice (NCI, USA)
NF-κB p52	Rabbit polyclonal Kind gift from Dr Nancy Rice (NCI, USA)
RELB	Rabbit polyclonal Kind gift from Dr Nancy Rice (NCI, USA)
IκBα	Rabbit polyclonal (C21) Santa-Cruz Biotechnology Inc (Santa Cruz, USA)
IκBβ	Rabbit polyclonal (S-20) Santa-Cruz Biotechnology Inc (Santa Cruz, USA)
Phospho-IκBα (serine 32)	Mouse monoclonal (B-9) Santa-Cruz Biotechnology Inc (Santa Cruz, USA)
IKKα	Rabbit polyclonal (M110) Santa-Cruz Biotechnology Inc (Santa Cruz, USA)
p53	Mouse monoclonal Oncogene research products
Cyclin D1	Mouse monoclonal (R124) from Santa-Cruz Biotechnology Inc (Santa Cruz, USA)
CDK1 p34	Rabbit polyclonal Santa-Cruz Biotechnology Inc (Santa Cruz, USA)
HMOX-1	Mouse monoclonal (OSA-110) from Stressgen Corp. (USA)
Nrf2	Rabbit polyclonal (H-300) Santa-Cruz Biotechnology Inc (Santa Cruz, USA)
Pin1	Rabbit polyclonal Oncogene research products

Phospho-ERK1/2	Mouse monoclonal (E4) Santa-Cruz Biotechnology Inc (Santa Cruz, USA)
ERK1	Rabbit polyclonal (K-23) Santa-Cruz Biotechnology Inc (Santa Cruz, USA)
Phospho-Akt (ser 473)	Mouse monoclonal New England Biolabs
XIAP (hIAP)	Mouse monoclonal (Clone 48) BD Biosciences/Transduction Laboratories (USA)
$P21^{Waf}$	Mouse monoclonal (C187) Santa-Cruz Biotechnology Inc (Santa Cruz, USA)
Tubulin α	Mouse monoclonal (CP06) Oncogene research products

Table 2.1 – list of primary antibodies

The secondary antibodies, anti-mouse and anti-rabbit IgG (whole molecule) horseradish peroxidase conjugates were obtained from Sigma-Aldrich; the anti-goat IgG HRP conjugate was purchased from Santa Cruz Biotechnology.

2.2.3 Radiochemicals

The radiochemical used in the gel-shift analyses was [γ^{32}P] adenosine 5'-triphosphate (ATP) purchased from Amersham Life Sciences Ltd (Little Chalfont, UK).

2.2.4 Reagent Kits

The RNA used in the micro-array analysis was isolated using the Qiagen RNeasy midi-RNA preparation kit obtained from Qiagen Ltd (Surrey, UK). The apoptotic studies using the annexin peptide were performed using the annexin kit obtained from Bender Medsystems (Vienna, Austria). The enhanced chemiluminescence (ECL) detection kit was obtained from Amersham Life Sciences.

2.3 Cell Lines

2.3.1 B Lymphoblast cell lines

The human B lymphoblast cell lines derived from normotensive (C143 and C149), and hypertensive (H308 and H242) subjects were a kind gift provided by Prof Leong Ng (Clinical Science Building, Leicester Royal Infirmary, UK). The cells were originally obtained from part of a study group which consisted of 23 normal healthy control subjects, 17 out of 61 randomly selected diabetic control patients, and 17 out of 43 randomly selected diabetic nephropathy patients as described in Ng *et al.* (1994).

The cell lines were established in 1991 by immortalisation with the Epstein Barr virus (EBV). In brief, peripheral blood lymphocytes were isolated from subjects using LeucoPREP tubes by density centrifugation, as described in manufacturer's protocol (Becton Dickinson, USA). Selected B-lymphocytes were suspended in 2 ml of Iscove's modified Dulbecco's growth medium and infected with 0.5 ml of EBV. Cyclosporin A (0.5 ml) was added to each of the tissue culture flasks as this increased the efficiency by which permanent B lymphoblast cell lines were established (Bird *et al.* 1981). The cells were incubated at 37°C for a 7-day period at which point 2 ml of complete growth medium was added to supplement the cell cultures. About 2-3 weeks after starting the cultures or when large clumps of cells appeared, they were supplemented with complete growth medium, every 3-4 days until they reached a volume of 50 ml with an approximate density of 10^6 cells/ml. The immortalised cells were then harvested by centrifugation and resuspended in complete growth medium (containing 10 % v/v cryoprotectant or DMSO) to be stored in liquid nitrogen.

2.3.2 Human umbilical vein endotheliai cells (HUVEC)

Ethical permission was obtained from Leicester Royal Infirmary to collect umbilical cords derived from normal placenta without hepatitis or HIV contamination. The cords were cut approximately 5-20 minutes after birth, after which they were placed in a sterile container containing sterile PBS supplemented with penicillin (P) (100 U/ml) and streptomycin (S) (100 μg/ml). The cords were then preserved at 4°C for up to 24 hours, until they were processed.

2.3.2.1 Preparation of HUVEC

At the time of processing, all areas of the cord with clamp and/or needle marks were excised. The cord was washed in sterile PBS (containing 100 U/ml P, and 100 μg/ml S) to remove all surface blood and tissue. The umbilical vein was cannulated with a sterile blunt needle and was fixed in place using sterile forceps. The vein was then perfused with sterile PBS (containing P and S) to remove any residual blood clots and debris. The other end of the cord was clamped using sterile forceps. The vein was filled with collagenase, and then incubated at 37°C in a large tissue culture dish, for 15 minutes. After incubation, the cord was removed and kneaded (i.e. massaged and squeezed) gently to promote cell detachment. The collagenase solution containing the endothelial cells was drained from the cord into a sterile 20 ml tube and was centrifuged (200 x g for 3 minutes at 4°C) to collect the cell fraction. The collagenase solution was aspirated off and cells were suspended in approximately 7 mls of complete endothelial growth medium (EGM) with supplements (containing heparin, hydrocortisone, human epidermal growth factor, human basic fibroblast growth factor and foetal bovine serum), (TCS cell works, UK).

The cell suspension was incubated overnight in a 25 cm^2 flask pre-coated with 1 % (w/v) gelatin at 37°C, in a humidified 95 % air, 5 % CO_2 incubator atmosphere to allow the endothelial cells to bed down on the gelatin layer. The medium was aspirated off, the flask was washed with sterile PBS and 10 ml of complete EGM was added to the flask. After approximately 2 days cells were ready to be split into larger tissue culture flasks.

2.3.3 Routine cell maintenance

2.3.3.1 Resuscitation of cell lines from storage

B lymphoblast cells that had been stored in liquid nitrogen, were rapidly thawed by incubating in a water bath at 37°C for approximately 5 minutes. The contents of the storage vial was then transferred to a fresh tube and resuspended in 10 ml of pre-warmed RPMI 1640 (Gibco BRL, UK) supplemented with 10 % foetal calf serum, 2mM glutamine (G), 100 U/ml P and 100 μg/ml S. The cells were collected by centrifugation (200 x g for 3 minutes), re-suspended in 2 ml of complete RPMI 1640 supplemented with 10% FCS plus P/S/G and transferred to a 25cm^2 flask. Cells were incubated in a 37 °C humidified 95 % air, 5 % CO_2 incubator, until they required sub-culturing.

2.3.3.2 Sub-culturing of cell lines

The B lymphoblast cell lines were cultured in complete RPMI 1640. Cells were kept at a density of between 2.0 x 10^5 and 2.0 x 10^6 per ml. This was achieved by routinely splitting cell cultures between 1:3 and 1:6 depending upon initial cell densities. Cultures were replaced from liquid nitrogen stocks after approximately 3 months of growth.
Before treatments, cells were washed and re-suspended (at the required densities) and were left in serum-free RPMI medium for 24 hours in a humidified 95 % air, 5 % CO_2 incubator at 37°C. At least 1 hour prior to treatment, cells were washed and resuspended in serum-free RPMI 1640 and placed at 37°C.

HUVEC were grown and routinely passaged in complete EGM in a humidified incubator with 95 % air, 5% CO_2 air at 37°C. Culture medium was replaced every two days. The confluent culture was washed with sterile PBS without Ca^{2+} and Mg^{2+}, and cells were incubated for 2 minutes with trypsin/EDTA to allow detachment from the flask. Trypsin activity was then stopped by adding 5 ml of complete EGM, and cells were collected by centrifugation. The supernatant was discarded and the cell pellet was re-suspended in fresh complete EGM. Cells were transferred at the required densities into new plates or flasks pre-coated with 1% gelatin and maintained at 37°C with 5% CO_2.

2.3.3.3 Storage of cell lines

B lymphoblast cells were collected by centrifugation (200 x g for 3 minutes). The cell pellet was re-suspended in 5-10 mls of freezing-down medium (50 % RPMI, 40 % FCS and 10 % cryoprotectant DMSO). Cell densities were established using a haemocytometer and volumes altered to give final cell densities of approximately 5.0 x 10^7 cells per ml. One ml of cells was then transferred to freezing cryovials and allowed to freeze gradually by leaving overnight at – 80°C. The cells were then transferred to permanent storage in liquid nitrogen.

HUVEC were not stored in liquid nitrogen as they were prepared from a cord and routinely passaged until they were used in an experiment.

2.4 Methods

2.4.1 Preparation of cytosolic protein fraction

B lymphoblast cells and HUVEC were processed in a very similar manner, with some minor differences. Following treatment, B lymphoblast cells were collected by centrifugation for 1 min, 14,000 rpm (eppendorf centrifuge 5415C) at 4°C. The supernatants were removed and the cell pellets washed twice in 1 ml ice-cold wash buffer. The samples were centrifuged as above, and the resultant pellets resuspended in 0.1 ml of ice cold H8 Buffer. The samples were sonicated (6-8 blasts, output 4 microns) and then centrifuged 100000 x g for 30 minutes at 4°C. The resultant supernatant containing the cytosolic protein fraction was stored at -20°C.

For HUVEC, medium including treatments was aspirated off and cells washed twice with 1 ml ice-cold wash buffer. The cells were then scraped into 1 ml of ice-cold wash buffer and collected by centrifugation (14,000 rpm eppendorf centrifuge 5415C for 1 minute at 4°C). Samples were then prepared as described for the B lymphoblast cell lines.

2.4.2 Preparation of nuclear protein fraction

B lymphoblast cells and HUVEC were again processed in similar manner. Following treatment, B lymphoblast cells were collected by centrifugation (14,000 rpm, at 4°C) (eppendorf centrifuge 5415C), and were re-suspended in 0.4 ml buffer A and incubated on ice for 15 minutes. Following incubation, 25 µl of buffer B was added to each sample, before vortexing vigorously for 15 seconds. Samples were then centrifuged again and the resultant pellets containing the nuclear extract were re-suspended in 50µl of Buffer C and mixed on a rotating platform for 20 minutes at 4°C. The samples were then sonicated (6-8 blasts, output 1 micron) and centrifuged as above for 5 minutes at 4°C. The resultant supernatants containing the nuclear proteins were stored at – 20° C.

For HUVEC, medium including treatments was aspirated and cells were washed twice in ice-cold PBS before being scraped and transferred into eppendorf tubes. Cells were centrifuged (14,000 rpm, at 4°C) and the resultant pellets resuspended in 0.4 ml buffer A. HUVEC nuclear protein fractions were then prepared as described above. Protein concentrations were determined as described below in 2.4.4.

2.4.3 Preparation of total cellular protein fraction

B lymphoblast cells and HUVEC were processed in a similar manner with some minor exceptions. Following treatment, B lymphoblast cells were collected by centrifugation (14,000 rpm, for 1 minute at 4°C). Supernatants were discarded and cell pellets were washed twice in 1 ml of ice-cold PBS. Samples were centrifuged and the resultant cell pellets resuspended in 100 µl of Triton X lysis buffer. Samples were left on ice for 15 minutes and then centrifuged 1000 x g for 5 minutes at 4°C. The resultant supernatant contained the purified total cellular protein fraction, which was removed and stored at – 20°C.

For HUVEC, cells were washed twice in ice-cold PBS and then scraped into 1 ml of triton-X 100 lysis buffer and prepared as for B lymphoblast cells.

2.4.4 Protein Determination using the Bradford Method

Determination of protein concentration was carried out using the Bradford protein assay method as described in the manufacturer's data sheet (Biorad, UK). In brief, 10 µl of each preparation was added to 990 µl of dH_2O (1:100 dilution). Of this diluted sample, 800 µl was taken and added to 200 µl of Bradford protein assay reagent. The absorbance (at λ 595nm) of each sample was determined using a spectrophotometer and the respective protein concentrations extrapolated from a protein standard curve. The standard curve was constructed using known concentrations of bovine serum albumin.

2.5 Detection of NF-κB binding by electrophoretic gel mobility shift assay (EMSA)

2.5.1 Reagents

- Consensus oligonucleotide (1.75 pmol/µl) (Promega, UK)
- T4 Polynucleotide kinase (PNK) (100U) (Promega, UK)
- T4 Polynucleotide kinase 10 X buffer (Promega, UK)
- TE buffer
- [γ-^{32}P]ATP

- 5 M NaCl
- Nap '5' column
- Nuclease free water
- 10 mM DTT
- 10 mM $MgCl_2$
- EtOH (100%)
- Poly DIDC (Sigma, UK)
- 5 X TBE buffer

Background

The EMSA analysis provided an ostensibly simple method of detecting DNA-binding proteins, such as transcription factors including NF-κB. The basis behind this assay was that protein:DNA complexes would migrate through the non-denaturing polyacrylamide gel more slowly than free DNA fragments.

The assay was performed by incubating a mixture of nuclear proteins containing NF-κB with a ^{32}P end-labelled DNA oligonucleotide containing putative NF-κB binding sites. The samples were left to react before finally being subjected to electrophoresis through a non-denaturing gel. Specificity of the interaction was established by competition experiments using an excess of non-end labelled (cold) DNA oligonucleotide containing a binding site for the protein of interest or another unlabelled unrelated DNA sequence.

2.5.2 Assay conditions

The B lymphoblast cells were left in serum-free RPMI at 37°C, 5% CO_2, overnight. Culture medium was replaced with serum-free RPMI at least one hour prior to treatment and cells were incubated in eppendorf tubes at 37°C.

HUVEC were seeded at a concentration of 1.25 X 10^6 cells on 9 cm plates in complete EGM medium and left overnight. Culture medium was replaced with serum-free EGM medium at least one hour prior to commencement of treatment.

Both B lymphoblast and HUVEC cultures were pre-treated with EGCG at the indicated concentrations for 45 minutes, followed by stimulation with PMA (0.15 μM for 15 minutes), TNFα (7.5ng/ml for 15 minutes) or mock stimulation with DMSO (0.003%) for 15 minutes. Nuclear protein fractions were then prepared as described in 2.4.2.

2.5.3 Labelling of the NF-κB oligonucleotide

Consensus NF-κB oligonucleotide (8.75 pmol), T4 polynucleotide kinase (1 μl), T4 polynucleotide kinase buffer (2 μl), deionised H_2O (7 μl) and [γ-^{32}P] ATP (5 μl) were incubated at 37°C, for 30 minutes. Following incubation, TE buffer (80 μl) was added to the oligonucleotide mix to stop the labelling reaction. The labelled oligonucleotide was dispensed on to a NAP '5' column and eluted into a screw top eppendorf. The labelled oligonucleotide was eluted with subsequent aliquots of 100 μl TE buffer and collected in 12 fractions. The disintegration per minute (DPM) was measured for each fraction and 2-3 fractions with the most activity were combined. The oligonucleotide was precipitated by adding 2.5 X v/v of 100% ethanol and 1/10 v/v of 5 M NaCl and incubated overnight at –20°C. The oligonucleotide was then collected by centrifugation (14000 g, 30 minutes at room temperature) and the resultant pellet re-suspended in an appropriate volume of deionised H_2O.

2.5.4 Preparation of samples for EMSA

Nuclear protein (5 μg) from each sample was added to fresh eppendorf tubes. The volume of each sample was adjusted to 4 μl by the addition of buffer C, and then to a final volume of 6 μl with deionised H_2O. To each of the tubes 14 μl of mastermix buffer, 10 μl binding buffer (8% ficoll, 40 mM Hepes pH 7.5), 1 μg poly DIDC, 10 nM $MgCl_2$, 10 nM DTT, 1.6 μl dH_2O) was added. For competitive and non-competitive control reactions 8.75 pmol of cold AP-1 or NF-κB oligonucleotides were added respectively. For the supershift reactions excess p50 of p65 (5 μl) were added respectively. The samples were then incubated for 15 minutes on ice before addition of 1 μl of the labelled oligonucleotide. The samples were mixed and then incubated for 30 minutes at room temperature to permit DNA oligonucleotide/protein interactions. To the tubes 2 μl of loading buffer was added and samples were transferred on to a 5 % acrylamide gel (8 ml 30 % acrylamide;bis acrylamide (37;5;1); 49.75 ml dH_2O; 3 ml 5 X TBE; 400 μl APS; 100 μl TEMED). Samples were then run through the gel at 240 V for 10 minutes followed by 120 V for 1.5-2 hours. The gel was then dried down on to Whatman blotting paper using a gel dryer (Hoeffer gel drier, USA). When completely dried, the gel was transferred either to a

phosphorimager cassette to scan the image (Molecular Dynamics) or exposed to hyperfilm (Amersham) which was developed after exposure to the gel for 1-2 days at –80°C.

2.6 Protein separation using SDS-PAGE

Polyacrylamide gels were prepared as described in manufacturer's applications guide ('Hoefer', Amersham Pharmacia Biotech, UK).

2.6.1 Sample and gel preparation

Background

During SDS polyacrylamide gel electrophoresis separation, the protein migration was determined by molecular weight and not by the polypeptide's intrinsic electrical charge. This was because SDS as well as denaturing the polypeptide conferred a net negative charge to the polypeptide in proportion to its length. When the polypeptide was run through the matrix i.e. the polyacrylamide gel, the migratory position of each protein gave a good estimation of its size and hence a clue to its identity. In addition to estimation of the proteins size, band intensity also gave an indication of the amount of sample present. The choice of acrylamide concentration was dependent upon the molecular weight of the protein that was being resolved. Generally, the higher the molecular weight of protein the lower the % acrylamide in the gel, which allowed for increased resolution.

Twenty to forty microlitres (10-50 μg) of each protein sample were combined with 1/3 v/v of sample buffer and boiled for 5 minutes.

Mini-gel or large-gel casting kits were set up according to the manufacturer's instructions (Biorad). The resolving gel solution was made up as detailed in table 2.2, and pipetted to a level about 4 cm from the top of the plates. It was left to polymerise covered by a layer of distilled water to prevent contact with the air and also to allow for the interface to set uniformly. After 15 min, the water layer was poured off and the stacking gel (table 2.3) poured on top of the resolving gel. A 10 or 15-well casting comb was inserted and the gel left to polymerise for 15 min. The casting combs were removed, gels were placed in the electrode assemblies and these placed in the tanks filled with 1 X electrophoresis buffer. Kaleidoscope and Blue broad-range protein standard ladders (Biorad, UK) were loaded to aid estimation of sample band sizes.

	10 %	12.5 %	15 %
Monomer solution	10 ml	12.5 ml	15 ml
4 X running gel buffer	7.5 ml	7.5 ml	7.5 ml
10 % SDS	0.3 ml	0.3 ml	0.3 ml
ddH_2O	12.1 ml	9.6 ml	7.1 ml
10% Ammonium persulfate	150 µl	150 µl	150 µl
TEMED[1]	10 µl	10 µl	10 µl

Table 2.2 - Running gel recipes for SDS-page

	4 %
Monomer solution	1.33 ml
4 X stacking gel buffer	2.5 ml
10 % SDS	0.1 ml
ddH_2O	6.0 ml
10% Ammonium persulfate	50 µl
TEMED[1]	5 µl

Table 2.3 - Stacking gel recipe for SDS-page

[1] TEMED; N,N,N′,N′-tetramethylethylenediamine.

Ten to fifty µg of protein per sample were loaded into the wells on each gel. Gels were run at 80 V until the samples had entered the resolving gel and then 150 V thereafter, until the bromophenol blue dye front had run off the end of the gel (approx 90 minutes for minigels and 5 hours for large gels). The gels were removed from the electrode assembly and the stacking gel discarded in preparation for transfer to a membrane.

2.6.2 Transfer of proteins to nitrocellulose membranes

The transfer of proteins to nitrocellulose membrane was performed as described in the manufacturer's protocol (Biorad, UK). Prior to transfer, the gel was allowed to equilibrate in 1 X transfer buffer for 10 minutes. Concurrently to this, nitrocellulose membrane was cut to size and soaked in 1 X transfer buffer along with two sponges and two pieces of Whatman blotting paper. The transfer apparatus was set up as depicted in figure 2.1. The transfer of proteins using the Biorad wet blotter occurred at either 100 volts for 90 minutes or 30 volts, overnight. Proteins were also transferred using the Biorad semi-

dry blotter, whereby the current used was calculated from the formula ; 0.3 ma X area of blot cm^2, for approximately 60 minutes (RT).

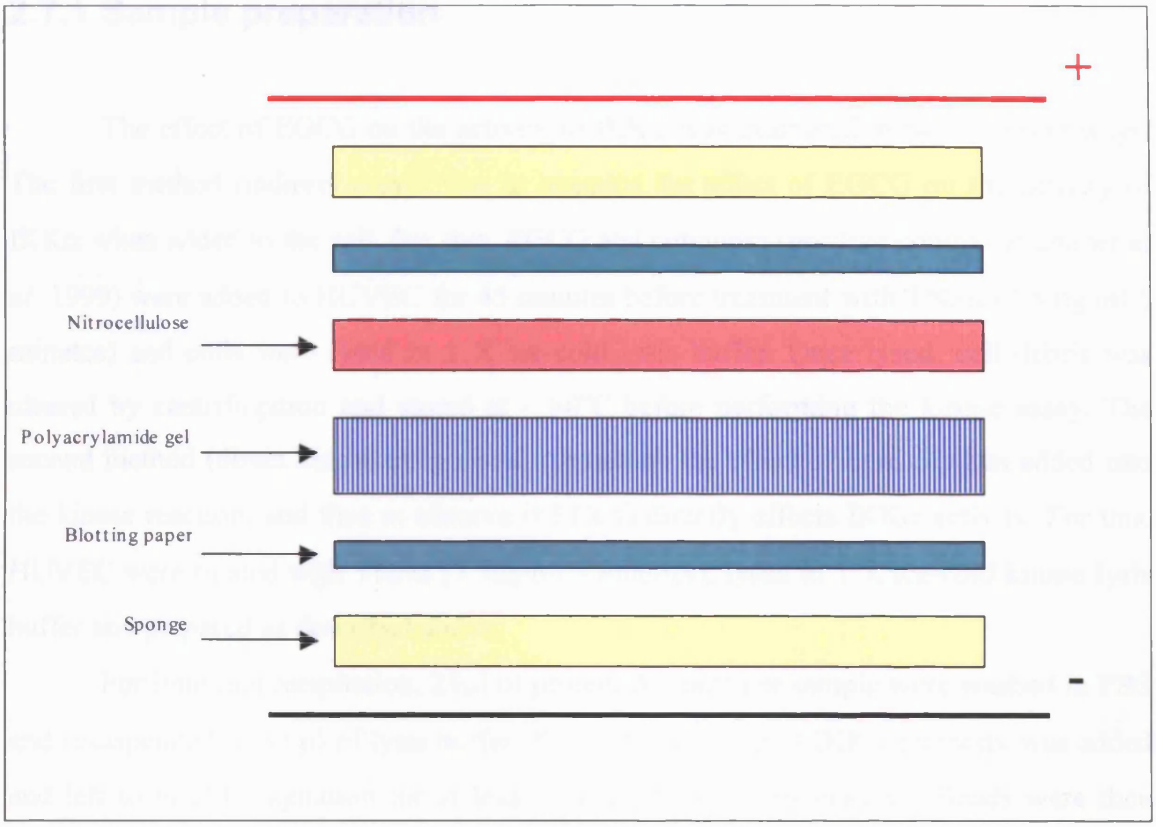

Figure 2.1 - Diagram of the assembly of an immunoblot sandwich for wet or semi-dry transfer

Protein transfer and equal loading were checked by Ponceau-S staining of membranes which were then washed in TBST and blocked for 1 h (RT) or overnight (4°C) with blocking buffer (either 5% BSA or 5% Marvel in TBS-T). Membranes were washed with TBS-T and probed with the primary antibody (in 5 % blocking buffer) for 1-2 h. Membranes were again washed with TBS-T (x 4) and then probed with the appropriate secondary antibody for 1-2 hours. The membranes were washed in TBS-T (x 4) and protein bands visualised using enhanced chemiluminescence system (Amersham) and film (Amersham) on an automatic developing machine (Compact 4, Xograph Imaging Systems). Films were then quantified using Syngene chemigenius multiimager.

2.7 Immunoprecipitation and IKKα kinase assay

2.7.1 Sample preparation

The effect of EGCG on the activity of IKKα was examined in two different ways. The first method (indirect assay) was to examine the effect of EGCG on the activity of IKKα when added to the cell. For this, EGCG and curcumin (positive control; Plummer *et al.* 1999) were added to HUVEC for 45 minutes before treatment with TNFα (7.5 ng/ml/5 minutes) and cells were lysed in 1 X ice-cold lysis buffer. Once lysed, cell debris was cleared by centrifugation and stored at – 80°C before performing the kinase assay. The second method (direct kinase assay) was to examine the effect of EGCG when added into the kinase reaction, and thus to observe if EGCG directly effects IKKα activity. For this, HUVEC were treated with TNFα (7.5ng/ml/5 minutes), lysed in 1 X ice-cold kinase lysis buffer and prepared as described above.

For immunoprecipitation, 25μl of protein-A beads per sample were washed in PBS and resuspended in 50 μl of lysis buffer. To the beads, 5 μg of IKKα antibody was added and left to bind by agitation for at least 1 hour at room temperature. Beads were then washed (X 2) in PBS to remove unbound antibody and resuspended in 25 μl kinase assay buffer (KAB).

Samples were removed from the – 80°C freezer, and 500 μl (1μg/μl) added to 25 μl of pre-bound IKKα/protein-A agarose beads. Samples were then agitated overnight at 4°C, before being collected by centrifugation. Beads were then washed (X2) in 1 X lysis buffer, resuspended in 1 ml KAB and 250 μl removed and incubated on ice for Western analysis. The remaining 750 μl were collected by centrifugation and resuspended in 34 μl KAB, containing 2.5 μl GST-IκBα and 3 μl (cold) ATP (final conc. 50 μM). To each of the samples, 5 μCi of [^{32}P]-labelled ATP was added and samples were incubated for 30 minutes at 30°C. For the direct assay, EGCG and curcumin (positive control) were added to the assay before the addition of [^{32}P]-labelled ATP. The reactions were stopped by the addition of 5 X sample buffer, and then boiled for 5 minutes. Samples were then centrifuged for 30 seconds (1000 xg) and the resultant supernatant was resolved by a 10 % SDS-PAGE. Gels were dried and incorporation of ^{32}P to the GST-IκBα substrate determined by exposing to autoradiorgaphy film (Amersham, UK) overnight at –20°C.

The 250 µl saved for the Western analysis were centrifuged to collect the beads, and resuspended in 5 X sample buffer. Samples were boiled for 5 minutes, centrifuged to remove beads and resolved by a 10 % SDS-PAGE. Proteins were transferred to a nitrocellulose membrane before immunoblotting with an antibody directed against IKKα. The Western/immunoblotting gave an idea of the concentration of IKKα immunoprecipitated from each sample.

2.8 Measurement of H_2O_2 using the ferrous oxidation in xylenol orange assay

Measurement of the peroxide (H_2O_2) generated in cell culture medium following addition of chemopreventive agents, was accomplished using the ferrous oxidation in xylenol orange (FOX) assay method as described in Long *et al.* (2001). A 1 X (working) FOX solution was made by adding 1 volume of 10 X FOX reagent to 9 volumes of methanol containing 4.4 mM butylated hydroxytoluene. For the time course experiments the chemopreventive agents EGCG, resveratrol, curcumin and I3C were added to RPMI cell culture medium and incubated at 37°C. At time points between 0 and 120 minutes, 90 µl of sample (RPMI medium plus chemopreventive agent) was removed and added to 10 µl of methanol, before a 30-minute incubation at room temperature. To this sample, 900 µl of 1 X FOX reagent was added, vortexed and incubated for a further 30 minutes. The solution was then centrifuged 14,000 x g for 10 minutes and the absorbance measured at 560 nm using a spectrophotometer. The concentration of generated peroxide was determined from a H_2O_2 standard curve.

The effect of catalase on this response was also examined. Exogenous catalase (4 units) or heat inactivated catalase were added concurrently with EGCG, and peroxide concentration established using the above assay. In addition, measurement of peroxide in other cell culture medium including endothelial growth medium (EGM) and Dulbecco's modified Eagle medium (DMEM) following a one-hour (37°C) incubation with the chemopreventive agents was also examined.

2.8.1 Measurement of reactive oxygen species (ROS) using chemiluminescence and fluorescence

Extracellular ROS measurement was based on the technique described by Liu *et al.* (1996), using the dye isoluminol. Lymphoblast cells were collected by centrifugation (200 x g) and resuspended in serum-free RPMI at a concentration of $1X10^6$ cells per ml. Cells were treated with EGCG (15, 30 or 50 μM) or mock treated with DMSO for 45 minutes, before being collected (200 x g) and resuspended in 1 ml of isoluminol buffer solution consisting of balanced salt solution (BSS), 56 μM isoluminol and 4 units per ml of horseradish peroxidase (HRP). Cells (100 μl) were added to a pre-warmed (37°C) bioluminescence plate, and incubated in the plate reader to allow warming of samples to 37°C. Cells were then stimulated with PMA (0.15μM) and chemiluminescence measured using a FluoStar plate reader according to the manufacturer's instructions (BMG Labtechnologies). Chemiluminescence was quantified according to Alexandrova *et al.* (2001) and was proportional to the area under the curve (AUC) with luminescence plotted against measurement time (40 mins) for each cell type. For HUVEC, the method was similar to that of B lymphoblasts, except for a few minor differences. HUVECs were seeded on a bioluminescent plate and were allowed to reach 70 % confluency before treatment with EGCG (as above). Medium was then washed off with BSS (x2) before the addition of isoluminol buffer solution and the protocol was carried out as for the B lymphoblasts, except TNFα (2.5 and 7.5 ng/ml) was used as a stimulus.

Intracellular H_2O_2 was determined using the fluorescent dye 2',7'-dichlorofluorescein diacetate (DCFH-DA) and was based on the method of Bass *et al.* (1984), with some modifications. B lymphoblast cells were collected by centrifugation (200 x g) and resuspended in serum free medium at a concentration of $1x10^6$ cells per ml. Cells were treated with EGCG (15, 30 or 50 μM) or GF109203X or mock treated with DMSO for 45 minutes, after which, they were collected by centrifugation (200 x g) and washed in 1ml of BSS, before finally being resuspended in 1 ml of BSS. The cells were added to a 37°C pre-warmed Nunc F98 plate, and left to reach this temperature, before the addition of 50 μl of DCF solution (consisting of 6 μM DCF-DA in 5 ml of BSS). The cells were allowed to adjust to 37°C before the addition of PMA (0.15 μM). BSS was added as a volume control for PMA, in order to ensure the DCF concentration was the same for all

cell samples. DCF fluorescence was then determined using a FluoStar plate reader according to the manufacturer's instructions (BMG Labtechnologies).

HUVECs were treated using a similar method to B lymphoblasts except for a few minor changes. HUVEC cells were seeded on to a Nunc F98 plate and were allowed to reach 70 % confluency before treatment with EGCG (as above). Medium was washed off with BSS (x2) before the addition of DCF solution and the protocol was carried out as for the B lymphoblasts, except TNFα (2.5 and 7.5 ng/ml) was used as a stimulus.

2.9 Characterisation of cell proliferation in B lymphoblast and HUVEC

2.9.1 Quantification of cell growth

B lymphoblast and HUVEC cells were seeded at densities of $2.5x10^5$ cells/ml and $5.0x10^5$ cells/ml respectively. Cells were incubated in the presence or absence of EGCG at the indicated concentrations. Cells were collected by centrifugation and to each cell treatment an appropriate volume of isoton solution (Coulter electronics, Luton) was added. Cell densities were established using a coulter counter ZM (Coulter electronics, Luton) as described in the manufacturer's protocol. Cell densities were measured on a 24-hour basis for the duration of 144 hours.

2.9.2 Cell cycle analysis

Cells were exposed to the chemopreventive agent EGCG at concentrations between 5 to 100 μM and time points between 24 to 96 hours. Cells were collected by centrifugation and were fixed in 2 ml of ice-cold 70 % ethanol v/v in PBS. Cells were then collected by centrifugation and resuspended in 1 ml of PBS containing 0.1 mg/ml ribonuclease (RNase A) and 5 mg/ml propidium iodide. The content of cellular DNA was established using a Becton Dickinson FACScan and the percentage of cells in each phase of the cell cycle calculated using Cell Quest software programme.

2.9.3 Determination of Apoptosis using Annexin V

The number of cells undergoing apoptosis was determined using annexin V-FITC/PI staining and the method was adapted from the manufacturer's instructions. Annexin V-FITC is a vascular protein that binds in a calcium dependent manner to the phospholipid, phosphatidylserine, which is expressed on the extracelluar cell surface of apoptotic cells. Propidium iodide (PI) is a cell impermeant dye that, under UV light, increases in fluorescence 20- to 30-fold when bound to nucleic acids. A combination of annexin V-FITC and PI were used to distinguish between apoptotic, dead or live cells respectively, as annexin binds to apoptotic cells, whereas PI is only taken up by cells that have lost plasma membrane integrity, binding only to the nuclei of dead cells.

B lymphoblast cells (1 x 10^6 cells/ml) were incubated in the presence or absence of EGCG (at the indicated concentrations) for a period of 24 hours. Following treatment, cells were collected by centrifugation (350 x g, 5 minutes at 4°C) and re-suspended in 6 mls of complete medium for 10 minutes. Cells were then collected by centrifugation (350 x g, 3 minutes at 4°C) and re-suspended in 1 ml of annexin V-binding buffer containing 100 ng/ml of fluorescin isothiocyanate (FITC)-conjugated annexin V for 8 minutes. To each of the samples 30 µl of propidium iodide stain (50 µg/ml) was added and samples were incubated for 1 minute at room temperature. Samples were then placed on ice and analysed using a fluorescent activated cell sorter (Becton Dickinson FACScan).

2.10 Gene expression analysis using cDNA microarray

2.10.1 Isolation of RNA from mammalian cells

Isolation of RNA was performed using the RNeasy midi kit as described in the manufacturer's protocol (Qiagen, UK). A phenol/chloroform extraction and re-precipitation step was included to improve the overall purity of the RNA. RNA was isolated from both the B lymphoblast and HUVECs using the same protocol except for a few minor differences.

After treatment cells were collected by centrifugation (200 x g, 3 minutes at 4°C), resuspended in ice-cold RLT buffer (containing 1% β-mercaptoethanol). Samples were kept on ice and sonicated (6 x 5 second pulses with 10 second intervals, output level 10 microns) before the addition of an equal volume of 70% EtOH. The suspension was placed

on an RNA binding column and centrifuged at 3000 x g for 5 minutes at room temperature. The column was washed with RW1 wash buffer and twice with RPE buffer and the RNA was eluted in 2 x 150 µl RNase-free water. Tri reagent (1 ml) and chloroform (200 µl) were added to each of the samples, which were then vortexed and centrifuged (3000 x g for 5 minutes at room temperature). The upper aqueous layer was recovered and to these 0.5 volumes of isopropanol was added to precipitate the RNA. The RNA was centrifuged (10000 x g for 10 minutes at room temperature) and the resultant pellet containing the RNA was resuspended in 200 µl RNase-free water. The RNA was reprecipitated overnight at – 70°C by adding 3 M sodium acetate (20 µl at pH 5.2) and 600 µl EtOH. The RNA was collected by centrifugation (10,000 x g, 5 minutes at room temperature) washed twice with 70 % EtOH and dried using a speed vac drier (Savant Instruments, USA). The dried pellet was re-suspended in 50 µl of RNase-free water and nucleic acid concentration and purity were determined.

2.10.2 Determination of RNA concentration and purity

The concentration and purity of RNA was determined by measuring the absorbance of each sample at wavelengths of 260 and 280 nm. Each sample of RNA was diluted appropriately (1:100 in RNAse free ddH_2O) and placed in a quartz cuvette. The spectrophotometer was zeroed using ddH_2O and the absorbance of the diluted RNA sample was measured at each wavelength. The concentration of the sample was then calculated using the following equation;

RNA concentration (µg/ml) = Absorbance (260 nm) x Dilution x 40

The relative purity of each RNA sample was established by comparing the absorbance values at 260 and 280 nm. A 260/280 ratio of 1.8 to 2.0 for RNA, indicated a relatively pure sample i.e. a low or protein free preparation.

2.10.3 cDNA synthesis and labelling reaction

Fifty micrograms of each RNA sample was taken and made up to 10 µl with ddH_2O (RNAse free). To the RNA 0.5 µl of oligo dT_{25} (8 µg/µl) was added and the mixture was

incubated at 70°C for 8 minutes, followed by a further incubation and reduction in temperature to 42°C over a period of 30 minutes.

After the 30 minutes incubation period, 0.3 µl RNAsin, 4 µl 5 x first strand buffer (1 x final concentration), 2 µl DTT (0.01 M), 0.5 µl dNTP mix (dGTP; dATP; dCTP, 0.5 mM and dTTP, 0.2 mM), 2 µl of Cy3-fluor-dUTP or Cy5-fluor-dUTP (0.1 mM) and 0.5 µl superscript II reverse transcriptase (100 units). The mixture was incubated for 60 minutes at 42°C, a further 0.5 µl of superscript II reverse transcriptase was added and the reaction incubated for a further 60 minutes. The reaction was made up to a final volume of 41 µl following the addition of 20.5 µl of dH_2O.

The labelling reaction was stopped following the addition of 1 µl 0.5 mM EDTA, followed by 1 µl of 10 % (w/v) SDS and 3 µl of 3 M NaOH. The mixture was incubated at 70°C for 10 minutes to hydrolyse residual RNA. The samples were neutralised following the addition of 3 µl of 2 M HCl, 10 µl of 1 M Tris/HCl (pH 7.5) and 1 µl of 4 µg/ml of tRNA to give a final volume 60 µl.

2.10.4 Purification of DNA and Pre-hybridisation of cDNA

The purification of the cDNA probes was performed using both centri-step and micron columns (Princetown Separations, USA). However, the majority of DNA purification was performed using Microcon TM-30 columns. To each of the labelled cDNA probes, 140 µl of TE buffer was added, and each probe combined at the top of a Microcon tube. The tube was centrifuged at 13000 x g for 7 minutes, and the flow-through again was discarded. To the column 450 µl TE buffer was added, and the column was centrifuged at 13000 x g for 7 minutes. The flow-through was discarded. To the column, 450 µl of TE buffer, 1 µl of 10 mg/ml human Cot 1 DNA and 1 µl of 1 µg/µl of poly A were added. The column was centrifuged at 13000 x g for 7 minutes and the flow-through discarded. The column was centrifuged for a further 2 minutes until the central filter was dry. The flow-through was discarded and the column inverted in a fresh tube. The column was then centrifuged at 13000 x g for 2 minutes and the resultant purified cDNA eluate dried in a speed vac (Savant Instruments, Farmingdale, USA) for approximately 10-15 minutes.

The dried down cDNA probes were re-suspended in 14 µl of hybridisation buffer, and 6 µl of 20 x SSPE. The entire probe was denatured by incubation at 100°C for 2 minutes followed by 42°C for 30 to 60 minutes.

2.10.5 Blocking, hybridisation and washing

The printed slides were blocked following heating at 100°C for two minutes. The slides were immediately washed in ddH_2O (to remove salts) and centrifuged to dryness (1000 x g for 5 minutes). The labelled probe (20 µl) was spotted on to the array slide and a coverslip was carefully lowered over the top. The array slide was then sealed in a hybridisation chamber and incubated overnight at 42°C overnight. After hybridisation, the slides were washed in 1 x SSC containing 0.03 % SDS for 10 minutes, followed by a 5 minute wash in 0.2 x SSC and a final wash in 0.05 x SSC for 5 minutes. The slide was then immediately dried by centrifugation at 1000 x g for 5 minutes.

2.10.6 Scanning and analysis

Methods used to analyse data are described by Turton *et al.* (2001). In brief, the fluorescence of the signals obtained from the microarray hybridisation was measured using GenePix 3.0 software version 3.0.6 and an Axon scanner 4000A (Axon Instruments, CA). The scanner contained two lasers that excited cyanine dyes at appropriate wavelengths, 635 nm for cy5 and 532 nm for cy3 respectively. Feature sizes were determined using the inbuilt automated parameters in the first instance and then adjusted manually where appropriate. The fluorescence of each pixel within the feature was determined and the median fluorescence of these pixel measurements taken as the measure of fluorescence for the whole feature. The local background fluorescence was measured using the default parameters of GenePix 3.0.

Data processing and manipulation was performed using ConvertData (version 3.1.2). The resultant data was then analysed using Genecluster 3 (Eisen *et al.* 1998) and clustered hierarchically using complete linkage analysis. The clustering data was then reviewed using Treeview imaging software.

The abbreviations for the genes were taken from a number of sources that included Weizmann Institute of Science (http://bioinfo.weizmann.ac.il), the National Cancer

Institute (NCI) Genecards website (http://nciarray.nci.nih.gov/cards), and the Jackson Laboratories (http://www.informatics.jax.org).

2.11 Statistical analysis

All statistical analysis was performed using the Minitab statistical software package. Significance of results was assessed by performing either one-way ANOVA (for a single variable), followed by Tukey's post hoc test, or balanced ANOVA (for two variables) followed by Fisher's least significant difference post hoc test. The significant differences between data were calculated from either the positive or vehicle controls where by a value of $p<0.05$ was classed as significant.

Chapter Three

Effect of four chemopreventive agents on the NF-κB signalling pathway

3.1 Introduction

As mentioned previously in chapter 1, the transcription factor NF-κB is central to many inflammatory and immune responses, and has been implicated in such responses during atherosclerosis. It is activated by over 150 different stimuli, including TNFα, PMA, and reactive oxygen species (ROS) (as reviewed in Singh *et al.* 2001; Pahl *et al.* 1999). Many of these agents are found to be involved in the initiation and progression stages of atherosclerosis. Therefore the aptitude of dietary agents to prevent or suppress activation of NF-κB may be beneficial in the therapy of atherosclerosis.

At the initial undertaking of the work contained in this chapter, very little had been reported concerning the effects of the four chemopreventive agents on the NF-κB signalling pathway in these particular cell types. Since then more work investigating the effects of various dietary compounds on the modulation of NF-κB pathway has been reported. For example, resveratrol has been shown to modulate the activity of NF-κB *in vitro*. Resveratrol inhibits iNOS production by suppressing NF-κB/DNA binding activity via blockade of IκBα degradation (Tsai *et al.* 1999). Resveratrol is also a potent inhibitor of IKK activity, accounting for its inhibition of chemoattractant protein-1 expression, a NF-κB regulated gene. Also Manna *et al.* (2000), have reported that resveratrol blocks the TNFα-, PMA-, LPS-, H_2O_2-induced activation of NF-κB in cultured U937, Jurkat, and HeLa cells. Interestingly, this response appeared to be independent of TNFα-induced phosphorylation and subsequent degradation of IκBα via IKK. More recently, Pendurthi *et al.* (2002b) in HUVEC, have shown that resveratrol inhibits expression of E-selectin (an adhesion molecule regulated by NF-κB) induced by IL-1β, TNFα, PMA, and LPS. In splenic lymphocytes, resveratrol also inhibited the production of IFNγ and IL-2, through inhibition of NF-κB/DNA binding (Gao *et al.* 2001).

The effect of I3C on NF-κB activation has been less investigated. One report by Howells *et al.* (2002), investigated the effect of I3C on NF-κB, in the tumor-derived breast cell line MDA MB468 and also in the immortalised breast line HBL100. I3C inhibited NF-κB DNA binding in the MDA MB468 cell line, but had no such effect in HBL100. This inhibition was independent of IKK activity, but may have been in part due to inhibition of an NF-κB upstream regulator known as protein kinase B (PKB)/Akt.

NF-κB appears to be a target also for curcumin. Curcumin has been shown to reduce the TNFα-induced expression of tissue factor gene in bovine aortic endothelial cells

via suppression of both AP-1 and NF-κB (Bierhaus *et al.* 1997). In this study, inhibition of NF-κB translocation was found to be due to the blockade of IκBα degradation. Using HUVEC, Pendurthi *et al.* (1997) have shown that curcumin blocks biological activity and as well as expression of tissue factor induced by PMA, LPS, and TNFα. Transcriptional inactivation of the tissue factor by curcumin appeared to be mediated via suppression of NF-κB and AP-1 activation. Curcumin blocked TNFα-, PMA- and H_2O_2-induced activation of NF-κB in human myeloid monoblastic leukaemia cells, via inhibition of IκBα phosphorylation and subsequent degradation (Singh *et al.* 1995). Curcumin has been shown to inhibit IKKα and IKKβ activity possibly acting on NIK, thus preventing IκBα degradation and subsequent NF-κB activation in human colonic epithelial cells (Plummer *et al.* 1999). Adhesion molecule expression induced by TNFα, including intracellular adhesion molecule-1, vascular cell adhesion molecule-1 and endothelial leukocyte adhesion molecule-1, was inhibited by curcumin in HUVEC. This was due to suppression of TNFα-induced NF-κB/DNA binding (Kumar *et al.* 1998).

It appears that EGCG can also impinge on NF-κB signalling. EGCG has been found to reduce iNOS gene expression in mouse peritoneal macrophages stimulated with LPS (Lin *et al.* 1997). This reduction in gene expression was due to the EGCG-induced suppression in NF-κB/DNA binding, which appeared to be mediated by blockade of IκBα degradation. Ahmad *et al.* (2000), have shown that EGCG lowered constitutive expression and activation of NF-κB in normal human epidermal keratinocytes (NHEK). EGCG inhibited LPS-induced gene expression in RAW 264.7 macrophage cells by suppressing nuclear DNA binding activity of NF-κB (Yang GY *et al.* 1998). Mukhtar *et al.* (1998) have shown in A431 human epidermoid carcinoma cells a 42 % decrease in nuclear NF-κB p65 subunit compared to control. This decrease in nuclear translocation was attributed to an EGCG-induced inhibition of IκBα degradation. In the JB6 murine epidermal cell line, PMA-induced transcriptional and DNA binding activity of NF-κB were inhibited by EGCG. This was through the blockade of IκBα serine 32 phosphorylation (Nomura *et al.* 2000).

The experiments described in this chapter were carried out to investigate the effects of four chemopreventive agents on the activity of the NF-κB signalling, in lymphoblast and HUVEC cells. Also of interest was the antioxidant capacity of these chemopreventive agents, as ROS are thought to contribute to atherosclerosis (As reviewed in Napoli *et al.* 2001), possibly through the modulation of NF-κB. As well as their antioxidant function,

some of these compounds have been shown to act as pro-oxidants under certain experimental conditions. For example, Long *et al.* (2000) have reported that the addition of EGCG and other tea polyphenols to cell culture medium (in the absence of cells), generated significant levels of H_2O_2, with the corollary that this 'artifactual' effect may account for some or all of the reported effects of EGCG in cell culture studies. Hence, the involvement of H_2O_2 in any EGCG-induced effects was also explored. Such data would help to elucidate the mechanisms of action of these chemopreventive agents with respect to NF-κB, and help to establish which of the agents may be suitable for further investigation as a potential cardiovascular chemopreventive compound.

In addition, these experiments might also highlight any differences between the normotensive and hypertensive B lymphoblast cell lines, which may explain why the disease of hypertension predisposes subjects to developing atherosclerosis. In humans there is evidence showing a relationship between an increased ROS production and hypertension (Pontremoli *et al.* 1989; Seifert *et al.* 1991). A report by Pettit *et al.* (2002) have shown that hypertensive B lymphoblast cell lines have an increased reactive oxygen species production in response to arachidonic acid (AA) and PMA stimulation, compared to normotensive B lymphoblast cell lines. Considering the above evidence it was deemed important to examine levels of ROS and how EGCG may affect these levels, between the different cell types.

3.2 Results

In all NF-κB activation experiments, unless otherwise stated, cells were serum-starved in 0 % FCS RPMI for B lymphoblasts or EGM for HUVEC, before treatment as described in the Materials and Methods chapter. Previous time courses and dose-response experiments had established that PMA (0.15 μM for 15 minutes) and TNFα (7.5 ng/ml for 15 minutes) treatments were the optimum concentration and time to induce maximum nuclear translocation of NF-κB (p65) in B lymphoblasts and HUVEC respectively (data not shown).

3.2.1 Comparison of constitutive and PMA-stimulated nuclear levels of NF-κB in B lymphoblasts

One of the aims of this thesis was to compare responses between normotensive and hypertensive B lymphoblasts. Therefore a comparison was made of the basal and stimulated levels of nuclear NF-κB p65 in each of the B lymphoblast cell lines. Cells were left untreated or exposed to DMSO or PMA (0.15 μM for 15 minutes). Equal concentrations of nuclear proteins were separated by SDS-PAGE and immunoblotted with an antibody directed against the p65 subunit of NF-κB. Data were normalised to the untreated sample of the C143 B lymphoblast cell line and were the result of 3 independent experiments.

There was evidence of constitutive basal nuclear NF-κB in each of the B lymphoblast cell lines, with C149 having the lowest levels (figure 3.1). DMSO treatment did not significantly affect nuclear levels of NF-κB (p65) compared to the untreated control. PMA stimulation significantly increased nuclear levels of NF-κB p65 nuclear translocation in each cell line ($p<0.05$). The percentage increase in nuclear NF-κB (p65) was 78 %, 73%, 64%, and 63% for C143, C149, H308 and H242 respectively.

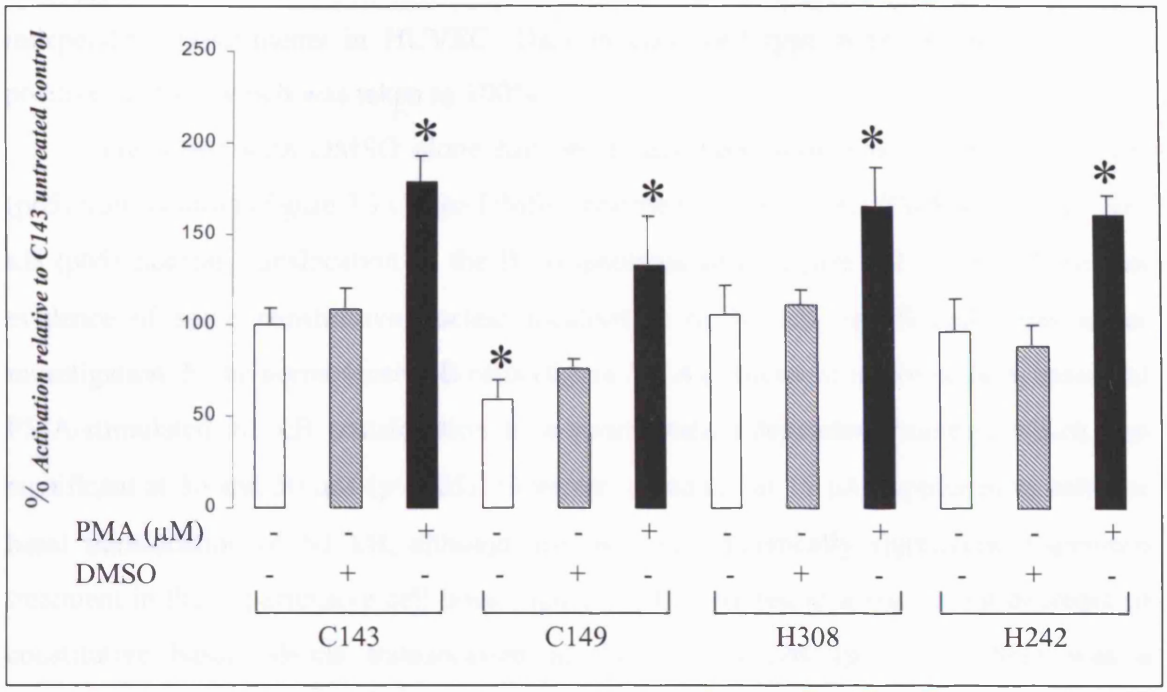

Figure 3.1 - Nuclear protein levels of NF-κB in the B lymphoblast cell lines. *Cells were untreated or treated with PMA (0.15 μM/15 mins) or mock treated with DMSO control. Nuclear proteins were extracted and equal amounts separated by SDS-PAGE, before immunoblotting with an antibody directed against the p65 subunit of NF-κB. DMSO treatment had no effect on NF-κB translocation. Data are the mean of 3 independent experiments.* indicates significant difference from the C143 control (untreated) group ($p<0.05$) as determined by the ANOVA balanced model followed by Tukey's least significant difference post hoc test.*

3.2.2 Curcumin inhibits constitutive and stimulated NF-κB nuclear translocation

To investigate the effect of curcumin on basal and stimulated NF-κB nuclear translocation, B lymphoblasts and HUVEC were stimulated with PMA (0.15 μM) and TNFα (7.5ng/ml) respectively, in the presence or absence of curcumin (15, 30, 50 μM for 45 minutes). Once treated, equal amounts of nuclear proteins were separated using SDS-PAGE and immunoblotted with an antibody directed against the p65 subunit of NF-κB. For B cells, as similar curcumin responses were noted, data for the normotensive (C143 and C149), and the hypertensive (H308 and H242) cell lines were combined respectively.

Figure 3.2 - illustrates the effect of curcumin on basal and stimulated nuclear translocation of NF-κB (p65) in normotensive (C143 and C149) (A) and hypertensive (H308 and H242) (B) B lymphoblast cell lines and HUVEC (C). Data for the normotensive

and hypertensive B cells were the result of 4 independent experiments respectively and 2 independent experiments in HUVEC. Data in each cell type were normalised to the positive control, which was taken as 100%.

Treatment with DMSO alone had previously been shown not to induce NF-κB (p65) translocation (figure 3.1). Also DMSO treatment did not effect PMA-stimulated NF-κB (p65) nuclear translocation in the B lymphoblast cells (figure 3.2 A, B). There was evidence of some constitutive nuclear localisation of NF-κB, in all cell types under investigation. In the normotensive B cells (figure 3.2 A), curcumin inhibited both basal and PMA-stimulated NF-κB translocation in a concentration-dependent manner, which was significant at 30 and 50 μM ($p<0.05$). However, curcumin at 15 μM, appeared to enhance basal translocation of NF-κB, although this was not statistically significant. Curcumin treatment in the hypertensive cell lines (figure 3.2 B) resulted in a significant decrease of constitutive basal NF-κB translocation at 30 and 50 μM ($p<0.05$). There was a concentration-dependent inhibition in PMA-stimulated nuclear NF-κB (p65), which was significant at 15, 30 and 50 μM curcumin ($p<0.05$).

Similar to the response in the normotensive B lymphoblast cells, curcumin-inhibited basal and TNFα-stimulated nuclear translocation of NF-κB in HUVEC (figure 3.2 C). The observed inhibition occurred in a concentration-dependent manner and was evident at 30 and 50 μM. However at a concentration of 15 μM, curcumin appeared to enhance basal NF-κB translocation. As noted in figure 3.5, although from a different experiment, treatment with DMSO had no significant effect on nuclear NF-κB (p65) levels.

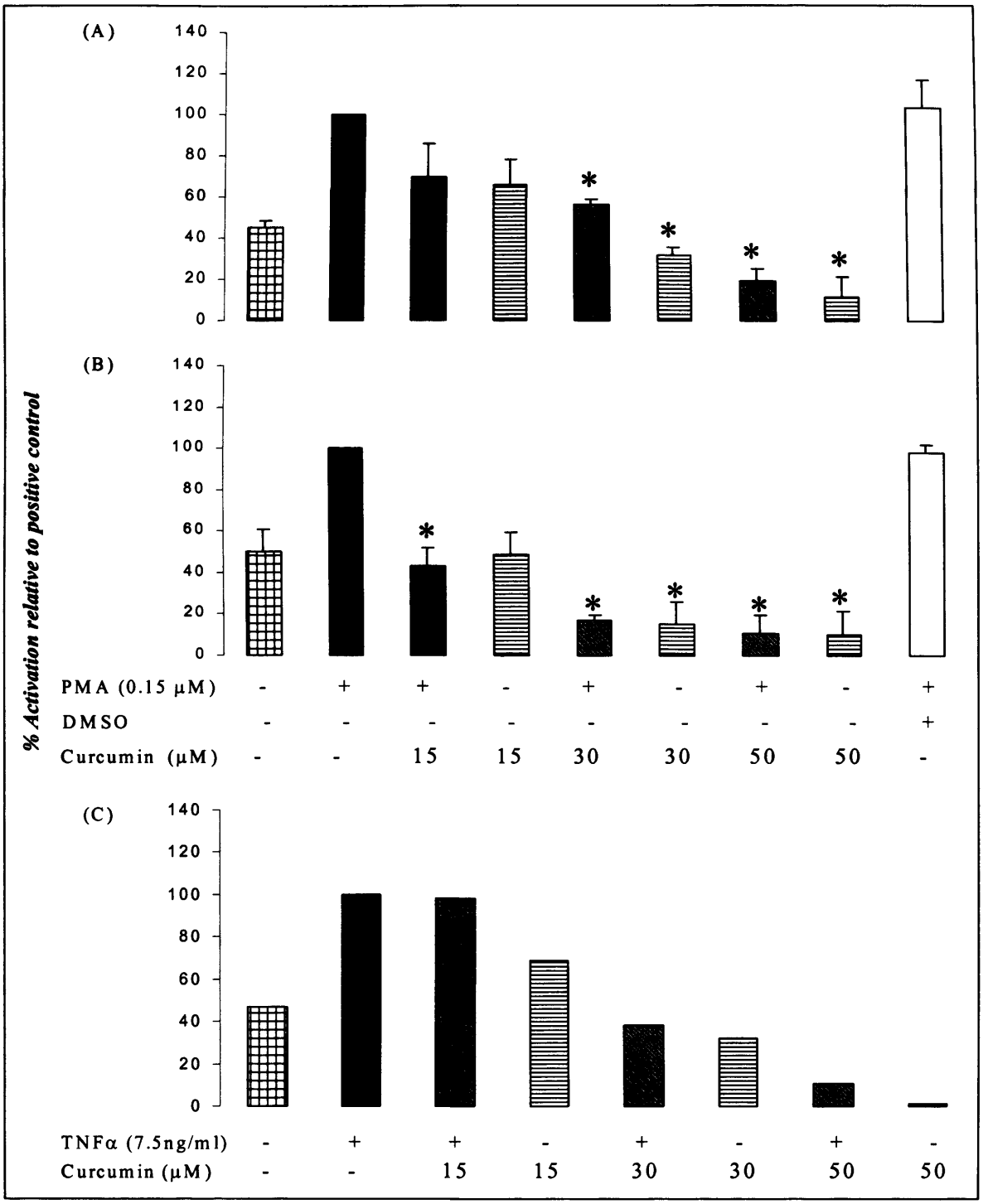

Figure 3.2 - Curcumin inhibits nuclear translocation in normotensive (C143 and C149) (A) and hypertensive (H308 and H242) (B) B lymphoblast cell lines, and HUVEC (C). *B lymphoblast cells or HUVEC were stimulated for 15 minutes with PMA (0.15 μM) or TNFα (7.5 ng/ml) respectively, in the presence or absence of a curcumin or DMSO (control), pretreatment for 45 mins. Nuclear proteins were extracted and equal amounts separated by SDS-PAGE, before immunoblotting with an antibody directed against the p65 subunit of NF-κB. Data for the normotensive and hypertensive B lymphoblasts are the mean of 4 independent experiments, and HUVEC are the mean of 2 independent experiments. * indicates significant difference from the control groups ($p<0.05$) as determined by the ANOVA balanced model followed by Tukey's least significant difference post hoc test.*

3.2.3 Resveratrol inhibits NF-κB p65 nuclear translocation

The effect of resveratrol on NF-κB nuclear translocation in B lymphoblast cell lines and HUVEC was investigated in a similar manner to that for curcumin. B lymphoblast cell lines and HUVEC were pre-treated for 45 minutes with resveratrol (15, 30, 50 μM concentrations), before exposure to 0.15 μM PMA (for B lymphoblast cell lines) or 7.5 ng/ml TNFα (for HUVEC). An equal amount of extracted nuclear protein for each treatment was separated on an SDS-PAGE gel, before being immunoblotted with an antibody against the p65 subunit of NF-κB. Figure 3.3 - illustrates the effect of resveratrol on basal and stimulated nuclear translocation of NF-κB (p65) in HUVEC and C143 and H308 B lymphoblast cell lines. Data were the result of 2 or 3 independent experiments for HUVEC and B lymphoblasts respectively, and were normalised to the positive control, which was taken as 100 %.

In each of the cell types, there was evidence of constitutive nuclear NF-κB (figure 3.3). The DMSO treatment had previously been shown not to affect NF-κB (p65) translocation (figure 3.1). Also DMSO treatment had no significant affect on stimulus-induced nuclear NF-κB (p65) levels (figure 3.3). In C143 (figure 3.3 A) data indicated that resveratrol significantly blocked basal and PMA-stimulated nuclear translocation of NF-κB at concentrations of 30 and 50 μM ($p<0.05$). It was also suggestive that 15 μM resveratrol might enhance basal and PMA-stimulated translocation, although these modest increases were not significant. Although only the result of one experiment, another normotensive cell line C149, indicated that 15, 30 and 50 μM resveratrol inhibited PMA-induced nuclear NF-κB (p65).

In H308, resveratrol significantly inhibited PMA-stimulated NF-κB nuclear translocation at 15 and 50 μM ($p<0.05$), but had no significant effect on basal constitutive levels (figure 3.3 B). Moreover, there is a suggestion that 30 μM resveratrol may have enhanced basal nuclear NF-κB, although this was not statistically significant. These results are similar when compared to the H242 hypertensive cell line, indicating that the effect was not cell-line specific (data not shown).

Although not dose-dependent it appeared that resveratrol blocked TNFα-stimulated NF-κB nuclear translocation in HUVEC (figure 3.3 C). However, basal levels of nuclear NF-κB were not noticeably affected by resveratrol. DMSO treatment had no significant effect on nuclear NF-κB (p65) levels as determined from figure 3.5.

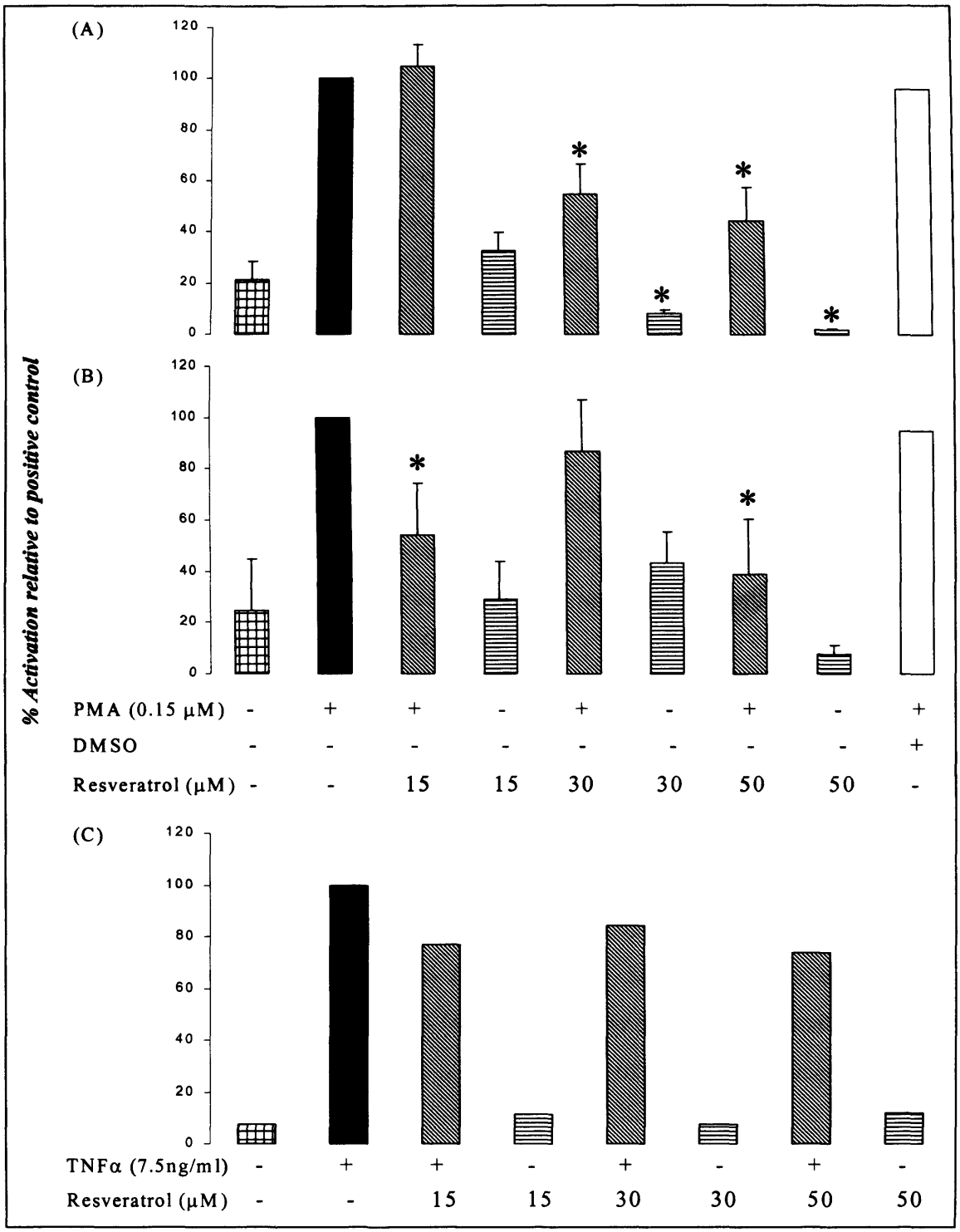

Figure 3.3 - Resveratrol inhibits nuclear translocation in the B lymphoblast cell lines C143 (A) and H308 (B), and HUVEC (C). *B lymphoblast cells or HUVEC were stimulated for 15 minutes with PMA (0.15 μM) or TNFα (7.5 ng/ml) respectively, in the presence or absence of a resveratrol or DMSO (control) pre-treatment for 45 mins. Nuclear proteins were extracted and equal amounts separated by SDS-PAGE, before immunoblotting with an antibody directed against the p65 subunit of NF-κB. (A) and (B) are the mean of 3 independent experiments, and (C) is the mean of 2 independent experiments. * indicates significant difference from the control groups ($p<0.05$) as determined by the ANOVA balanced model followed by Tukey's least significant difference post hoc test.*

3.2.4 Effect of I3C on constitutive and stimulated NF-κB p65 nuclear translocation

The effect of I3C on NF-κB nuclear translocation in B lymphoblast cell lines and HUVEC was investigated. B lymphoblast cell lines and HUVEC were pre-treated for 45 minutes with I3C (50, 100, and 500 μM concentrations), before exposure to 0.15 μM PMA (for B lymphoblast cell lines) or 7.5 ng/ml TNFα (for HUVEC). An equal amount of nuclear protein for each treatment was separated on an SDS-PAGE gel, before immunoblotting with an antibody directed against the p65 subunit of NF-κB. Figure 3.4 - shows histograms representing the effect of I3C on basal and stimulated nuclear translocation of NF-κB (p65) in HUVEC and C143 and H308 B lymphoblast cell lines. In B lymphoblasts, data were derived from 4 independent experiments, whereas in HUVEC, data were the result of a single experiment. In each instance data were normalised to the positive control, which was taken as 100 %.

DMSO treatment alone, had no significant effect on basal (figure 3.1) or PMA-induced NF-κB translocation (figure 3.4). In all cell types there was evidence of constitutive nuclear NF-κB. I3C had no significant effect on basal NF-κB nuclear translocation in C143 ($p<0.05$) (figure 3.4 A). However, I3C enhanced PMA-induced nuclear translocation of NF-κB at concentrations of 50, 100, and 500 μM, with statistical significance reached at 50 and 100 μM. The responses in H308 (figure 3.4 B) cell line were different than those observed in the C143 cell line. There was evidence of a significant decrease in PMA-stimulated NF-κB translocation following treatment with 50 μM I3C ($p<0.05$). Interestingly, I3C at a concentration of 50 μM significantly enhanced basal nuclear NF-κB ($p<0.05$).

In HUVEC, I3C did not affect the constitutive levels of NF-κB p65 (figure 3.4 C). However, although only a result of one experiment, it did appear that I3C inhibited the TNFα-induced increase in NF-κB p65. This inhibition was concentration-dependent and was most obvious at 500 μM I3C. As I3C was the least effective of the four agents, it was decided not to follow this experiment up further in HUVEC.

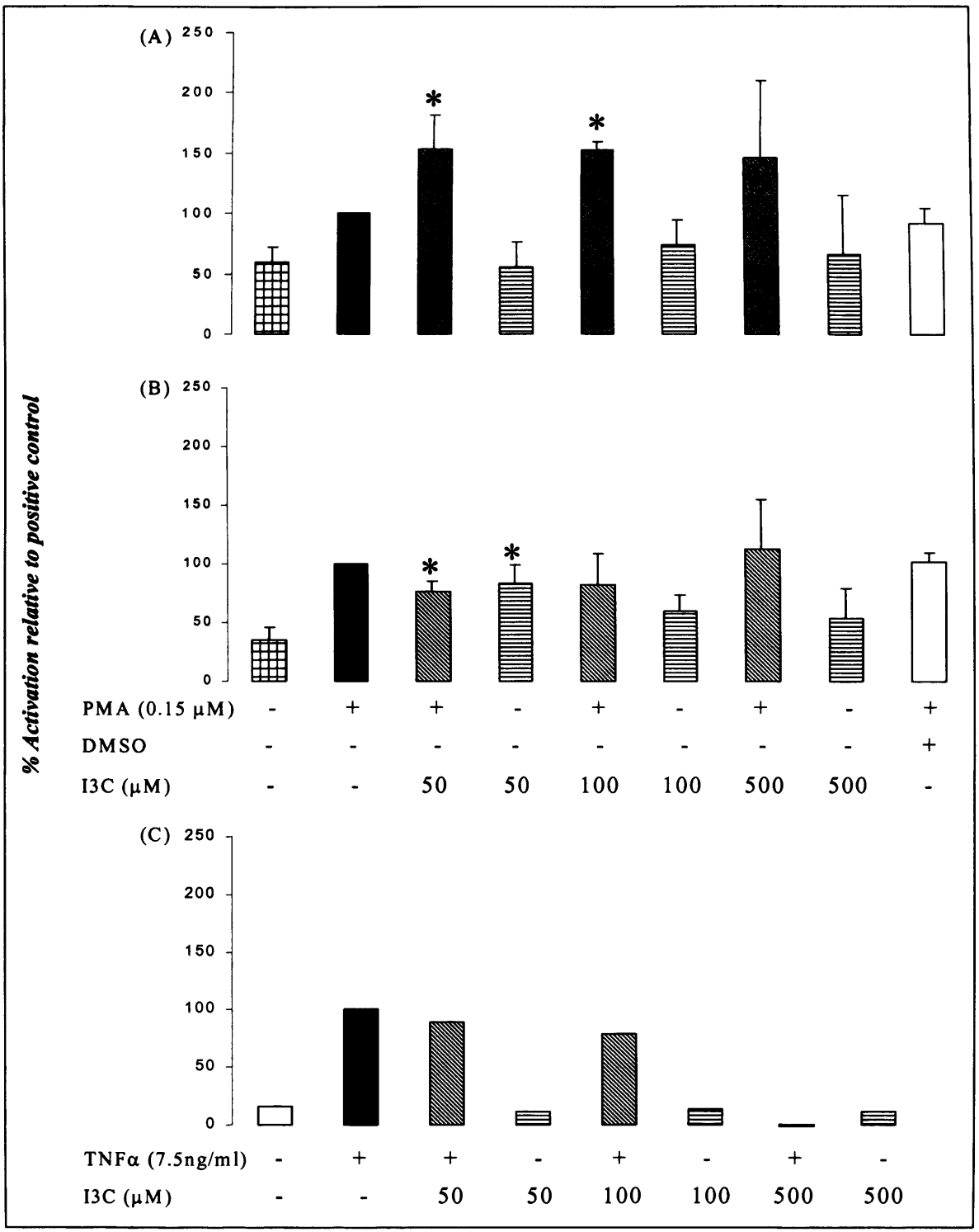

Figure 3.4 - Effect of I3C on nuclear NF-κB translocation in C143 (A), and H308 (B), and HUVEC (C). *Cells were stimulated for 15 minutes with PMA (0.15 µM), in the presence or absence of a I3C or DMSO (control) pretreatment for 45 mins. Nuclear proteins were extracted and equal amounts separated by SDS-PAGE, before immunoblotting with an antibody directed against the p65 subunit of NF-κB. Data are the mean of 3 independent experiments for B lymphoblasts and 1 experiment for HUVEC. * or * indicate significant difference from the PMA-treated or untreated control groups respectively, ($p<0.05$) as determined by the ANOVA balanced model followed by Tukey's least significant difference post hoc test.*

3.3 EGCG inhibits constitutive and stimulated NF-κB p65 nuclear translocation

The effect of EGCG on basal and stimulated NF-κB nuclear translocation was investigated. NF-κB was stimulated with PMA (0.15 μM) for 15 minutes and TNFα (7.5 ng/ml), in B lymphoblasts and HUVEC respectively. Nuclear proteins were isolated, subjected to Western blotting and probed with an antibody directed against the p65 subunit of NF-κB. Figure 3.5 - shows histograms representing the effect of EGCG on basal and stimulated nuclear translocation of NF-κB (p65) in HUVEC and C143 and H308 B lymphoblast cell lines. For each cell type data were derived from 3 independent experiments. In each example data were normalised to the positive control, which was taken as 100 %.

Figure 3.5 represents the effects of EGCG on basal and PMA-stimulated NF-κB nuclear translocation in the B lymphoblast cell lines, C143 (A) and H308 (B). Data were normalised as a percentage of the PMA treatment, the positive control. The DMSO treatment (which was a control for the EGCG treatment), did not affect PMA-induced translocation indicating that any inhibitory effect was likely to be due to EGCG alone. There was some constitutive nuclear NF-κB as evidenced by the detection of a p65 NF-κB immunoreactive band in the untreated samples. In both C143 and H308, EGCG inhibited PMA-induced NF-κB translocation in a concentration-dependent manner, and was significant at 30 and 50 μM in C143 and 15, 30 and 50 μM in H308 ($p<0.05$). In addition 50 μM EGCG in C143, and 30 and 50 μM EGCG in H308 significantly inhibited constitutive nuclear NF-κB ($p<0.05$). A similar response was observed in additional normotensive (C149) and hypertensive (H242) B lymphoblast cell lines, suggesting that this effect was not cell line specific (data not shown).

Figure 3.5 (C) shows the effect of 45 minutes pre-treatment of EGCG (at concentrations of 15, 30, 50 μM) on basal and TNFα-stimulated NF-κB nuclear translocation in HUVEC. DMSO treatment had no significant effect. However, significant inhibition of TNFα-induced NF-κB (p65) translocation was observed at EGCG concentrations of 30 and 50 μM ($p<0.05$).

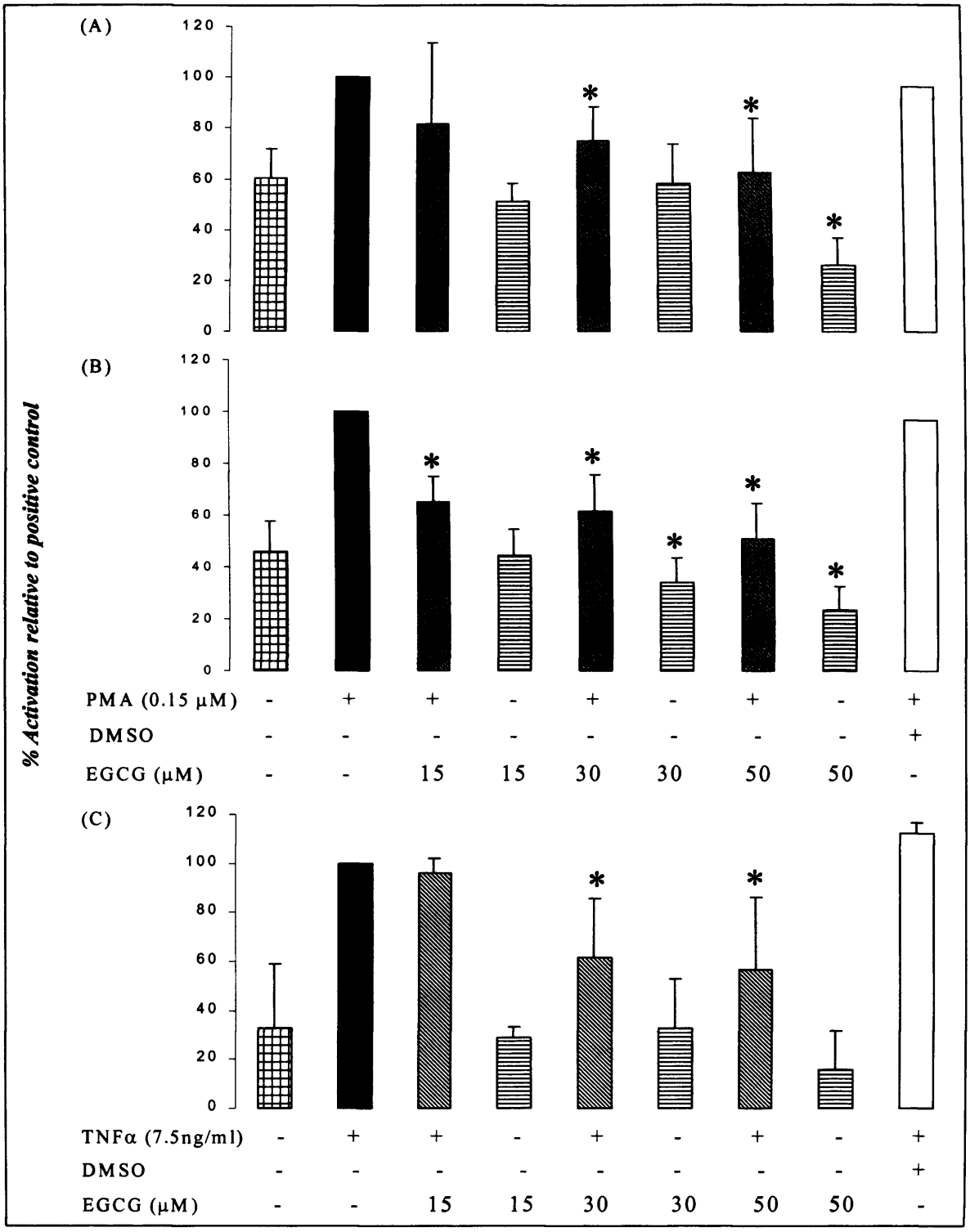

Figure 3.5 - EGCG inhibits nuclear translocation in B lymphoblast cell lines C143 (A) and H308 (B), and HUVEC (C). *B lymphoblast cells or HUVEC were stimulated for 15 minutes with PMA (0.15 μM) or TNFα (7.5 ng/ml) respectively, in the presence or absence of a EGCG or DMSO (control) pre-treatment for 45 mins. Nuclear proteins were extracted and equal amounts separated by SDS-PAGE, before immunoblotting with an antibody directed against the p65 subunit of NF-κB. Data are the mean of 3 independent experiments.* indicates significant difference from the control groups ($p<0.05$) as determined by the ANOVA balanced model followed by Tukey's least significant difference post hoc test.*

3.3.1 Effect of EGCG on constitutive NF-κB p52, RELB and p50 subunits in C143 B lymphoblasts

It was decided to examine the effect of EGCG on other subunits of NF-κB. This preliminary study was performed only in the C143 cell line (figure 3.6). C143 cells were treated with EGCG 30, 50 and 100 μM at times of 30, 40, 60, 90 and 120 minutes. Nuclear proteins were isolated, equal amounts separated by SDS-PAGE analysis, before immunoblotting with an antibody directed against NF-κB p50, p52 or RELB.

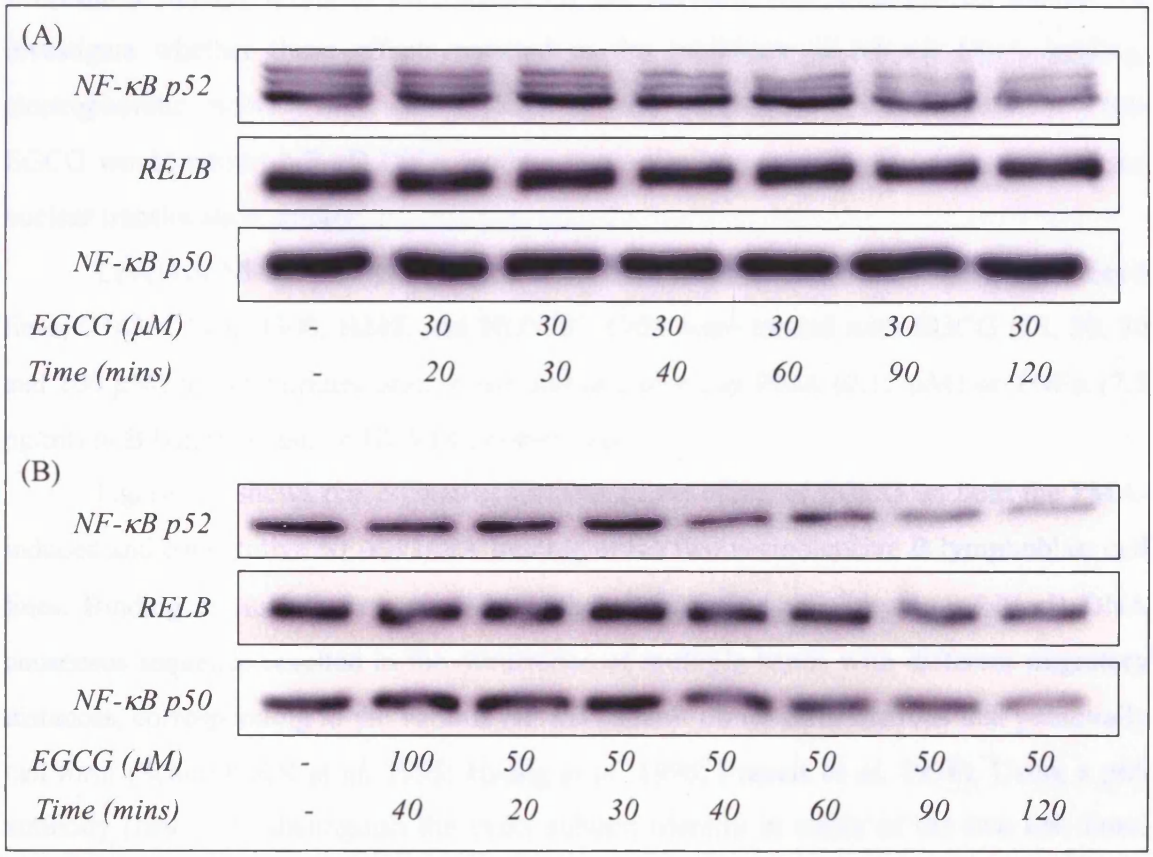

Figure 3.6 - Effect of EGCG on basal levels of nuclear p52, RELB and p50 in C143 B lymphoblast cells*. Cells were treated with EGCG 30 μM (A), 50 or 100 μM (B). Nuclear proteins were isolated, separated by SDS-PAGE, and immunoblotted with antibodies directed against p52, RELB, and p50 subunits of NF-κB. Data are the representative of two independent experiments.*

It was evident that the p52, RELB, and p50 subunits of NF-κB were present in the nucleus of non-stimulated C143 B lymphoblast cells. Basal levels of nuclear p52 decreased following 90 and 120 minutes of treatment with 30 μM EGCG (figure 3.6 A) and40, 60, 90, and 120 minutes with 50 μM EGCG (figure 3.6 B). There was a small decrease in

RELB at 90 and 120 minutes treatment with 50 μM EGCG, and in p50 protein at 40, 60, 90 and 120 minutes treatment with 50 μM EGCG. With no significant change in p50 at 30 μM, this blot also acted as a loading control.

3.3.2 EGCG inhibits NF-κB DNA binding in B lymphoblasts cell lines and HUVEC

The data from Western analyses demonstrate that NF-κB nuclear translocation and constitutive nuclear levels of the various NF-κB subunits were inhibited by EGCG. To investigate whether these effects resulted in the inhibition of NF-κB DNA binding, electrophoretic mobility shift assays (EMSA) were performed. It was hypothesised that EGCG would inhibit NF-κB DNA binding at similar concentrations to those used in the nuclear translocation studies.

Levels of NF-κB DNA-binding activity were investigated in the B lymphoblast cell lines, C143, C149, H308, H242, and HUVEC. Cells were treated with EGCG (15, 30, 50 and 100 μM) for 45 minutes prior to the addition of either PMA (0.15 μM) or TNFα (7.5 ng/ml) to B lymphoblasts or HUVEC, respectively.

Figure 3.7 shows representative EMSAs of the effect of EGCG on both the PMA-induced and constitutive NF-κB DNA binding in the two normotensive B lymphoblast cell lines. Binding of nuclear extracts from both normotensive cell lines to a NF-κB DNA consensus sequence resulted in the occurrence of multiple bands with different migratory distances, corresponding to the various NF-κB subunit dimer combinations that potentially can form (Schmidt KN *et al.* 1995; Huang *et al.* 1996; Francis *et al.* 1998). Using a p65 antibody (lane 3) to distinguish the exact subunit identity in either of the two cell lines, suggested that at least one of the bands contained p65 (lanes 2 in C143 and C149). Excess unlabelled NF-κB oligonucleotide competitively inhibited NF-κB binding, as evidenced by the absence of bands in treatment lanes 14. Conversely the excess unlabelled non-specific AP-1 oligonucleotide did not affect binding, indicating binding specificity (lanes 15).

PMA did not significantly alter the binding of NF-κB to the DNA in the C143 normotensive B lymphoblast cell line (lane 5), when compared to the untreated (lane 3) or DMSO (lane 4) control samples (figure 3.7 A). This may have been due to high constitutive levels of NF-κB DNA binding present.

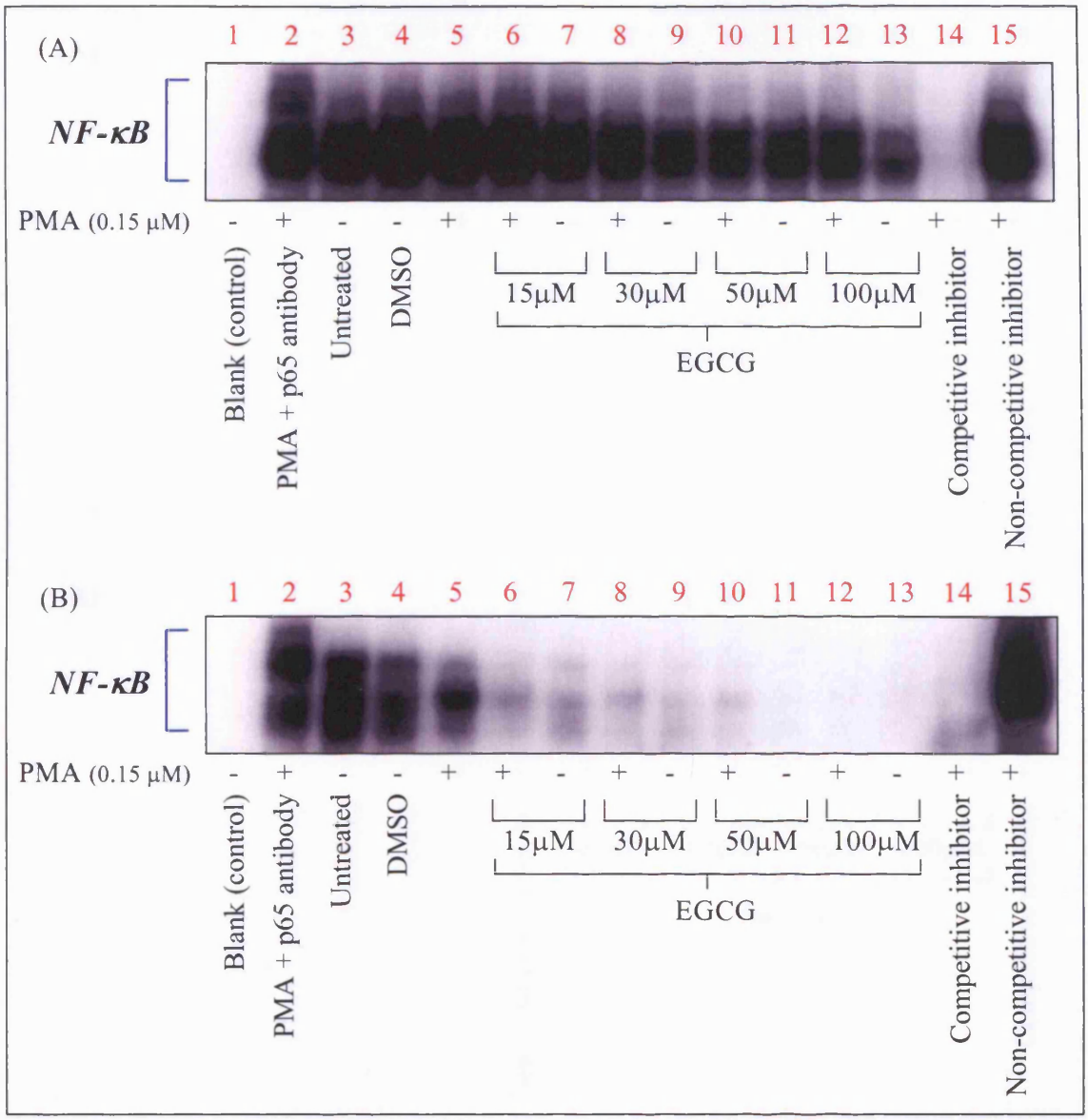

Figure 3.7 - EGCG inhibits NF-κB/DNA binding in C143 (A) and C149 (B) normotensive B lymphoblast cell lines. *Cells were serum-starved for 24 hours. Serum-free medium was changed again at least 1 hour prior to treatment. EGCG was added at the indicated concentrations, 45 minutes before incubation with PMA (0.15μM) for 15 minutes. Nuclear extracts were isolated as described in materials and methods. Four μg of these extracts were incubated with a ^{32}P-labelled NF-κB consensus oligonucleotide and a NF-κB (p65) antibody in lane 2, before resolving the DNA/NF-κB complex by acrylamide gel electrophoresis. Non-labelled NF-κB oligonucleotide competitively inhibited the band of interest and unlabelled AP-1 as a non-competitive oligonucleotide indicated the specificity of NF-κB DNA binding. The images shown are representative of three independent experiments.*

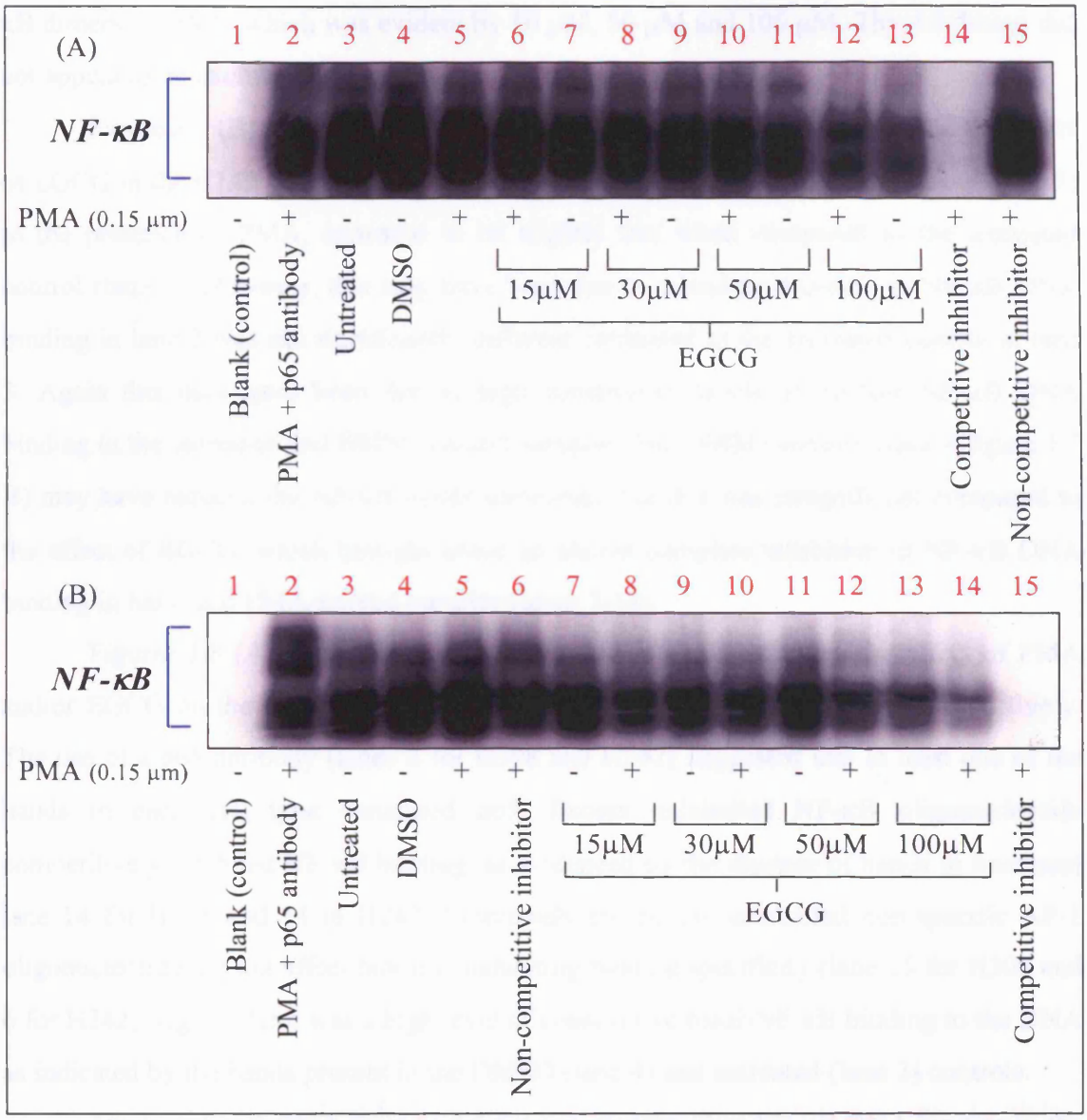

Figure 3.8 - EGCG inhibits NF-κB/DNA binding in H308 (A) and H242 (B) hypertensive B lymphoblast cell lines. *Cells were serum-starved for 24 hours. Serum-free medium was changed again at least 1 hour prior to treatment. EGCG was added at the indicated concentrations, 45 minutes before incubation with PMA (0.15μM) for 15 minutes. Nuclear extracts were isolated as described in materials and methods. Four μg of these extracts were incubated with a ^{32}P-labelled NF-κB consensus oligonucleotide and a NF-κB (p65) antibody in lane 2, before resolving the DNA/NF-κB complex by acrylamide gel electrophoresis. Non-labelled NF-κB oligonucleotide competitively inhibited the band of interest and unlabelled AP-1 as a non-competitive oligonucleotide indicated the specificity of NF-κB DNA binding. The images shown are representative of 3 independent experiments.*

Importantly the DMSO control did not reduce the basal levels of NF-κB DNA binding (lane 4). However in lanes 8-13, EGCG brought about a decrease in the binding of all NF-

κB dimers to DNA, which was evident by 30 μM, 50 μM and 100 μM. This inhibition did not appear to be exclusively concentration-dependent.

Figure 3.7 (B) shows the PMA-induced NF-κB DNA binding pattern and the effect of EGCG in the C149 normotensive B lymphoblast cell line. NF-κB DNA binding (lane 5) in the presence of PMA, appeared to be slightly less when compared to the untreated control (lane 3). However, this may have been due to a loading problem as NF-κB DNA binding in lane 2 was not significantly different compared to the untreated control in lane 3. Again this may have been due to high constitutive levels of nuclear NF-κB DNA binding in the untreated and DMSO control samples. The DMSO control (lane 4 figure 3.7 B) may have reduced the NF-κB bands somewhat, but this was insignificant compared to the effect of EGCG, which brought about an almost complete inhibition in NF-κB DNA binding in basal and PMA-treated samples (lanes 7-14).

Figures 3.8 (A) and (B) are representative EMSAs illustrating the effect of PMA and/or EGCG on the induction of NF-κB DNA binding in H308 and H242 respectively. The use of a p65 antibody (lanes 2 for H308 and H242) suggested that at least one of the bands in each cell type contained p65. Excess unlabelled NF-κB oligonucleotide competitively inhibited NF-κB binding, as evidenced by the absence of bands in treatment lane 14 for H308 and 15 in H242. Conversely the excess unlabelled non-specific AP-1 oligonucleotide did not affect binding, indicating binding specificity (lane 15 for H308 and 6 for H242). Again, there was a high level of constitutive basal NF-κB binding to the DNA as indicated by the bands present in the DMSO (lane 4) and untreated (lane 3) controls.

Treatment with PMA (lane 5) did not significantly alter NF-κB DNA binding in the H308 B lymphoblast cell line (figure 3.8 A) when compared to the untreated control (lane 3). DMSO did not significantly alter NF-κB DNA binding (lane 4). However, EGCG appeared to reduce both PMA and basal NF-κB binding at 15 to 50 μM (lanes 6 and 11) and was more obvious at 100 μM (lanes 12 and 13).

There was evidence of a slight increase in NF-κB DNA binding in H242 following PMA stimulation (lane 5), when compared to the untreated control (lane 3) (figure 3.8 B). There was no difference in binding between the untreated control (lane 3) and the DMSO (lane 4) control. There was a decrease in NF-κB DNA binding in basal and PMA-treated samples at 15 (lanes 7 and 8), 30 (lanes 9 and 10), 50 (lanes 11 and 12) and 100 μM (lanes 13 and 14) EGCG, compared to the controls.

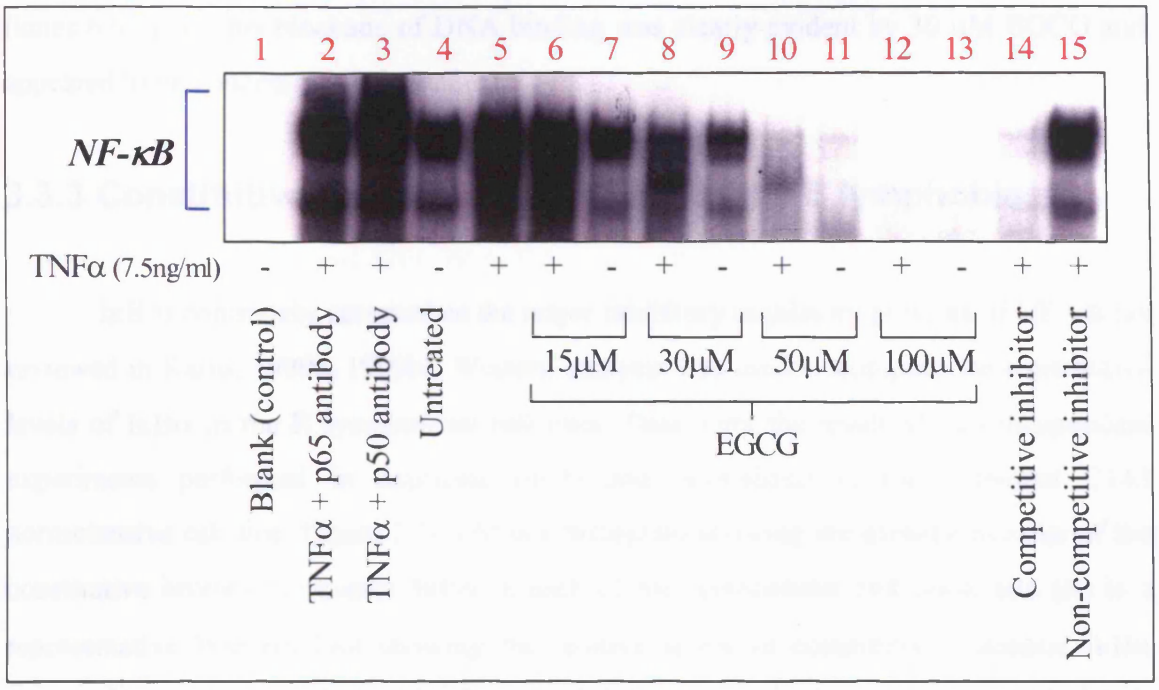

Figure 3.9 - EGCG inhibits the TNFα-induced NF-κB/DNA binding in HUVEC. *Cells were grown to 90 % confluency before serum starvation for 1 hour. EGCG was added at the indicated concentrations, 45 minutes before incubation with TNFα (7.5 ng/ml) for 15 minutes. Nuclear extracts were isolated as described in materials and methods. Four μg of these extracts were incubated with a ^{32}P-labelled NF-κB consensus oligonucleotide and antibodies directed against p65 and p50 subunits of NF-κB (lanes 2 and 3) before resolving the DNA/NF-κB complex by acrylamide gel electrophoresis. Non-labelled NF-κB oligonucleotide competitively inhibited the band of interest and unlabelled AP-1 a non-competitive oligonucleotide indicated the specificity of NF-κB DNA binding. The image shown is representative of 3 independent experiments.*

Figure 3.9 is a representative EMSA showing the effect of EGCG on TNFα-induced NF-κB/DNA binding in HUVEC. TNFα treatment alone in lane 5, increased the binding of NF-κB to the DNA oligonucleotide, compared to the untreated control (lane 4). It was noted that there was some NF-κB DNA binding in the untreated sample, indicating constitutive nuclear NF-κB DNA binding (lane 4). Antibodies to the p65 and p50 subunit were included (lanes 2 and 3 respectively) and indicated that some of the dimer combinations contained p65 and p50. The results show that the NF-κB bands were competitively inhibited following the addition of excess unlabelled (cold) NF-κB oligonucleotide (lane 14), but not by the addition of the non-specific (AP-1) oligonucleotide (lane 15). Taken together these two results indicate the specificity of binding of NF-κB to the DNA. It was clear from the data that EGCG reduced both the basal and TNFα-induced binding of NF-κB subunits in a concentration-dependent manner

(lanes 6 to 13). This blockade of DNA binding was clearly evident by 30 μM EGCG and appeared to be concentration dependent.

3.3.3 Constitutive protein levels of IκBα in the B lymphoblasts

IκB is commonly accepted as the major inhibitory regulatory proteins of NF-κB (as reviewed in Karin, 1999a, 1999b). Western analysis was used to compare the constitutive levels of IκBα in the B lymphoblast cell lines. Data were the result of two independent experiments performed in duplicate (n=4) and normalised to the untreated C143 normotensive cell line. Figure 3.10 (A) is a histogram showing the meaned average of the constitutive levels of cytosolic IκBα in each of the lymphoblast cell lines, and (B) is a representative Western blot showing the relative levels of constitutive cytosolic IκBα protein. It appeared that the C149 cell line had the least constitutive cytosolic protein levels of IκBα. There were no obvious differences in the cytosolic levels of IκBα protein between C143, H308 or H242 cell lines.

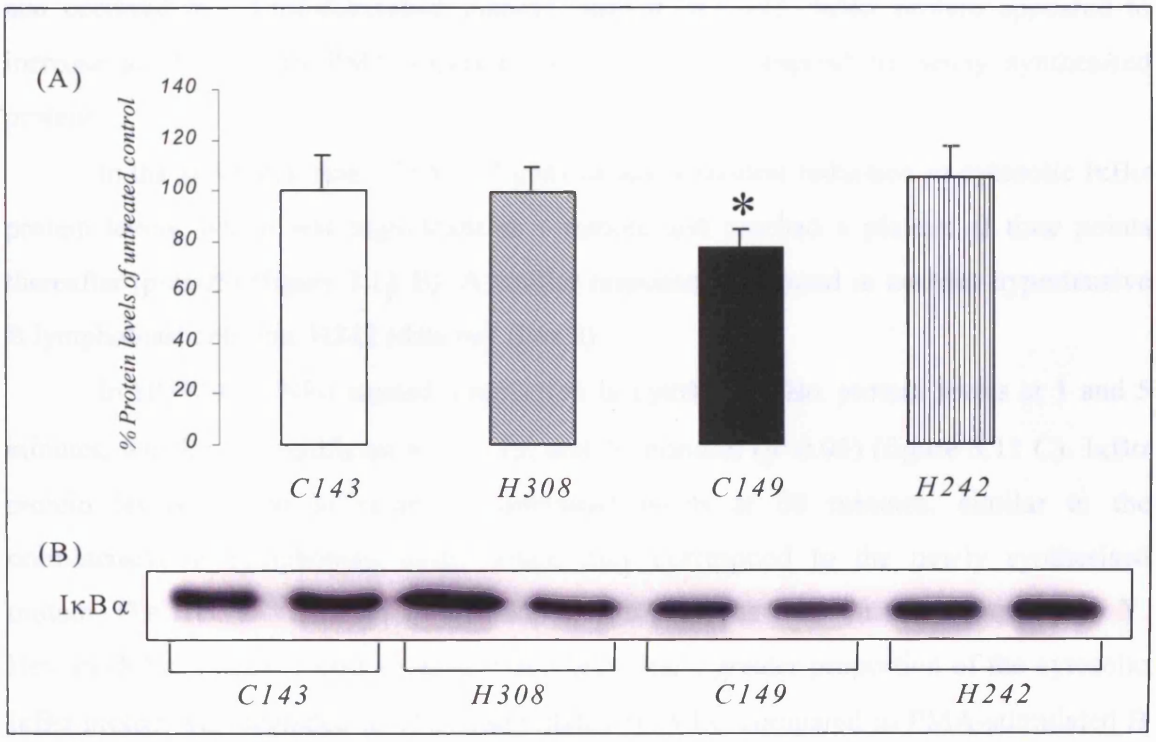

Figure 3.10 - The levels of IκBα in unstimulated B lymphoblast cell lines. *Cytosolic proteins were isolated, separated by SDS-PAGE and immunoblotted with an antibody directed against IκBα, as described in materials and methods. (A) represents the averaged protein levels of IκBα in each cell type, (B) representative Western blot of IκBα in each cell line. Data are the mean of two independent experiments performed in duplicate (N=4).*

3.3.4 PMA- and TNFα-induced IκBα degradation

The IκBα protein is one of the main inhibitors (along with other IκB family members) sequestering NF-κB in the cytosol, in an inactive state. To examine whether PMA- and TNFα-stimulated NF-κB nuclear translocation involved IκB, cytosolic proteins were isolated and an equal amount of protein separated by SDS-PAGE, before immunoblotting with an antibody directed against IκBα. Data from each cell type were normalised as a percentage of the untreated control. Data from the B lymphoblasts and HUVEC were the result of three independent experiments.

PMA (0.3 μM) induced a decrease in IκBα protein in a time-dependent manner, which was evident by 5 minutes and significant at 15 minutes in the C143 B lymphoblast cell line ($p<0.05$) (figure 3.11 A). Although not significantly different from the protein levels at 60 minutes, there may have been a modest increase in IκBα protein at 90 minutes PMA exposure, which may correspond to newly synthesised protein. In the C149 cell line (data not shown), IκBα degradation was evident at 10 minutes PMA (0.3 μM) exposure, and occurred in a time-dependent manner, similar to C143. IκBα protein appeared to increase at 60 minutes PMA exposure, which may correspond to newly synthesised protein.

In the H308 cell line, PMA (0.3 μM) caused a modest reduction in cytosolic IκBα protein levels, which was significant at 1 minute and reached a plateau at time points thereafter ($p<0.05$) (figure 3.11 B). A similar response was found in another hypertensive B lymphoblast cell line, H242 (data not shown).

In HUVEC, TNFα caused a reduction in cytosolic IκBα protein levels at 1 and 5 minutes, which was significant by 10, 15, and 30 minutes ($p<0.05$) (figure 3.11 C). IκBα protein levels started to return to untreated levels at 60 minutes, similar to the normotensive B lymphoblast cells, which may correspond to the newly synthesised protein. The HUVEC data are in agreement with previous work in the laboratory by Y. Heidari (MSc. Thesis, 1998). Overall it appeared that a greater proportion of the cytosolic IκBα protein was degraded in TNFα-stimulated HUVEC compared to PMA-stimulated B lymphoblast cells.

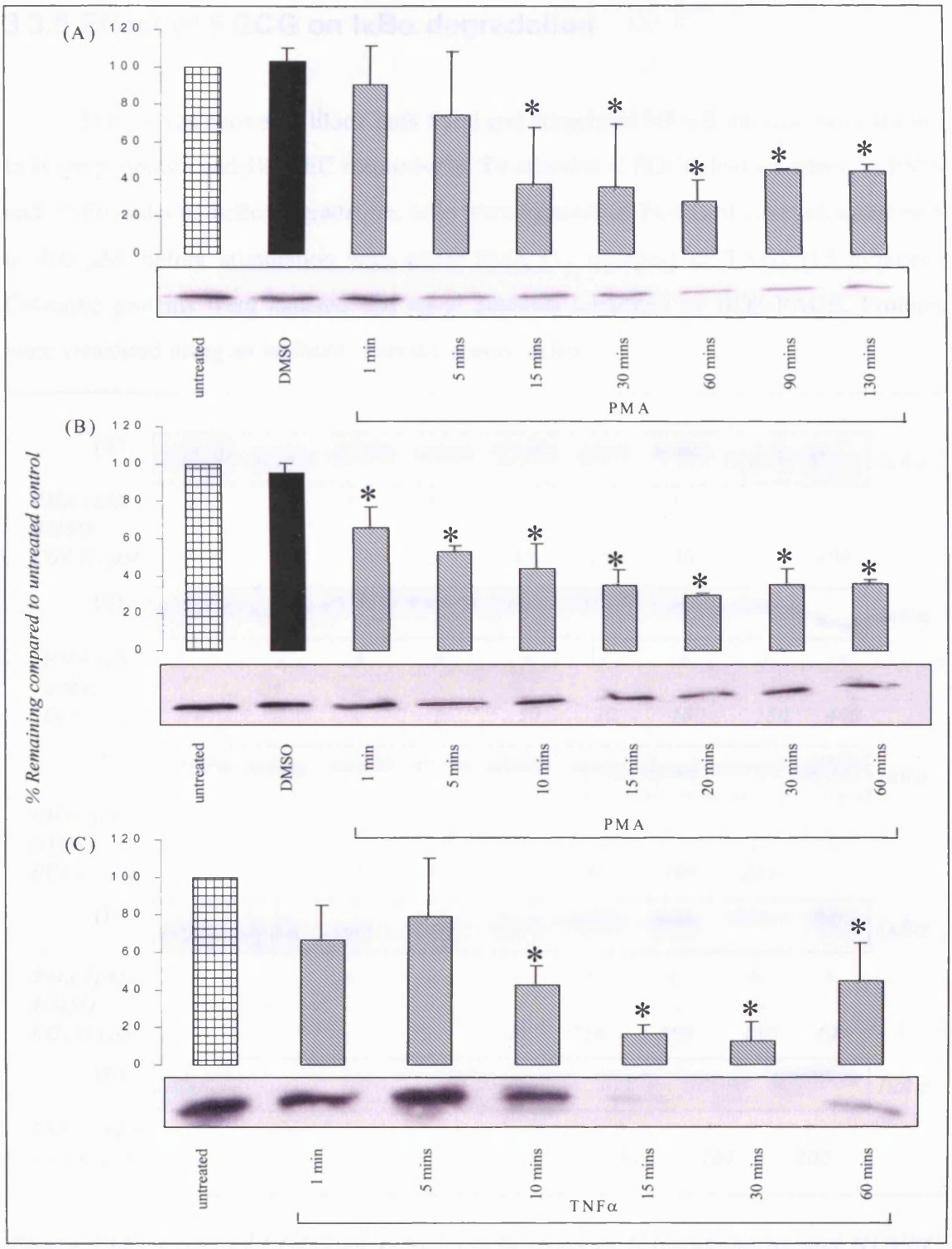

Figure 3.11 - Stimulus-induced degradation of IκBα in C143 (A), H308 (B) B lymphoblasts and HUVEC (C). *B lymphoblast cells or HUVEC were stimulated with PMA (0.3 μM) or TNFα (7.5 ng/ml) at the indicated treatment times, respectively. Cytosolic proteins were extracted and equal amounts separated by SDS-PAGE, before immunoblotting with an antibody directed against the IκBα. Data are the mean of 3 independent experiments.* indicates significant difference from the control (untreated) group ($p<0.05$) as determined by the ANOVA balanced model followed by Tukey's least significant difference post hoc test.*

3.3.5 Effect of EGCG on IκBα degradation

EGCG was shown to block both basal and stimulated NF-κB nuclear translocation in B lymphoblasts and HUVEC respectively. To examine if EGCG had an effect on PMA and TNFα-induced IκBα degradation, cells were exposed to EGCG at concentrations of 5 to 400 μM, before stimulation with either PMA (15 minutes) or TNFα (15 minutes). Cytosolic proteins were isolated and equal amounts separated by SDS-PAGE. Proteins were visualised using an antibody directed against IκBα.

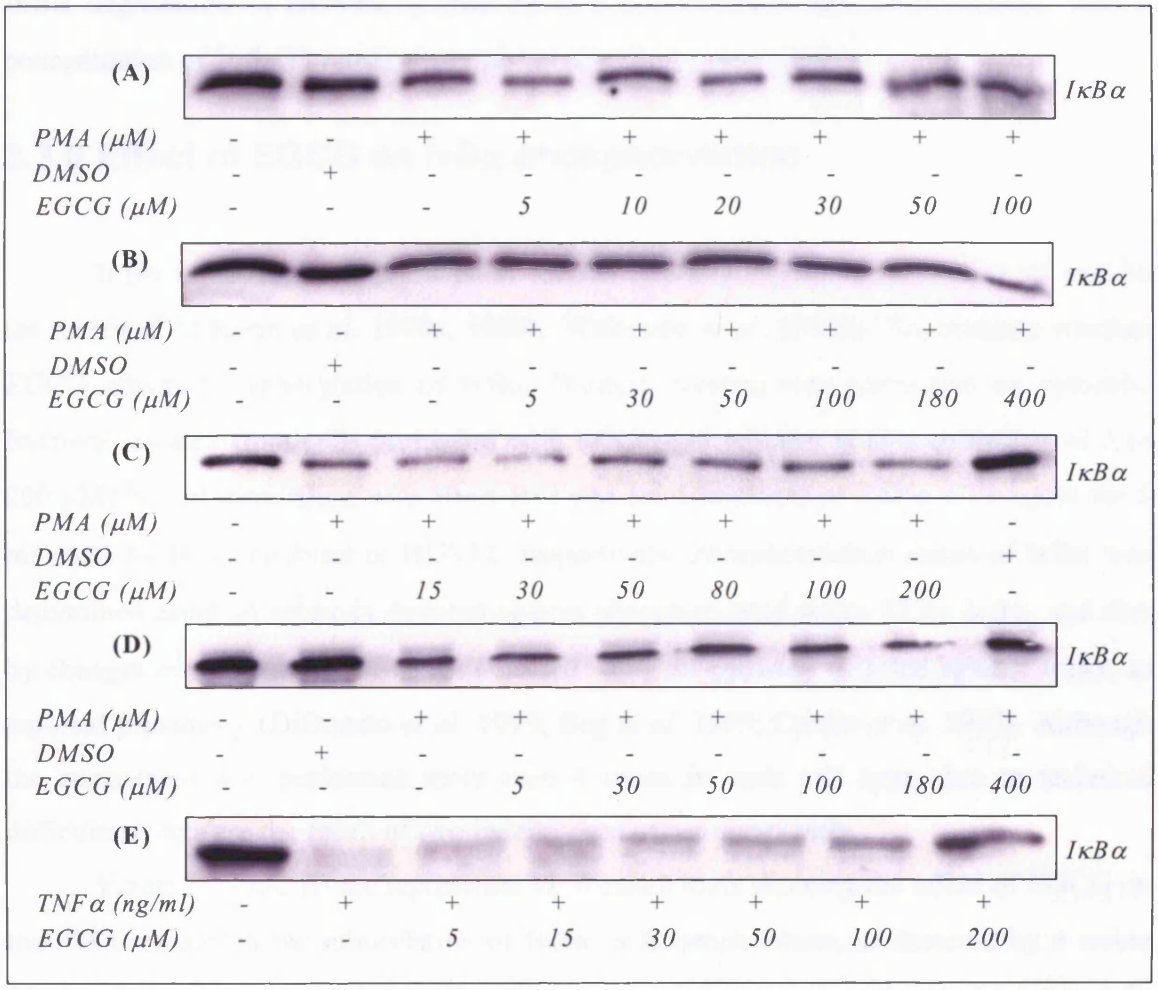

Figure 3.12 - Effect of EGCG on IκBα protein levels in B lymphoblasts and HUVEC. *Cells were pretreated with EGCG (45 minutes) before stimulation with either PMA (15 minutes) or TNFα (15 minutes) or mock stimulated with DMSO. Cytosolic proteins were isolated, separated by SDS-PAGE and immunoblotted with an antibody directed against IκBα, as described in materials and methods. (A, B, C, D) are representative Western blots corresponding to C143, C149, H308, and H242 B lymphoblasts respectively, from two independent experiments in each cell line. (E) is representative of 3 Western blots for HUVEC.*

For B lymphoblasts, these experiments were performed 4 different times, however due to technical difficulties the data here are representative of two independent experiments in each cell type. In HUVEC data were the result of 3 independent experiments. Figure 3.12 contains representative Western blots, showing the effect of EGCG on PMA- and TNFα-induced IκBα degradation in the B lymphoblasts, C143 (A), C149 (B), H308 (C), H242 (D) and HUVEC (E), respectively. EGCG does not appear to fully protect IκBα from degradation in the B lymphoblast cell lines following stimulation with PMA except at 400 μM in H242. However, EGCG appears to prevent TNFα-induced IκBα degradation in HUVEC (figure E) in a concentration-dependent manner, with a concentration of 200 μM required to prevent significant degradation.

3.3.6 Effect of EGCG on IκBα phosphorylation

IκBα is targeted for degradation following phosphorylation on serines 32 and 36 (as reviewed in Karin *et al.* 1999a, 1999b, Whiteside *et al.* 1997b). To examine whether EGCG effects phosphorylation of IκBα, Western blotting was performed on cytosolic fractions isolated from cells pretreated with EGCG (45 minutes at concentrations of 5 to 200 μM) before stimulation with PMA (0.3 μM for 5 minutes) or TNFα (7.5 ng/ml for 5 minutes) for B lymphoblast or HUVEC respectively. Phosphorylation status of IκBα was determined using an antibody directed against phosphorylated serine 32 on IκBα, and also by changes in the mobility of bands detected using an antibody directed against IκBα, as reported previously (DiDonato *et al.* 1995; Beg *et al.* 1993; Cordle *et al.* 1993). Although the experiment was performed more than 4 times in each cell type, due to technical difficulties, data are the result of two independent experiments only.

Figure 3.13 (A, B) are representative Western blots showing the effect of EGCG on the PMA-stimulated phosphorylation of IκBα in B lymphoblasts, as detected by a serine 32 phosphorylated IκBα antibody. Equal protein loads were confirmed as indicated by LC, which represents the protein levels of a cytosolic protein (present on the same gel) to which the antibody bound non-specifically. PMA (0.3 μM, 5 minutes) induced serine 32-phosphorylation of IκBα in both C143 (A) and H308 (B) lymphoblasts respectively. Data indicated that EGCG at the concentrations up to 100 μM, did not inhibit phosphorylation of serine 32, in C143 (A) or H308 (B) B lymphoblast cell lines. These observations were supported by further Western analyses using an antibody directed against IκBα, which

recognises both the phosphorylated and non-phosphorylated form of the protein (figure 3.13 C, D). Using this antibody both faster and slower mobility bands were detected in B lymphoblasts and HUVEC, following stimulation with either PMA or TNFα respectively.

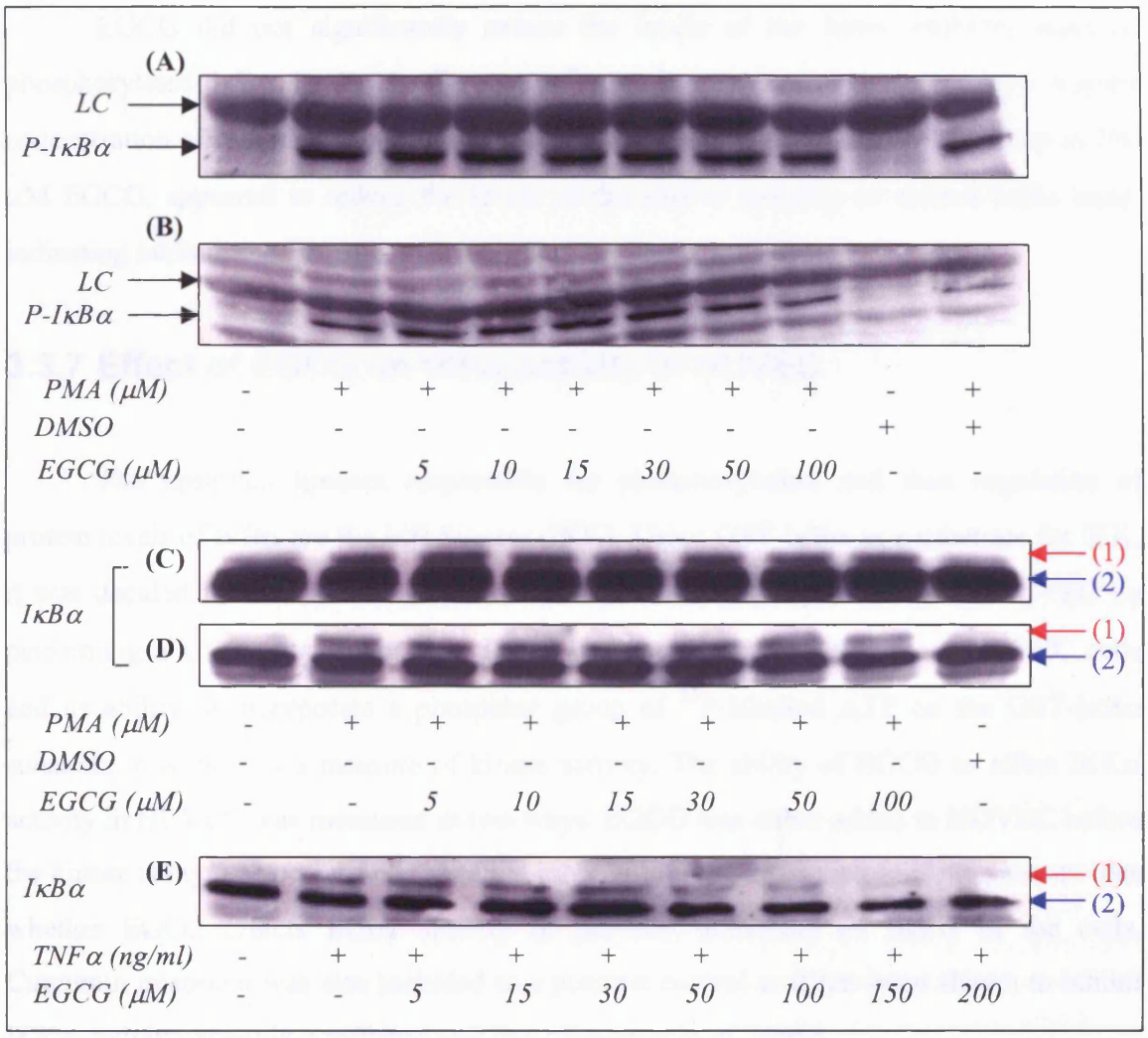

Figure 3.13 - Effect of EGCG on PMA and TNFα-induced IκBα phosphorylation in B lymphoblasts and HUVEC respectively. *B lymphoblast cells and HUVEC were pretreated with EGCG (at the indicated concentrations for 45 minutes) before stimulation with either PMA (0.3 μM for5 mins) or TNFα (7.5 ng/ml for 5 mins) respectively. Cytosolic proteins were isolated, and equal amounts of protein separated by SDS-PAGE. C143 (A) and H308 (B) B lymphoblasts were immunoblotted with an antibody directed against phosphorylated serine 32-IκBα. LC indicates the presence of a non-specific band, which acts as a loading control for phosphorylated-IκBα. C143 (C), H308 (D) B lymphoblasts and HUVEC (E) were immunoblotted with an antibody directed against IκBα. (1) indicates shifted or slower mobility band corresponding to phosphorylated IκBα, (2) the faster mobility band corresponds to non-phosphorylated IκBα, and also acts as a loading control for (1). Data are representative Westerns and are the result of at least two independent experiments.*

This slower mobility or shifted band was reported to correspond to the phosphorylated form of IκBα (DiDonato *et al.* 1995; Beg *et al.* 1993; Cordle *et al.* 1993). In C, D, and E there was no apparent difference in total IκBα protein, indicating equal protein loads in these experiments.

EGCG did not significantly reduce the levels of the lower mobility band or phosphorylated IκBα in C143 (C) or H308 (D) B lymphoblasts, even at the highest concentration of 100 μM. Conversely in HUVEC, a concentration of 50 μM and up to 200 μM EGCG, appeared to reduce the levels of the slower mobility or shifted IκBα band, indicating inhibition of phosphorylation of IκBα (figure 3.13 E).

3.3.7 Effect of EGCG on IKKα activity in HUVEC

The upstream kinases responsible for phosphorylation and thus regulation of protein levels of IκBα are the IκB kinases (IKK). Using GST-IκBα as a substrate for IKK, it was decided to examine the effect of EGCG on the activity of IKKα in HUVEC by performing a kinase assay. For this, IKKα was immunoprecipitated from HUVEC cells and its ability to incorporate a phosphate group of ^{32}P-labelled ATP on the GST-IκBα substrate, was used as a measure of kinase activity. The ability of EGCG to affect IKKα activity in HUVEC was measured in two ways. EGCG was either added to HUVEC before the kinase assay was performed, or added directly into the kinase assay. This gave an idea whether EGCG affects IKKα activity or prevents activation of IKKα in the cells. Curcumin treatment was also included as a positive control as it has been shown to inhibit IKKα activity, albeit in a different cell line (Plummer *et al.* 1999).

Due to a high background signal on the kinase gels the data obtained from the assays were difficult to analyse and thus quantify. Although not obvious from the figure, taking into consideration the loading, it appeared that TNFα induced a modest increase in the activity of IKKα in both the indirect and direct assay. It was also noted that in the untreated samples there was a constitutive level of IKKα activity in both the direct and indirect assay (lanes 1 in A and B). In the direct kinase assay (figure 3.14 A), it appeared that EGCG 10, 30 50 and 100μM, and curcumin 30 μM inhibited phosphorylation of GST-IκBα, indicating that EGCG and curcumin inhibit IKKα activity when added directly to the assay. Results from the indirect kinase assays were not as clear. In two of the three assays there was a suggestion of an inhibition of IKKα activity at a concentration of 100

μM. However referring to figure 3.14 B, inhibition of IKKα activity might have been evident at concentrations as low as 10 μM. These data imply that EGCG inhibits IKKα activity either directly, but also maybe indirectly by acting upstream of IKKα.

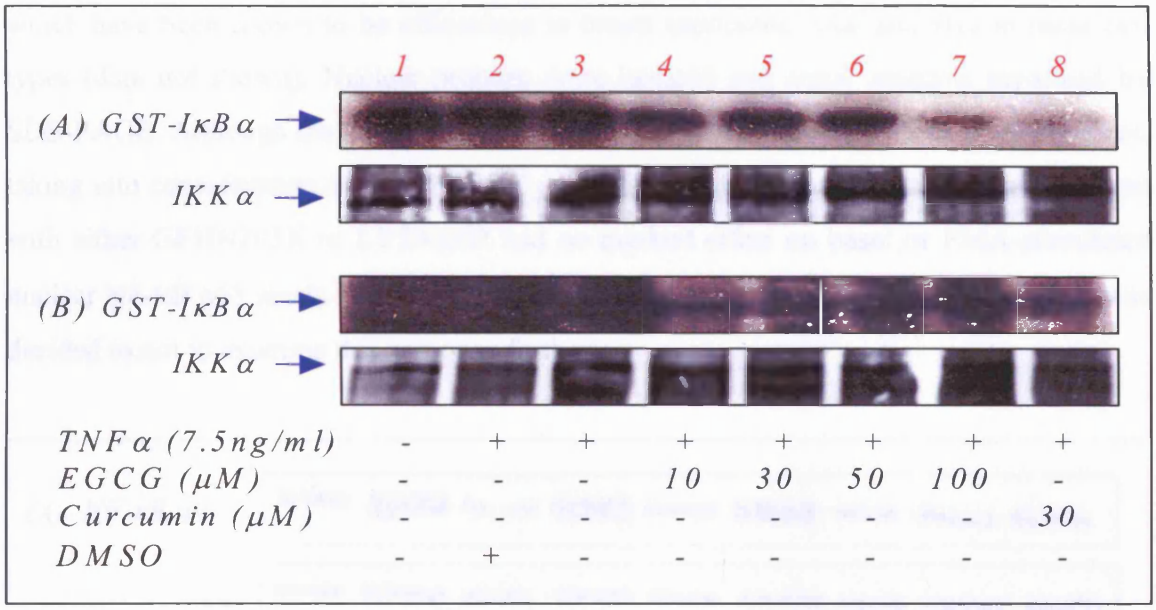

Figure 3.14 – Effect of EGCG and curcumin on IKKα activity in HUVEC. *For the direct assay (A) untreated cells were exposed to TNFα (7.5 ng/ml for 5 minutes) or DMSO before the immunoprecipitation step. In the indirect assay (B) cells were pretreated with EGCG (10, 30, 50 and 100 μM/45 minutes) or curcumin (30 μM /45 minutes) before exposure to TNFα (7.5 ng/ml for 5 minutes) or DMSO. In A and B cells were then lysed and IKKα was immunoprecipitated from equal concentrations of protein in each sample. IKKα activity was measured by the ability of the purified kinase to phosphorylate and incorporate ^{32}P-labelled ATP on a GST-IκBα substrate. For the direct assay (A), samples were treated with EGCG (10, 30, 50 and 100 μM) and curcumin (30 μM) before addition of ^{32}P-labelled ATP. IKKα Westerns, which were run from samples of the immunoprecipitated IKKα, were an indication to the relative amounts of immunoprecipitated kinase and thus a form of loading control. Figures are the representative of 3 independent experiments.*

3.3.8 LY294002 or GF109203X do not inhibit NF-κB translocation in C143 and H308 B lymphoblasts

Both PI3K and PKC are signalling intermediates, which can be important in cell growth and/or the regulation of NF-κB activity (Pan *et al.* 1999; Lallena *et al.* 1999; Dominguez *et al.* 1993; Folgueira *et al.* 1996). To investigate the significance of the PKC and PI3K pathways on the activity of NF-κB, the effects GF109203X (an inhibitor of various PKC isoforms) and LY294002 (an inhibitor of PI3K) on the nuclear protein levels

of NF-κB p65 in C143 and H308 B lymphoblasts were examined. For this series of experiments, cells were pre-treated with GF109203X (1 μM for 30 minutes) or LY294002 (30 and 50 μM for 30 minutes) before stimulation with PMA (0.15 μM/15 minutes). The concentrations of the inhibitors were selected from those previously used in the group, which have been shown to be efficacious in breast carcinoma cells and also in these cell types (data not shown). Nuclear proteins were isolated and equal amounts separated by SDS-PAGE. Although the data contained in figure 3.15 were the result of one experiment, taking into consideration the loading (LC or loading control), it appears that pre-treatment with either GF109203X or LY294002 had no marked effect on basal or PMA-stimulated nuclear NF-κB p65 levels in either C143 (A) or H308 (B) B lymphoblast cells. Thus it was decided to not to examine this response further.

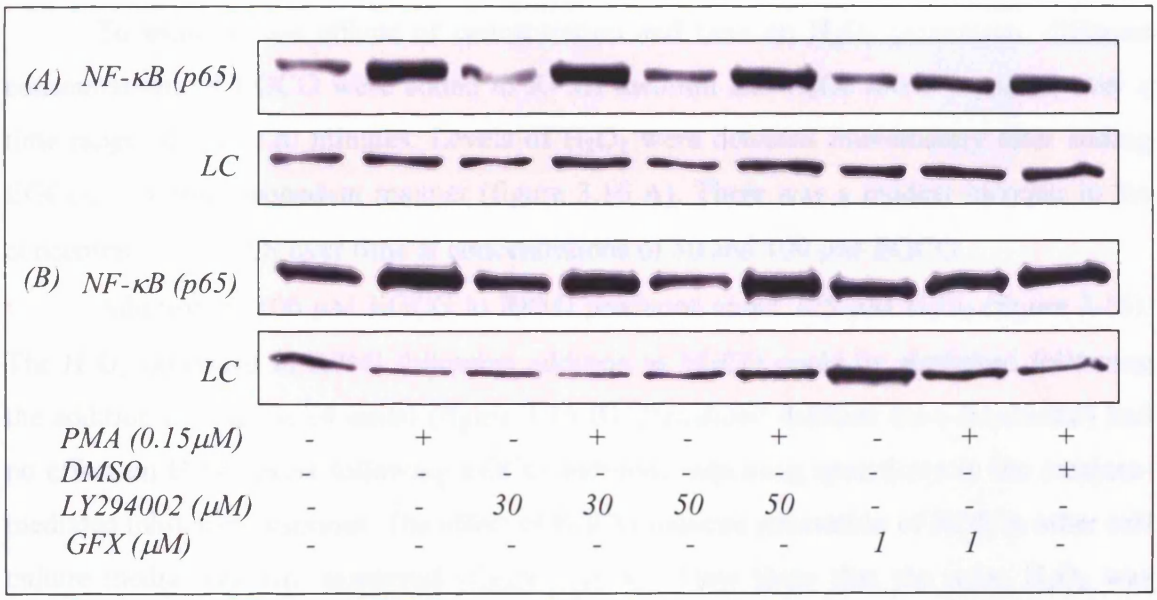

Figure 3.15 - LY294002 or GF109203X do not affect nuclear protein levels of NF-κB p65 in C143 (A) and H308 (B) B lymphoblasts. *Cells were pre-treated with either GF109203X (1 μM for 30 minutes) or LY294002 (30 and 50 μM for 30 minutes) before stimulation with PMA (0.15 μM). Nuclear samples were then isolated, separated by SDS-PAGE and immunoblotted with an antibody directed against NF-κB p65. LC is a non-specific band on the same gel, indicating the relative protein loads on each gel. Westerns are the result of only one experiment, performed in two individual lymphoblast cell lines.*

3.3.9 EGCG, but not resveratrol or I3C, produces H_2O_2 in cell culture medium

As mentioned previously, evidence in the literature demonstrates the pro-oxidant nature of EGCG. The generation of H_2O_2 by EGCG in certain cell culture medium has been investigated by a number of different groups (e.g. Long *et al.*, 2000). The effect of EGCG (and other chemopreventive agents) on the generation of H_2O_2 was investigated in cell culture medium (in the absence of cells) particular to these series of experiments. The detection of H_2O_2 in medium was accomplished using the ferrous oxidation in xylenol orange or FOX assay (see Materials and Methods). These experiments were carried out in collaboration with Mr Toryn Poolman (Cancer Biomarkers and Prevention Group, University of Leicester).

To examine the effects of concentration and time on H_2O_2 generation, different concentrations of EGCG were added to RPMI medium and H_2O_2 levels recorded over a time range of 0 to 120 minutes. Levels of H_2O_2 were detected immediately after adding EGCG, in a dose-dependent manner (figure 3.16 A). There was a modest increase in the concentration of H_2O_2 over time at concentrations of 50 and 100 μM EGCG.

Addition of 100 μM EGCG to RPMI produced about 125 μM H_2O_2 (figure 3.16). The H_2O_2 generated in RPMI following addition of EGCG could be abolished following the addition of catalase (4 units) (figure 3.16 B). Denatured catalase (non-functional) had no effect on H_2O_2 levels following EGCG addition, indicating specificity in the catalase-mediated inhibitory response. The effect of EGCG-induced generation of ROS in other cell culture media was also examined (figure 3.16 C). Data show that the most H_2O_2 was produced following EGCG addition (100 μM) to Dulbecco's modified Eagle medium (DMEM), followed by RPMI, with the least amount in endothelial growth medium (EGM) (figure 3.16 C). Under similar conditions, curcumin, but not I3C or resveratrol, was also found to generate H_2O_2 when added to cell culture medium, but was not as potent as EGCG (data not shown)

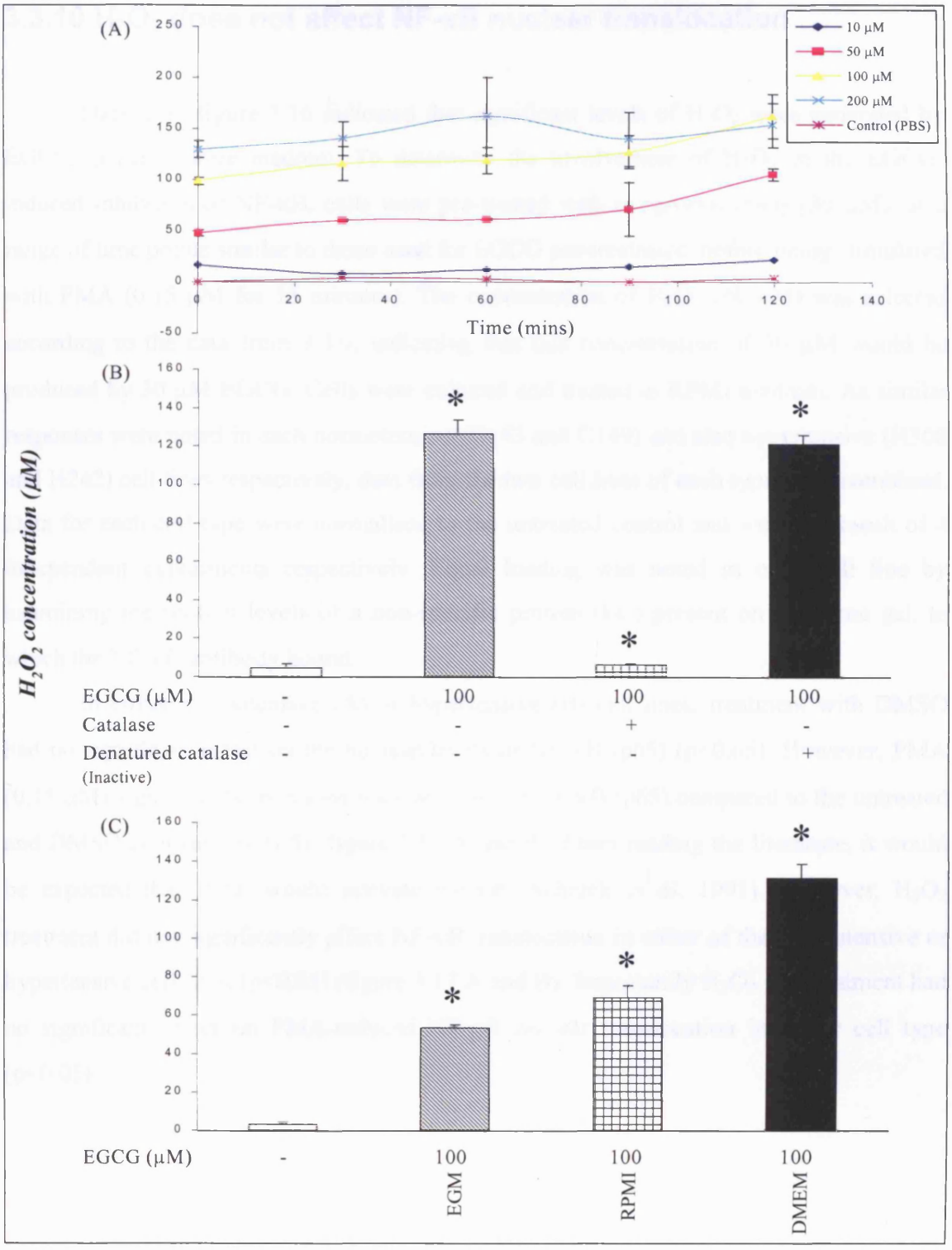

***Figure 3.16 - EGCG generates H_2O_2 in cell culture medium**. EGCG was added to cell culture medium (RPMI) at different concentrations and time points (A) and the levels of H_2O_2 produced were measured using the FOX assay (see Materials and Methods). The H_2O_2 generated by EGCG could be blocked following catalase treatment (B). The EGCG-induced generation of H_2O_2 was examined in different cell culture media (C). Data are the mean of 3 independent experiments.* indicates significant difference from the PBS control (untreated) group ($p<0.05$) as determined by the ANOVA balanced model followed by Tukey's least significant difference post hoc test.*

3.3.10 H_2O_2 does not affect NF-κB nuclear translocation

Data from figure 3.16 indicated that significant levels of H_2O_2 were generated by EGCG in cell culture medium. To determine the involvement of H_2O_2 in the EGCG-induced inhibition of NF-κB, cells were pre-treated with exogenous H_2O_2 (30 μM), at a range of time points similar to those used for EGCG pre-treatment, before being stimulated with PMA (0.15 μM for 15 minutes). The concentration of H_2O_2 (30 μM) was selected according to the data from 3.3.9, indicating that this concentration of 30 μM would be produced by 30 μM EGCG. Cells were cultured and treated in RPMI medium. As similar responses were noted in each normotensive (C143 and C149) and also hypertensive (H308 and H242) cell lines respectively, data from the two cell lines of each type were combined. Data for each cell type were normalised to the untreated control and were the result of 4 independent experiments respectively. Equal loading was noted in each cell line by examining the protein levels of a non-specific protein (LC) present on the same gel, to which the NF-κB antibody bound.

In either normotensive (A) or hypertensive (B) cell lines, treatment with DMSO had no significant effect on the nuclear levels of NF-κB (p65) ($p<0.05$). However, PMA (0.15 μM) significantly increased nuclear levels of NF-κB (p65) compared to the untreated and DMSO controls ($p<0.05$) (figure 3.17 A and B). From reading the literature, it would be expected that H_2O_2 would activate NF-κB (Schreck *et al.* 1991). However, H_2O_2 treatment did not significantly affect NF-κB translocation in either of the normotensive or hypertensive cell lines ($p<0.05$) (figure 3.17 A and B). Importantly H_2O_2 pre-treatment had no significant effect on PMA-induced NF-κB nuclear translocation in either cell type ($p<0.05$).

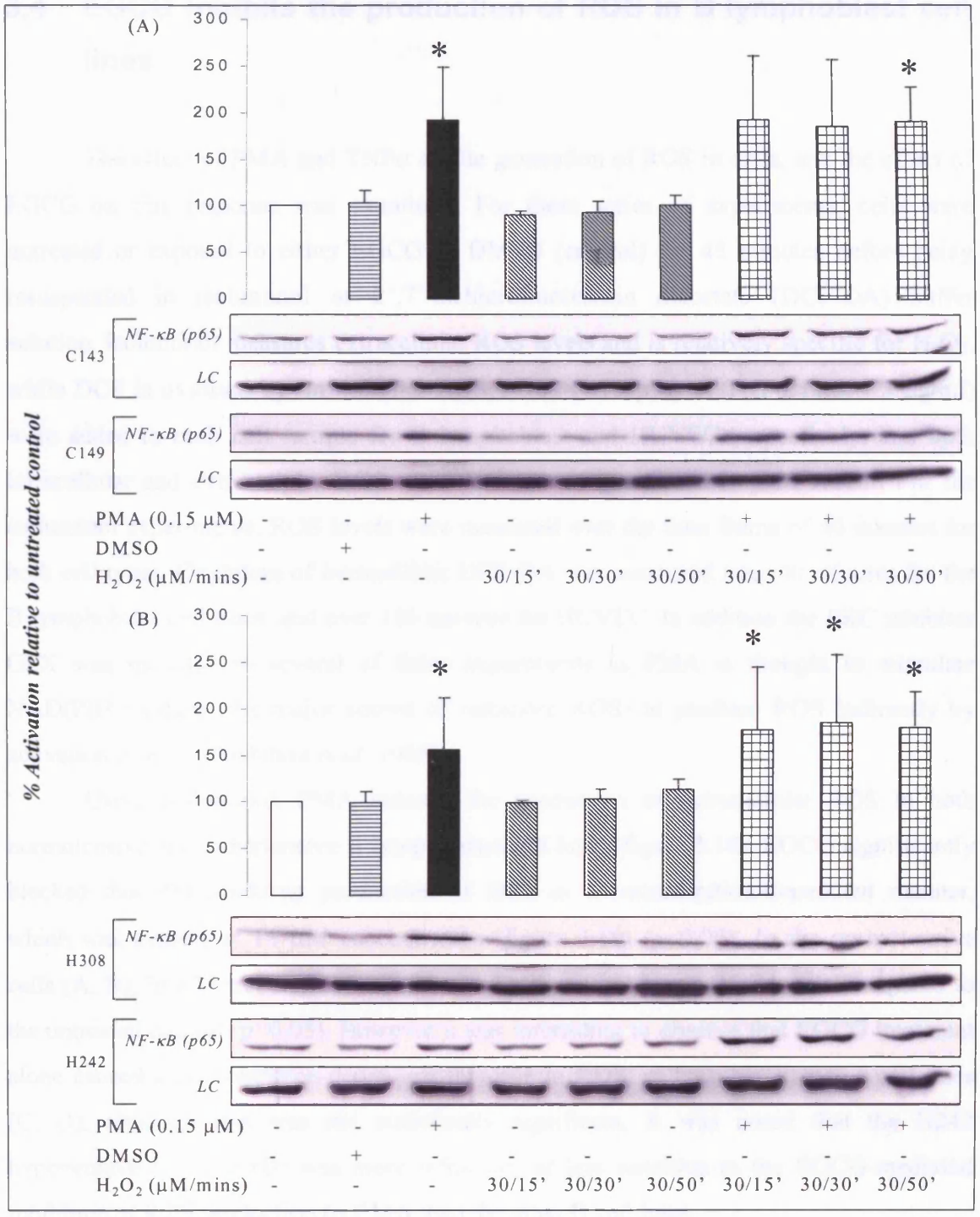

Figure 3.17 - H_2O_2 does not affect NF-κB (p65) nuclear translocation in B lymphoblasts. *Normotensive (C143 and C149) (A) and hypertensive (H308 and H242) (B) B cells were pre-treated with exogenous H_2O_2 (30 μM) in RPMI medium, before stimulation with PMA (0.15 μM) or with just H_2O_2 (30 μM). Nuclear proteins were isolated and immunoblotted with an antibody directed against the p65 subunit of NF-κB. Collective data for each cell type are the result of 4 independent experiments, with * indicating significant difference from the control (untreated) group ($p<0.05$) as determined by the ANOVA balanced model followed by Tukey's least significant difference post hoc test. LC is a non-specific band present on the same gel respectively, indicating equal protein loading.*

3.4 EGCG inhibits the production of ROS in B lymphoblast cell lines

The effect of PMA and TNFα on the generation of ROS in cells, and the effect of EGCG on this response was examined. For these series of experiments, cells were untreated or exposed to either EGCG or DMSO (control) for 45 minutes before being resuspended in isoluminol or 2',7'-dichlorofluorescein diacetate (DCF-DA) buffer solution. Isoluminol measures extracellular ROS levels and is relatively specific for H_2O_2, while DCF is oxidised by intracellular ROS. PMA (0.15 μM) or TNFα (2.5, 7.5 ng/ml) were added to each cell sample for B lymphoblast and HUVEC respectively, and both intracellular and extracellular ROS were detected using a FluoStar plate reader. For the isoluminol experiments, ROS levels were measured over the time frame of 40 minutes for both cell types. Oxidation of intracellular DCF-DA was measured over 40 minutes for the B lymphoblast cell lines, and over 185 minutes for HUVEC. In addition the PKC inhibitor GFX was included in several of these experiments as PMA is thought to stimulate NAD(P)H oxidase (the major source of inducible ROS) to produce ROS indirectly by activation of PKC (Nishihira *et al.* 1985).

Using isoluminol, PMA induced the production of extracellular ROS in both normotensive and hypertensive B lymphoblast cell lines (figure 3.18). EGCG significantly blocked this PMA-induced production of ROS in a concentration-dependent manner, which was evident at 15 μM concentration (figure 3.18) ($p<0.05$). In the normotensive cells (A, B), EGCG treatment alone did not significantly change ROS levels compared to the untreated control ($p<0.05$). However it was interesting to observe that EGCG treatment alone caused a concentration-dependent increase in ROS, in both hypertensive cell lines (C, D), although this was not statistically significant. It was noted that the H242 hypertensive cell line (D) was more refractory or less sensitive to the EGCG-mediated inhibition of ROS production by PMA than the other B cell lines.

PMA induced an increase in the oxidation of DCF indicating an increased intracellular level of ROS in C143 and H308 B lymphoblast cells (figure 3.19). EGCG significantly blocked this response, returning ROS levels to the background oxidation levels of the untreated sample. In addition the protein kinase C inhibitor, GF109203X, also suppressed the PMA-induced DCF oxidation, indicating suppression in ROS production. It was also noted that the DMSO control samples exhibited a minor decrease in the PMA-

induced oxidation of DCF, although this was not as significant as the comparable EGCG treatment, which was dissolved in DMSO.

In HUVEC (figure 3.20), treatment with 2.5 or 7.5 ng/ml TNFα did not significantly affect isoluminol fluorescence compared to the untreated control ($p<0.05$). There was no significant change in isoluminol fluorescence following treatment with EGCG ($p<0.05$).

DCF oxidation (figure 3.21) was not significantly ($p<0.05$) altered in HUVEC following treatment with 2.5 ng/ml (A) or 7.5 ng/ml (B) TNFα or PMA (0.15 μM) (C). Also the DMSO treatment had no effect on DCF oxidation. However, EGCG caused a significant concentration-dependent decrease in DCF oxidation ($p<0.05$), below that of the untreated control, following treatment with 2.5 ng/ml, and 7.5 ng/ml TNFα. EGCG had no significant affect on DCF oxidation following treatment with PMA (C). Treatment with GF109203X significantly inhibited DCF oxidation ($p<0.05$) (A), but this was not evident in (B) or (C).

Considering the B lymphoblast and HUVEC data collectively, these data are suggestive of an anti-oxidant function of EGCG both inside and outside of the cell.

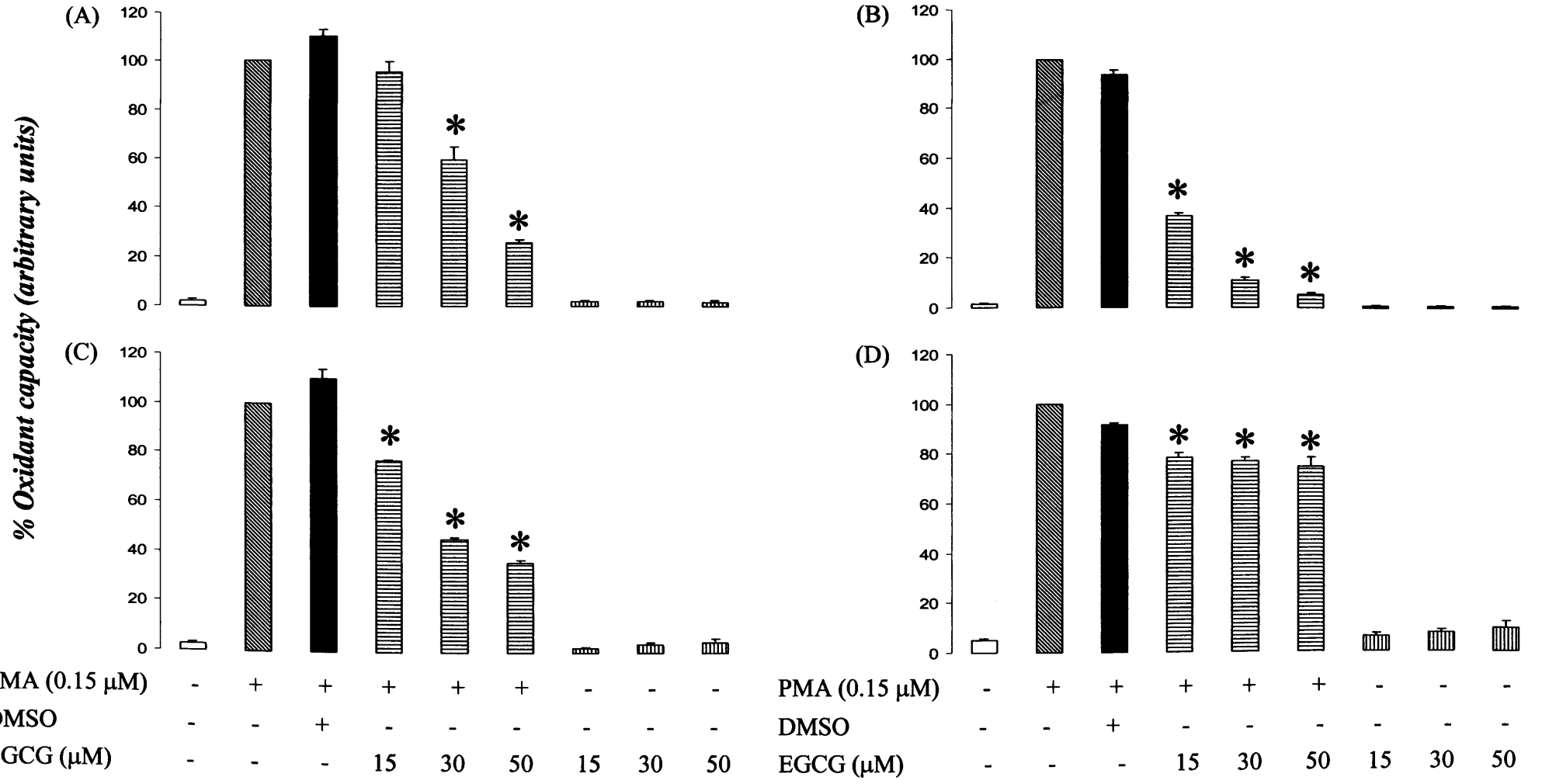

Figure 3.18 – EGCG inhibits PMA-induced increases in isoluminol luminescence in B lymphoblast cells. *C143 (A), C149 (B), H308 (C), and H242 (D) cells were incubated in the presence or absence of EGCG (15, 30, 50 μM) or DMSO control for 45 minutes before being treated with PMA (0.15 μM). Isoluminol luminescence was measured every 2 minutes over a 40-minute period and a graph plotted of luminescence against time. From this graph the area under the curve (AUC) was proportional to the luminescence. * indicates significant difference from the control (untreated) group ($p < 0.05$) as determined by the ANOVA balanced model followed by Tukeys least significant difference post hoc test.*

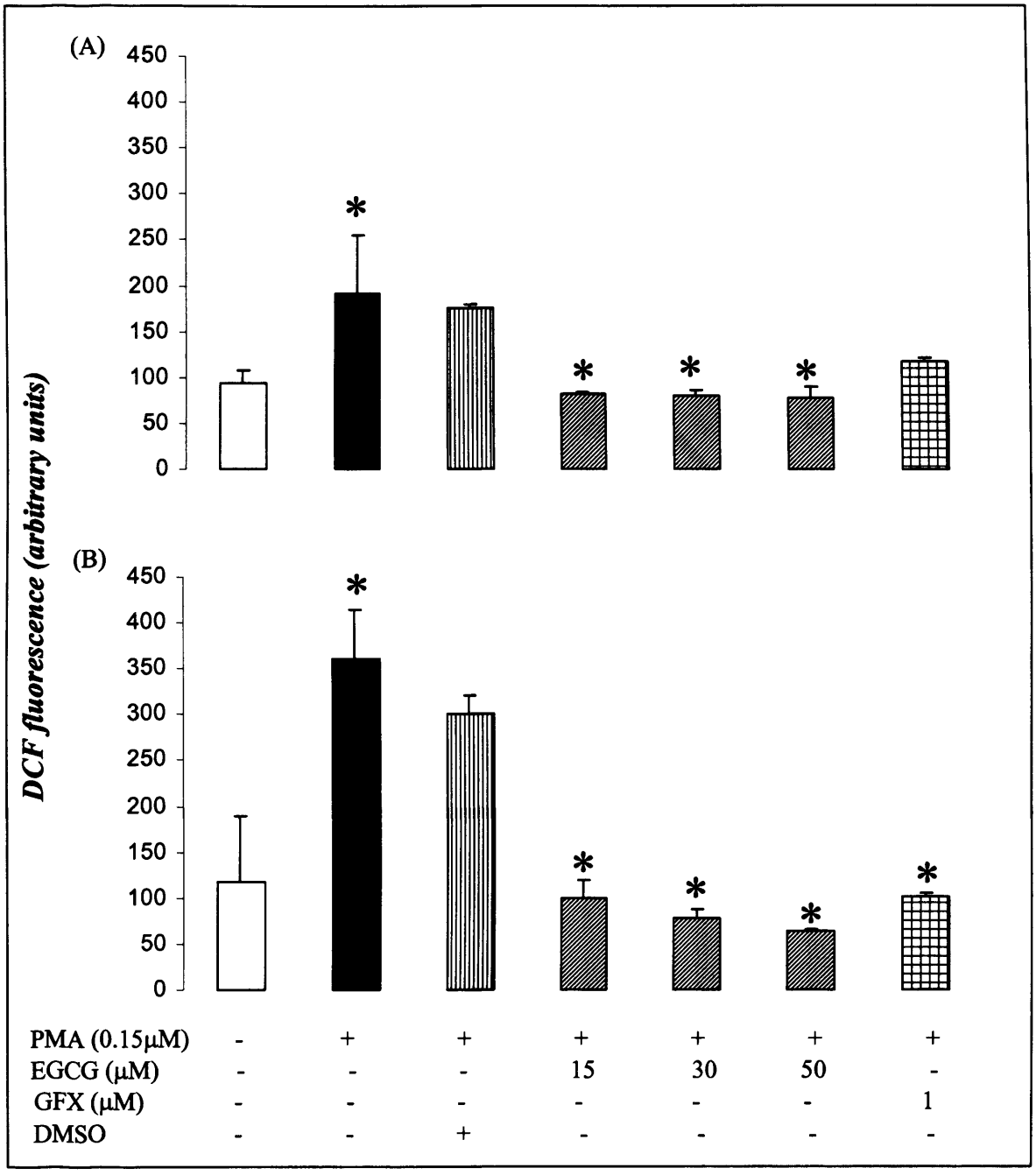

Figure 3.19 – Effect of EGCG on DCF fluorescence in C143 (A) and H308 (B) B lymphoblasts exposed to PMA (0.15μM). *Cells were incubated in the presence or absence of EGCG (15, 30, 50 μM) or GF109203X (1μM) or DMSO control for 45 minutes before being treated with PMA. DCF fluorescence was measured over a 40 minute period and final fluorescence for each treatment plotted on a graph. * indicates significant difference from the control (untreated) group ($p < 0.05$) as determined by the ANOVA balanced model followed by Tukey's least significant difference post hoc test.*

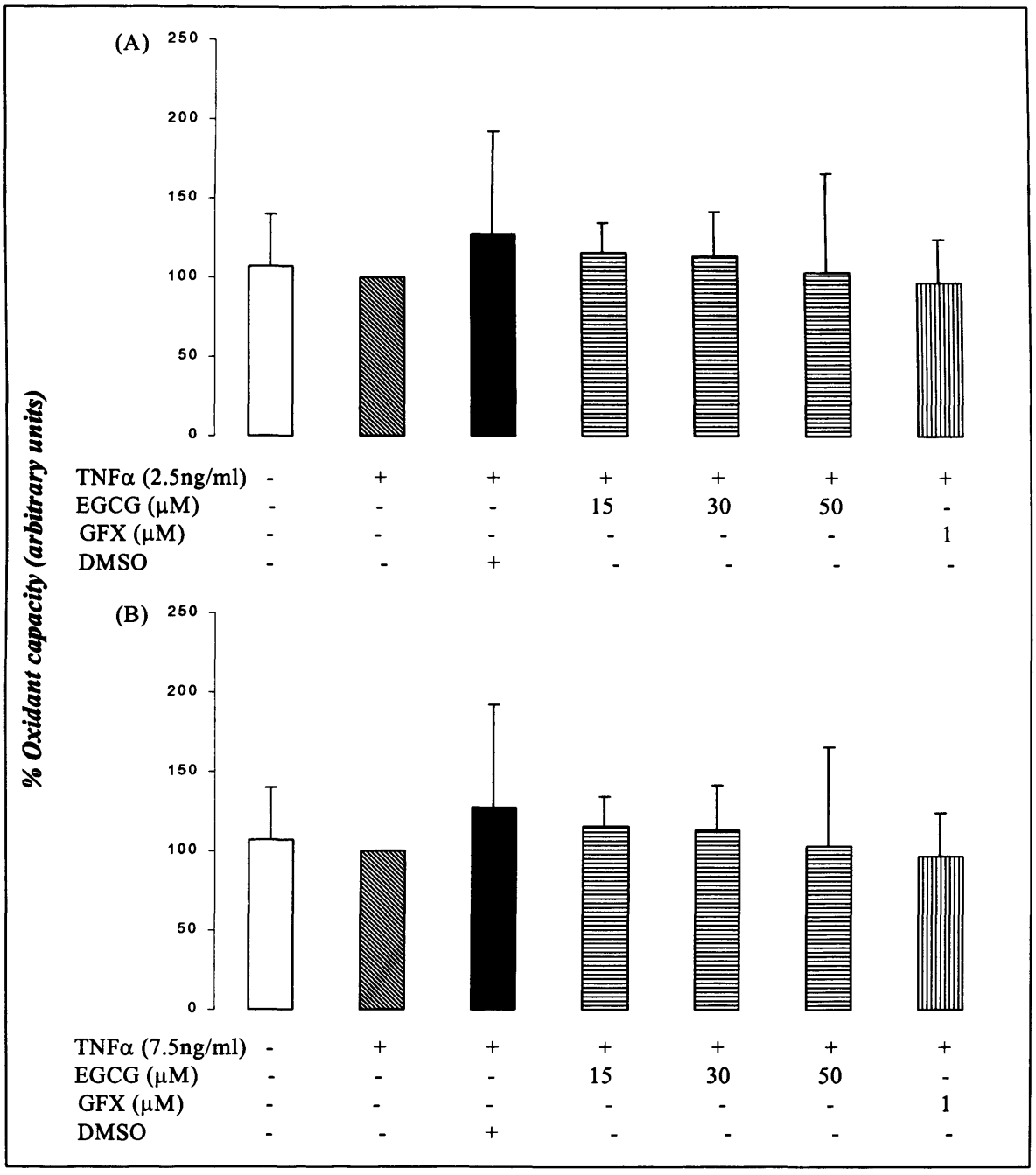

Figure 3.20 – Effect of EGCG on isoluminol luminescence in HUVECs exposed to 2.5 ng/ml (A) and 7.5 ng/ml (B) TNFα. *Cells were incubated in the presence or absence of EGCG (15, 30, 50 μM) or GF109203X (1μM) or DMSO control for 45 minutes before being treated with TNFα. Isoluminol luminescence was measured every 2 minutes over a 40-minute period and a graph plotted of luminescence against time. From this graph the area under the curve (AUC) was proportional to the luminescence.*

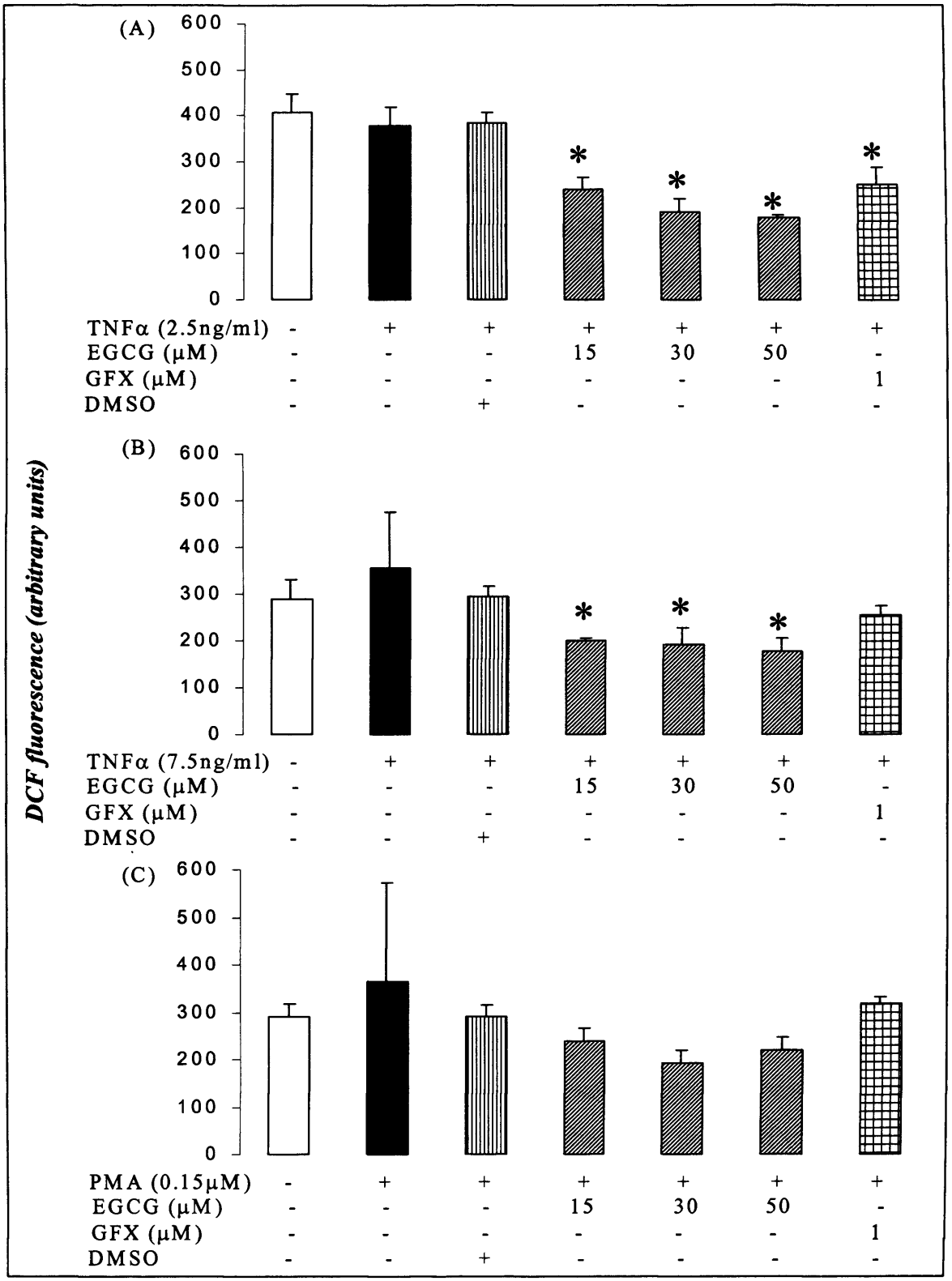

Figure 3.21 – Effect of EGCG on DCF fluorescence in HUVECs exposed to TNFα (2.5 ng/ml, 7.5 ng/ml) and PMA (0.15μM). *Cells were incubated in the presence or absence of EGCG (15, 30, 50 μM) or GF109203X (1μM) or DMSO control for 45 minutes before being treated with TNFα or PMA. DCF fluorescence was measured over a 40 minute period and final fluorescence for each treatment plotted on a graph. Data were the mean of 3 independent experiments.*

3.5 Discussion

The aim of the experiments described in this chapter was to characterise the effect four chemopreventive agents on the activity of the NF-κB pathway. After the initial experiments it was decided to focus in on one of the chemopreventive agents. EGCG was preferred as it was one of the more efficacious agents, but also as a potential chemopreventive agent it was considered to be one of the more bioavailable agents for human consumption (refer to chapter 1).

In all cells examined, nuclear levels of NF-κB p65 were detected in the absence of stimulus. It was also noted that constitutive p52, RELB, and p50 were present in the nucleus of C143 in the absence of any stimulus. The nuclear levels of NF-κB p65 could be increased in B lymphoblasts and HUVEC following treatment with PMA or TNFα, respectively. Interestingly constitutive nuclear NF-κB p65 was more obvious in the B lymphoblast cell lines than in HUVEC. The constitutive NF-κB p65 in HUVEC was a little surprising, but may have been present because the HUVEC, being a primary cell type, were possibly more stressed than the B cell lines under these culture conditions. However, this observation in B cells was not as unexpected as it has been documented that mature B lymphocytes are unusual in that they contain nuclear Rel proteins prior to cell stimulation. In B cells, this constitutive nuclear activity typically consists of p50/c-Rel heterodimers (Grumont *et al.* 1994; Kistler *et al.* 1998; Liou *et al.* 1994), although in c-Rel deficient mice, p50/p65 heterodimers in the splenic B cells are elevated, and thus are thought to compensate. The constitutive nuclear activity is important to B cell function as exemplified by the lower viability of B cells from p50 deficient and XID mice, both of which have decreased constitutive NF-κB (Bajpai *et al.* 2000; Ellmeier *et al.* 2000; Kerner *et al.* 1995; Snapper *et al.* 1996). There are several alternate proposals that explain constitutive nuclear NF-κB in B cells. One mechanism is thought to be due to an increased turnover rate of IκBα (Miyamoto *et al.* 1994), which leaves more free NF-κB to translocate to the nucleus. Miyamoto and colleagues have provided much of the support for the turnover model, and in particular the recent demonstration that intracellular calcium controls IκBα degradation in mature B cells (Fields *et al.* 2000; Miyamoto *et al.* 1998). An alternative mechanism is that nuclear NF-κB is protected by hypophosphorylated IκBβ (Phillips *et al.* 1997), which competes for nuclear NF-κB members thus preventing sequestration to the cytoplasm by IκBα. A third proposed mechanism attributes constitutive nuclear NF-κB to constitutively

active IκBα kinase (Naumann *et al.* 1994). This hypothesis was recognised when examining constitutive nuclear NF-κB in Namalwa B cells. It is thought that the observed phosphorylation-dependent mobility shifts of IκBα were due to chronic infection by Epstein Barr virus (EBV).

The latter theory is certainly pertinent to this investigation, as the B cells used in these experiments have been transformed with EBV as a requisite step of the immortalisation process. The EBV infects and transforms resting primary B cells *in vitro* and is associated with several human malignancies *in vivo*, such as lymphoproliferative disorders in immunocompromised individuals. Studies have shown that *in vitro* EBV transformation of B cells is associated with expression of a restricted set of proteins, so called latency proteins. Of these, latent membrane protein 1 (LMP1) is one of the more important proteins in the transformation of cells. Data from several groups demonstrate that LMP1 interacts with TNF receptor associated factors (TRAF) (Mosialos *et al.* 1995), TNF receptor-associated death domain (TRADD) and TNF receptor-interacting protein (RIP) (Eliopoulos *et al.* 1999; Izumi *et al.* 1999). Genetic analyses of the C-terminal domain of LMP1 in conjunction with B lymphocyte transformation assays revealed that the TRAF interaction site and the TRADD and RIP interaction site are critical for efficient outgrowth of transformed B lymphocytes (Izumi *et al.* 1997; Izumi and kieff, 1997; Kaye *et al.* 1993). Interaction and thus an association of TRADD, RIP and TRAF are essential steps in the transduction pathway of various members of the TNFα receptor (TNFR) family such as receptors type I and II, CD30 and CD40. Thus the LMP1 protein induces multiple downstream effects of the TNFR pathway, which includes activation of IKK and thus activation of Rel/NF-κB proteins (Laherty *et al.* 1992). It would be of interest in future studies to examine some of these upstream signalling intermediates.

Curcumin suppressed constitutive levels and PMA-induced increases in nuclear NF-κB p65 in both the normotensive and the hypertensive cell lines. Curcumin had a similar effect in HUVEC, where TNFα-induced nuclear translocation of NF-κB p65 was inhibited at concentrations of 15, 30, and 50 μM. It was unlikely that this response was cell type specific as inhibition was observed in endothelial cells and a range of B lymphoblast cells with heterogenetic backgrounds. These data are in agreement with reports in the literature, where curcumin is known to inhibit the activation of NF-κB in a number of different cell types to the ones used here, following stimulation with PMA (Singh *et al.* 1995) or TNFα (Bierhaus *et al.* 1997; Plummer *et al.* 1999).

Precisely how curcumin functions to inhibit NF-κB activation is open to question. Both PMA and TNFα are known to induce reactive oxygen species (ROS) (as reviewed in Siebenlist *et al.* 1994). Moreover, NF-κB is a known transcription factor responsive to various ROS such as H_2O_2 (Schreck *et al.* 1991). Therefore, it is possible that the NF-κB inhibitory effect is through the quenching or antioxidant function of curcumin. However, curcumin was found to produce H_2O_2 in cell culture medium, implying that curcumin may block NF-κB activation through a signalling mechanism. Given the fact that curcumin generated H_2O_2 in cell culture medium, this may explain the modest increase (which was not statistically significant) in constitutive nuclear NF-κB p65 at 15 μm curcumin, in the normotensive and HUVEC cell types.

Curcumin has been shown to inhibit PKC activity, induced by PMA, in NIH 3T3 mouse embryo fibroblast cells (Liu *et al.* 1993). Several PKC isoforms have been shown to activate NF-κB (Lallena *et al.* 1999; Dominguez *et al.* 1993; Folgueira *et al.* 1996). In addition PI3K has been shown to activate NF-κB (Pan *et al.* 1999). However in the B lymphoblasts, it is unlikely that curcumin (or resveratrol, I3C and EGCG) mediates an effect through either PKC or PI3K, as GF109203X (a PKC inhibitor) or LY294002 (a PI3K inhibitor) had no significant effect on PMA-induced or constitutive nuclear levels of NF-κB p65. These inhibitors were effective at blocking the respective pathways in these and other cells used by the group (data not shown).

Other kinases that can activate NF-κB include MEKK1 (Yin *et al.* 1998), MEKK2, MEKK3 (Zhao *et al.* 1999), NIK (Malinin *et al.* 1997), and NAK (Tojima *et al.* 2000). Curcumin has been shown to block NF-κB activation through JNK via MEKK1 and 2 suppression (Chen YR *et al.* 1998; Squires *et al.* 2003) and also through NIK (Jobin *et al.* 1999). However, data from the HUVEC kinase assays, indicated that curcumin blocked IKKα activity following treatment with TNFα. This suggests that curcumin in HUVEC blocks activation of NF-κB by acting on IKKα. This is consistent with Plummer *et al.* (1999) who have shown inhibition of IKKα and IKKβ activity by curcumin, thus preventing IκBα degradation and subsequent NF-κB activation in human colonic epithelial cells.

The effect of resveratrol on the NF-κB pathway was examined in B lymphoblasts and HUVEC. Data indicated a number of subtle differences between cell types. In C143, 30 and 50 μM resveratrol inhibited basal and PMA-stimulated translocation of NF-κB. However in H308, resveratrol 15 and 50 μM inhibited PMA-stimulated nuclear

translocation of NF-κB (p65). HUVEC responded in a similar manner to B lymphoblasts, that is resveratrol blocked TNFα-stimulated NF-κB nuclear translocation. However, basal levels of NF-κB translocation were not noticeably affected by resveratrol. These data agree with several more recent reports showing inhibition of NF-κB at different concentrations and in different cell types (Tsai *et al.* 1999; Manna *et al.* 2000). As with curcumin, resveratrol may block activation of NF-κB via the quenching of ROS. In addition resveratrol also blocks activation of upstream signalling pathways, which can impinge on the NF-κB pathway. It is a possibility that resveratrol acts on the IκB family of inhibitory proteins, thus preventing translocation of NF-κB. This supposition is based on several reports in the literature that demonstrate resveratrol, at similar concentrations to those used here, inhibits NF-κB activation through suppression of IκBα phosphorylation and/or degradation (Tsai *et al.* 1999; Manna *et al.* 2000). A study by Tsai *et al.* (1999) have shown that resveratrol inhibits iNOS production by suppressing NF-κB/DNA binding activity via blockade of IκBα degradation. Interestingly, Manna *et al.* (2000) has shown that resveratrol blocks TNFα-activation (also PMA, LPS and H_2O_2) of NF-κB in cultured U937, Jurkat and HeLa cells, independent of any TNFα-induced phosphorylation and subsequent degradation of IκBα via IKK. It may be that resveratrol acts on ERK, JNK and/or p38 MAPK pathway as demonstrated by El-Mowafy *et al.* (1999). These reports indicate that resveratrol may have a number of biological targets *in vitro*, dependent on the type of NF-κB inducing signal or cell.

The effect of I3C on basal and stimulated NF-κB nuclear translocation was examined. I3C was found to have differential effects in each cell type, which also appeared to be dependent on the concentration used. I3C enhanced PMA-stimulated NF-κB nuclear levels in the C143 cell line, but may have reduced nuclear levels in the H308 cell line. In HUVEC, I3C had no significant effect on basal/constitutive NF-κB p65 levels. However the results indicated that I3C may have inhibited TNFα-induced translocation in a concentration-dependent manner, which was more obvious at 500 μM I3C. These data support the notion of a cell type specific response of I3C. This proposal may not be surprising when considering the work by Howells *et al.* (2002). In this report, treatment with I3C had no effect on IKK activity or NF-κB nuclear levels in either HBL100 or MDA MB468 breast cell lines. Interestingly, I3C suppressed the levels of NF-κB DNA binding in the MDA MB468 cells but increased the levels of NF-κB DNA binding in the HBL100

cells, suggesting it may alter DNA binding of various NF-κB subunits independent of IKK activity.

The effect of EGCG on basal and stimulated NF-κB (p65) translocation was examined in HUVEC and B lymphoblasts. EGCG blocked basal and TNFα or PMA stimulated NF-κB nuclear translocation in HUVEC and B lymphoblast cells respectively. The HUVEC data are in agreement with previous early work by the group, demonstrating the inhibition of TNFα-induced nuclear translocation of NF-κB by EGCG, using Western analyses and immunofluorescence (Yasin Heidari, MSc. Thesis, 1998). In addition, the blockade of stimulated and constitutive nuclear NF-κB was associated with an inhibition of NF-κB sequence-specific DNA binding as demonstrated using EMSA. This implies that EGCG as well as inhibiting NF-κB p65 translocation, may also affect NF-κB-dependent gene transcription by impairing the DNA binding potential of NF-κB. There was also a decrease in the constitutive nuclear levels of p50, RELB, and p52 in C143, following treatment with EGCG. These findings concur with a number of more recent reports showing the EGCG-induced inhibition of NF-κB activation following treatment with PMA (Nomura *et al.* 2000), and TNFα (Chen *et al.* 2002; Mukhtar *et al.* 1998), albeit in different cell types. EGCG has also been shown to inhibit NF-κB activation following stimulation with LPS in a murine model (Yang *et al.* 2001) and UVB exposure in murine and guinea pig models and human dermal fibroblast cells (Kim *et al.* 2001).

A central tenet of the NF-κB pathway is the release of NF-κB due to degradation of its inhibitor IκB. In most cells, NF-κB activation is transient because NF-κB induces transcription of IκBα (among other target genes) (Baeuerle *et al.* 1994; Siebenlist *et al.* 1994) and by a negative feedback loop the newly synthesised IκBα sequesters nuclear NF-κB back in the cytoplasm. However, as previously mentioned, control of NF-κB activity through IκBα regulation in B cells is not as straightforward as in other cells. It therefore seemed rational to examine the involvement of IκB family of proteins in the activity of constitutive and stimulated NF-κB activity.

The data from HUVEC indicated that cytosolic IκBα was rapidly degraded following stimulation with TNFα, which complemented the increase in nuclear NF-κB. This implicates the IκB family of proteins in the TNFα induced response and thus NF-κB is regulated in the classical manner as reviewed in Karin *et al.* (1999a, 1999b). Interestingly, it was evident that in HUVEC almost all the cytosolic pool of IκBα was degraded following TNF treatment, compared to PMA-treated B lymphoblasts, whereby

PMA induced a decrease in only a proportion of the cytosolic IκBα pool (at a treatment time complementing the increased nuclear NF-κB p65). The fact that PMA did not induce degradation of the total cytosolic pool of IκBα would suggest that a proportion of the cytosolic IκBα protein (hence NF-κB) is refractory to the effect of PMA. The dissimilarity in the responses between B cells and HUVEC may be due to the different stimuli used to activate the NF-κB pathway in each cell type. However, it is more likely due to the mechanisms regulating IκBα and hence NF-κB activity, which are different between the cells. For example the phosphorylation status and the type of phosphorylation of IκB can be very important in regulating IκBα protein levels (see below).

Although these data suggest that IκBα regulates NF-κB translocation, it is important to realise that other members of the IκB family may also play a role. For example, in response to extracellular stimuli the IκBβ protein can be phosphorylated on serines 19 and 23, ubiquitinated and degraded by the proteosomal pathway (Thompson *et al.* 1995; DiDonato *et al.* 1996). These processes tend to be much slower for IκBβ than with IκBα and occur in response to stimuli that induce a sustained NF-κB activation. IκBβ protein levels were also examined, but the data from these studies were more ambiguous than for IκBα, and thus were omitted from this chapter.

It was evident that EGCG blocked TNFα-induced degradation of IκBα in HUVEC, but had no significant effect in the B cells. The HUVEC data are in agreement with a number of independent groups that have documented the fact that EGCG, at concentrations similar to those used here, can block IκBα degradation induced by a number of different agents, but also in a number of alternate cell types (Lin *et al.* 1997; Chen *et al.* 2002; Mukhtar *et al.* 1998). In agreement with Nam *et al.* (2001), it may be that EGCG acts as a proteosome inhibitor in HUVEC, thus preventing degradation of IκBα.

The difference in the protective responses between B lymphoblasts and HUVEC could be due to a number of possible reasons. It is unlikely that the absence of any protection against PMA-induced IκBα degradation was due to inappropriate concentrations of EGCG, as EGCG protected IκBα degradation in HUVEC and in other cell systems at similar concentrations (Lin *et al.* 1997; Chen *et al.* 2002; Mukhtar *et al.* 1998). It is evident from reading the literature that the phosphorylation status, and type of phosphorylation of IκB are important in the regulation of IκB protein levels, and thus NF-κB activity. Typically phosphorylation on serines 32 and 36 consequently targets the IκBα protein for ubiquitination and eventual degradation. More recently, it is recognised that

IκBα can be phosphorylated on different residues, which in turn can affect NF-κB activity. For example, treatment with phosphatase inhibitors or reoxygenation of hypoxic cells can lead to IκBα phosphorylation on tyrosine 42 (Imbert *et al.* 1996). Phosphorylation of tyrosine 42 leads to the dissociation of IκBα from the NF-κB complex without degradation of the inhibitor protein IκBα. It may be that in B cells a proportion of cytosolic IκBα is tyrosine phosphorylated and thus is not degraded, which would explain the high constitutive nuclear NF-κB. Therefore the phosphorlyation status and type of phosphorylation of IκBα following stimulation with PMA or TNFα was examined. This was accomplished by detecting changes or shifts in the migratory distance of IκBα using total IκBα antibody. This slower mobility or shifted band was reported to correspond to the phosphorylated form of IκBα (DiDonato *et al.* 1995; Beg *et al.* 1993; Cordle *et al.* 1993). Phosphorylation of IκBα was also examined using an antibody specific for phosphorylated serine 32 on IκBα.

In B lymphoblasts and HUVEC, the levels of the shifted or slower mobility band of IκBα protein increased following treatment with PMA and TNFα respectively, indicating phosphorylation of IκBα protein. Phosphorylation in B lymphoblasts was confirmed using the phosphospecific-(serine-32)-IκBα antibody, when a band of similar molecular weight to IκBα was detected following treatment with PMA. These data indicated that IκBα was phosphorylated in B lymphoblasts and HUVEC following treatment with PMA or TNFα respectively. In addition these data demonstrate that IκBα in the B cells is phosphorylated on serine 32. Pre-treatment with EGCG did not affect phosphorylation of IκBα in B lymphoblasts, as the levels of either the shifted band or phospho-serine 32 band of IκBα were unaffected by EGCG pre-treatment. As EGCG did not prevent phosphorylation of IκBα in B lymphocytes, this may explain why it did not prevent degradation of cytosolic IκBα. However in HUVEC, EGCG decreased the intensity of the shifted band of IκBα at a concentration of 100 μM, indicating a decrease in the levels of phosphorylated IκBα. This may explain the reason behind the EGCG-induced protection of TNFα-induced IκBα degradation in HUVECs. The phosphorylation status of HUVEC was also examined using the phospho-serine 32 antibody, however due to technical difficulties no satisfactory data were obtained.

IKK controls the phosphorylation and thus stability of IκB (Karin *et al.* 1999). To investigate whether inhibition of IκBα phosphorylation by EGCG in HUVEC, was through the modulation of the IKKs, an IKKα kinase assay using GST-IκBα as a substrate was

performed. The data obtained from the kinase assays were difficult to analyse due to a high background signal on the kinase gel. However it appeared that TNFα induced a modest increase in the activity of IKKα. In all three direct kinase assays, it appeared that EGCG (10 μM) inhibited phosphorylation of GST-IκBα, indicating that EGCG inhibits IKKα activity directly. In the indirect kinase assays (i.e. EGCG added to cells before stimulation with TNFα) there was a suggestion of an IKKα inhibitory effect at a concentration of 10 μM. These data are in agreement with a number of more recent reports that show inhibition of IKK by EGCG in a number of cell types different from the ones used here (Yang *et al.* 2001; Pan *et al.* 2000b; Chen *et al.* 2002). It is likely that EGCG inhibits IKKα activity directly, but this does not exclude a possible indirect mechanism by acting on upstream regulators of IKKα. This is consistent with the work by Chen *et al.* (2002) who suggests that EGCG blocks IKKα activity by acting on an upstream regulator of IKK family of kinases.

Phosphatidylinositol 3'-kinase (PI3K) and the serine/threonine kinase AKT have been shown to have important roles in activating the p65 subunit of NF-κB in response to various stimuli (Kane *et al.* 1999). The inhibitor LY294002 was used to examine whether the PI3K pathway may have been involved in the PMA-induced translocation of NF-κB in B lymphoblasts. LY294002 was shown to inhibit the PI3K pathway in the B cells at similar times and concentrations to those used in these experiments (data not shown). However, it was clear that nuclear levels of NF-κB p65 appeared to be unaffected in C143 or H308 B lymphoblasts, following treatment with LY294002 (30 and 50 μM). This implies that the PI3K pathway does not regulate NF-κB p65 nuclear translocation. However it has been shown that PI3K can bring about activation of NF-κB (through phosphorylation) by a pathway distinct from the classical IκBα degradation and NF-κB translocation pathway (Sizemore *et al.* 1999; Bird *et al.* 1997). Therefore it remains a possibility that the PI3K pathway is involved in the PMA-induced activation of NF-κB.

The protein kinase C (PKC) family has been implicated in regulating NF-κB activation (Lallena *et al.* 1999; Dominguez *et al.* 1993; Folgueira *et al.* 1996). Using the inhibitor GF109203X, the role of PKC was investigated in the B lymphoblast cell lines. The result is from one experiment performed in two different B lymphoblast cell types, and thus only offers a preliminary insight to the effect of PKC inhibition on the levels of nuclear NF-κB p65. However it was evident from the Western blots that nuclear levels of NF-κB p65 were unchanged following treatment with GF109203X. This would strongly

imply that PKC does not effect NF-κB p65 translocation under these particular conditions and cell types. These data do not exclude the effect of PKC on phosphorylation of NF-κB, which has been positively demonstrated by Anrather *et al.* (1999).

Recently, it has been reported that the addition of EGCG and other tea polyphenols to cell culture medium (in the absence of cells), generated significant levels of H_2O_2, with the corollary that this 'artifactual' effect may account for some or all of the reported effects of EGCG in cell culture studies (Long *et al.* 2000). The effect of H_2O_2 generation by EGCG in cell culture medium was investigated. Addition of 100 μM EGCG to cell culture medium, resulted in the production of significant levels of H_2O_2. DMEM gave the highest H_2O_2 levels (131 μM), then RPMI (68 μM), and finally EGM (53 μM). The addition of catalase prevented the formation of H_2O_2, indicating the specificity of peroxide generation. These results are consistent with the findings of Long *et al.* (2000), who also found that H_2O_2 was produced in cell culture medium, with the most in DMEM, followed by RPMI.

To further elucidate the involvement of H_2O_2 in the EGCG-induced inhibition of NF-κB, the effect of exogenously added H_2O_2 (to replicate EGCG treatment) on NF-κB translocation in B lymphoblasts was examined. H_2O_2 did not affect either basal or PMA-stimulated NF-κB nuclear translocation in the B lymphoblast cell lines. In addition, previous work by the group has shown that H_2O_2 at concentrations of 50, 100 and 200 μM, had no effect on nuclear translocation of NF-κB in HUVEC (data not shown). Collectively these data would argue that the effect of EGCG was unlikely to be an artifact of H_2O_2 generated in cell culture medium under these assay conditions. This conjecture is supported by Dashwood *et al.* (2002), who report that physiologically relevant concentrations of EGCG (< or = 25 μM) generated H_2O_2 (10-12 μM) in cell culture medium with or without serum, and in growth medium containing HEK293 human embryonic kidney cells plus serum. However the addition of 20 μM H_2O_2 directly to HEK293 cells that had been transiently-transfected with wild-type or mutant β-catenin constructs and TCF-4, had no effect on β-catenin/TCF-4 reporter activity or β-catenin expression levels. Conversely, 2-25 μM EGCG inhibited β-catenin/TCF-4 reporter activity in a concentration-dependent manner, with a concomitant reduction in β-catenin protein levels without changes in TCF-4 expression.

A number of animal models have demonstrated a relationship between ROS and hypertension (Heitzer *et al.* 1999; Swei *et al.* 1997). In humans, there is evidence showing a relationship between an increased ROS production and hypertension (Pontremoli *et al.*

1989; Seifert *et al.* 1991). A report by Pettit *et al.* (2002) has shown an increased free radical production in B lymphoblasts derived from hypertensive subjects, associated with an increased expression of the NAD(P)H oxidase subunit $p22^{phox}$. It is believed that elevated levels of ROS in hypertension is the link with the premature development of atherosclerosis. This correlation is supposed, as ROS are thought to contribute to vascular dysfunction and atherogenesis (As reviewed in Napoli *et al.* 2001).

ROS generation was detected in B lymphoblasts following stimulation with PMA using isoluminol and DCF. In agreement with Pettit *et al.* (2002), PMA induced the production of ROS in both normotensive and hypertensive B lymphoblast cell lines. There was no significant difference in the concentrations of ROS produced between the normotensive and hypertensive cell lines using isoluminol. However, in agreement with Pettit *et al.* (2002) there was an increased PMA response in the H308 compared to the C143 B lymphoblasts as detected using DCF. It is important to point out that to compare between a small number of normotensive and hypertensive cell lines would be statistically invalid. Therefore these differences may simply be due to insufficient number of B lymphoblast cell lines examined, as a larger number of hypertensive and normotensive cell lines were used by Pettit *et al.* (2002) (12 hypertensive and 12 normotensive cell lines).

In this present study, EGCG significantly blocked PMA-induced production of extracellular and intracellular ROS in B lymphoblasts. This is consistent with reports in the literature that demonstrate inhibition of ROS generation by EGCG (Lin *et al.* 2000; Alvarez *et al.* 2002; Feng *et al.* 2001; Kondo *et al.* 1999). The mechanisms behind the extracellular inhibition are unlikely to be due to the scavenging mechanisms of EGCG, as this was washed from the cell culture media before measurements were made. This does not exclude a scavenging function inside the cell, as EGCG may still be present in the intracellular environment. However, it is hypothesised as well as a ROS scavenger, that EGCG may act at an earlier stage by acting on ROS generating systems. In fact PMA is thought to stimulate NAD(P)H oxidase to produce ROS indirectly by activation of PKC (Nishihira *et al.* 1985). As the PKC inhibitor GF109203X suppressed PMA-induced increases in ROS production (as measured by DCF), this would suggest that PMA might enhance ROS production partly through a PKC-dependent and NAD(P)H dependent mechanism. Therefore, it may be possible that EGCG acts through this mechanism. However these data do not exclude other potential mechanisms of action of EGCG. PMA can also trigger ROS production through the activation of xanthine oxidase (XO).

Therefore EGCG may inhibit PMA-induced ROS production by acting on the XO system as demonstrated by Lin *et al.* (2000).

In endothelial cells, increased ROS production can lead to dysfunction, a contributing factor in the early stages of atherosclerosis. Potential alternative sources of endothelial ROS production include NAD(P)H-dependent oxidases (as reviewed in Van Heerebeek *et al.* 2002), XO (Michiels *et al.* 1992), cyclooxygenases (Gierse *et al.* 1995), and NOS (Vasquez-Vivar *et al.* 1998). Intracellular and extracellular ROS were measured using DCF and isoluminol respectively. TNFα did not induce a significant increase in either intracellular or extracellular ROS production in HUVEC. The lack of a significant response in endothelial cells following treatment with TNFα, contradicts a number of reports in the literature (Peng *et al.* 2000; Corda *et al.* 2001). A possible reason for the differences in the data may be due to the concentrations of TNFα used to induce ROS. TNFα concentrations of 2.5 ng/ml and 7.5 ng/ml were used in this investigation, whereas 60 ng/ml was used in Peng *et al.* (2000). Further studies using a range of concentrations may resolve some of these differences.

Pre-treatment with EGCG appeared to reduce DCF fluorescence following treatment with TNFα and PMA. The suppression of DCF oxidation by EGCG may be due to the scavenging ability of the compound. This was proposed as DCF gradually oxidises over time in the absence of any stimulus. Although much of the extracellular EGCG was removed from the extracellular environment, it is feasible that some EGCG remained inside the cell preventing basal DCF oxidation.

Figure 3.22 is a diagram showing the proposed pathways leading to NF-κB activation and indicates where the chemopreventive agents act in B cells and HUVEC. To conclude, curcumin, resveratrol and EGCG inhibit NF-κB translocation into the nucleus. Also EGCG blocked NF-κB/DNA specific binding. I3C appeared to increase PMA-induced NF-κB p65 translocation in the C143, but had no significant effect in H308 B lymphoblast cell lines. EGCG may function to block IKK or upstream regulators of IKK in HUVEC, but appears to act on NF-κB in B lymphoblasts. Although EGCG was a prooxidant in cell medium generating H_2O_2, this oxidant had no significant effect on NF-κB translocation. In addition EGCG functioned as an antioxidant when cells were stimulated PMA or TNFα. These data have helped to characterise the chemopreventive properties of these four agents with particular attention to EGCG, which is useful when designing future *in vitro* and *in vivo* studies.

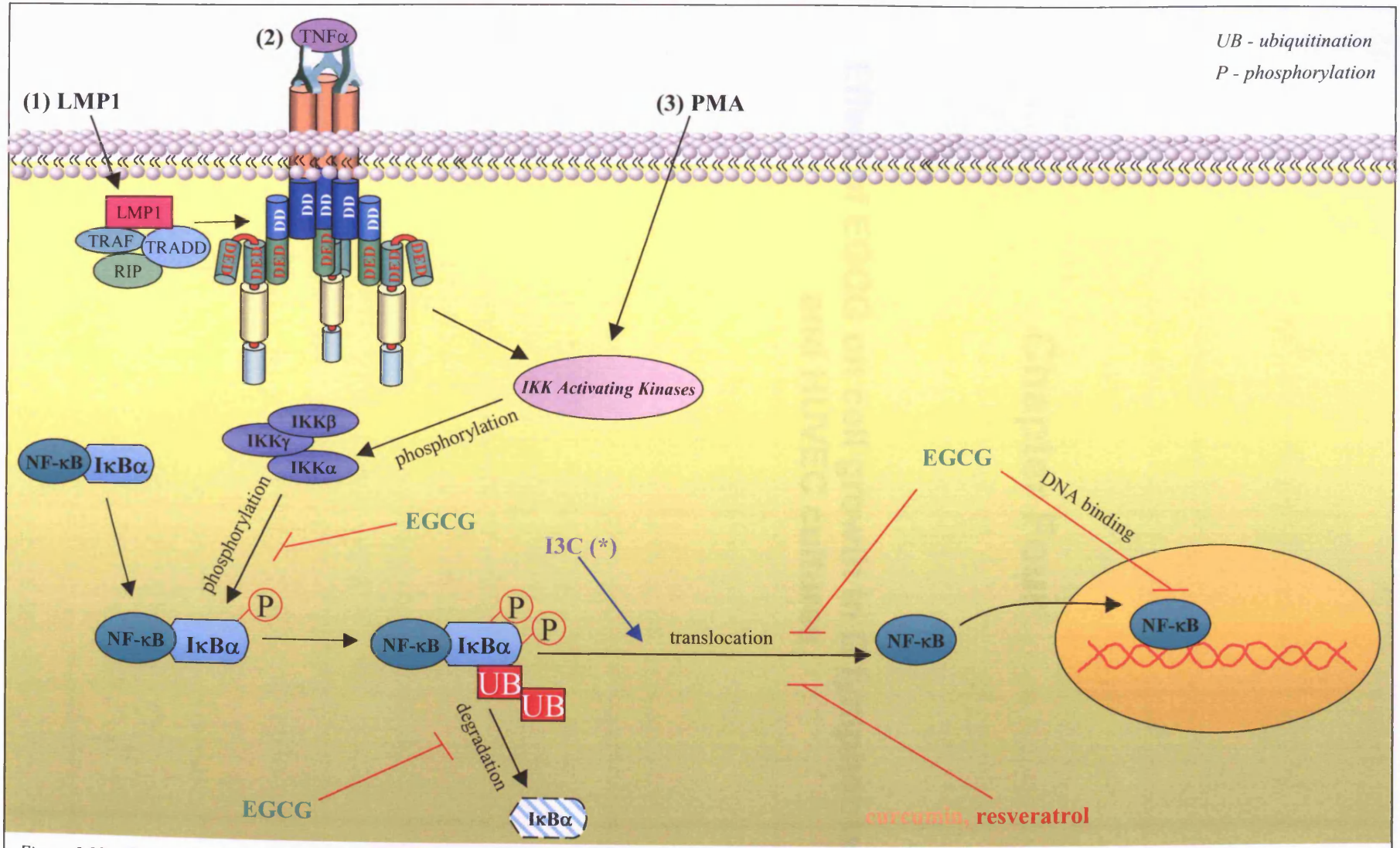

Figure 3.22 - The proposed mechanisms of action of EGCG, curcumin, resveratrol, and I3C on the NF-κB signalling pathway in B lymphoblasts and HUVEC. Constitutive activation of NF-κB in B lymphoblasts (1) may occur through EBV-upregulated LMP1 interaction with TNF receptor associated factor (TRAF), TNF receptor associated death domain (TRADD) and receptor interacting protein (RIP). In HUVEC TNFα binding to the TNF receptor leads to activation of NF-κB via TRAF/TRADD/RIP signalling (2) In B lymphoblasts the signalling mechanisms leading to activation of NF-κB by PMA have not been clearly defined (3). The blue and red lines represent promotion or inhibition, respectively. DD and DED correspond to death domain and the death effector domain of the TNF receptor. (*) represents an effect in C143 B lymphoblasts.

Chapter Four

Effect of EGCG on cell growth in B lymphoblast and HUVEC cultures

4.0 Introduction

4.1 Proliferation and apoptosis in cardiovascular disease

One of the downstream consequences of NF-κB regulation is the modulation of cell growth and/or cell survival pathways. As mentioned in chapter 1, NF-κB can be both pro- or anti-apoptotic dependent on a number of factors, such as the cell and type of ambient signal. Many of etiological changes associated with the atherosclerosis are characterised by phases/stages of hyperproliferation and/or apoptosis. Early plaque progression is correlated with continual proliferation, and recruitment of circulating monocytes and in the commencement of vascular remodelling by the laying down of collagenous tissue. Plaque size is increased as more smooth muscle cells and monocytes migrate into and proliferate within the plaque, and secrete more extra-cellular matrix proteins. Intriguingly, this increase in proliferation is also associated with an increase in apoptosis of cells present within the lipid-rich core of the plaque, such as T lymphocytes, endothelial cells, VSMC's and monocytes. In these early stages it remains unclear whether apoptosis is a trigger or conversely a consequence of the disease process.

Theoretically, it would be beneficial to prevent or induce regression of thickened arterial walls in atherosclerotic plaques by suppressing cellular proliferation and/or inducing apoptosis. Moreover, apoptosis of certain cells may help to suppress the immune response/reactions that are pivotal in the initiation and progression of plaque formation. It is also conceivable that regulating cell numbers by inhibiting the cell cycle or inducing apoptosis, may provide a means for the conversion a cellular-rich lesion to a more fibrous hypocellular stable plaque. However, the benefit of cell cycle or apoptosis regulation in atherosclerosis depends on the stage of plaque localisation and cell types involved. Proliferation early on in the disease process is thought to contribute to plaque stability, through the 'laying-down' of matrix proteins, whereas induction of apoptosis in the later stages of atherosclerosis could in fact have deleterious effects. It is plausible that apoptosis of VSMCs and to a lesser extent endothelial cells, which are present in the shoulder region of the plaque may result in selectively weakening the fibrous coat, which in turn would result in plaque erosion and eventual rupture. This would culminate in one or more acute coronary events such as thrombosis, stroke or myocardial infarction, and hence would be a less desirable therapeutic outcome. However, in terms of chemoprevention, blocking or suppressing the early stages of proliferation would be the ideal objective.

To date there has been only a limited investigation into the role of cell cycle and apoptosis in the pathology of vascular disease (As reviewed in Zettler *et al.* 2000). In a rat model of arterial injury, an up-regulation and activation of CDK2 and cyclins E and A were observed between 1 and 2 days post-balloon injury (Wei *et al.* 1997). This was correlated with PCNA (proliferating cell nuclear antigen) expression (an indicator of cell proliferation) up to 10 days post-injury, declining after 18 days. CDK2 and cyclin E expression was noted in medial VSMCs 36 hours post-injury which then became undetectable in the media and confined to luminal surface of the intima. Basal p21 expression was not observed in uninjured vessels.

Expression of other cell cycle proteins in primary atherectomy specimens has been assessed. The presence of p53 in the nuclei of VSMCs, ECs and macrophages in human atherosclerotic tissue has been demonstrated by Ihling *et al.* (1997). In this report p53 co-localised with $p21^{WAF1}$. The same cells were negative for a marker of proliferation Ki-67, providing evidence for the role of p53 and $p21^{WAF1}$ in abrogating cellular proliferation. Control tissue had very low levels of p53 and $p21^{WAF1}$ and no positive staining for Ki-67. Tanner *et al.* (1998) observed $p27^{KIP1}$ expression in non-replicating cells of both control and atherosclerotic lesions. However, the degree of $p21^{WAF1}$ expression was correlated with the severity of the disease.

A number of growth regulatory proteins are investigated in this chapter including cyclin D1, cyclin dependent kinase 1 (CDK1), prolyl *cis-trans* isomerase (Pin1), X-linked inhibitor of apoptosis (XIAP) and p53. Several of these proteins were selected as they have been shown to be modulated by EGCG in certain cell types but more importantly because they are targets of NF-κB, either through protein:protein interaction or via transcriptional regulation.

There are many reports in the literature demonstrating the importance of cyclin D1 in cellular proliferation. For example, expression of anti-sense cyclin D1 constructs in human cancer cell models resulted in a marked inhibition of cell proliferation and the cells reverted toward normal phenotype losing their tumourigenicity in nude mice (Arber *et al.* 1997; Zhou *et al.* 1995). Conversley, Pagano *et al.* (1994) have demonstrated cyclin D1 can have an inhibitory rather than a growth stimulatory effect. They reported that microinjection of cyclin D1 into human lung fibroblasts resulted in cell cycle arrest in late G1. Cyclin D1 can also play a role in apoptosis as reported by Freeman *et al.* (1994) who found that cyclin D1 levels were induced about 10 fold during neuronal cell death. Cyclin D1 when ectopically over-expressed in serum starved rat fibroblasts led to an increase in

apoptosis (Sofer-Levi *et al.* 1996). These studies support the concept that cyclin D1 promotes apoptosis in these particular cell systems. In contrast suppression of cyclin D1 has also been associated with an increase in apoptosis (Lahti *et al.* 1997). Cyclin D1 may impinge on cell growth and apoptotic pathways through its regulation by or interaction with NF-κB (Ahmad *et al.* 2000; Guttridge *et al.* 1999; Hinz *et al.* 1999; Joyce *et al.* 1999) or p53 (Chen XB *et al.* 1995; Hiyama *et al.* 1999).

The protein CDK1 (also known as Cdc2) is thought to be an essential cell cycle regulatory protein (Nurse *et al.* 1994). In yeast cells $p34^{CDC2}$, via the interaction with cyclins, promotes the entry into the S phase and mitosis (Forsburg *et al.* 1991). In mammalian cells CDK1 binds to cyclin B and phosphorylates Cdc25c, essential to the progression of the cell cycle from G2-M (Nurse *et al.* 1994). As well as its role in G2/M phase progression, there is conflicting evidence in the literature suggesting that CDK1 is also involved in G1-S phase transition (Furukawa *et al.* 1990; Krek *et al.* 1991). As well as acting on Cdc25c, CDK1 may facilitate a growth regulatory function through its interaction with p53 (Taylor *et al.* 1999, Azzam *et al.* 1997; Passalaris *et al.* 1999).

The protein Pin1 plays an important role in the cell cycle through specific interaction with proteins that are phosphorylated at serine/threonine-proline motifs (Lu *et al.* 1996; Yaffe *et al.* 1997; Shen *et al.* 1998). Increasing evidence suggest a role of Pin1 in breast cancer tumorigenesis (MacLachlan *et al.* 2000). Inhibition of Pin1 function in human tumor cells using expression of Pin1 antisense RNA, or dominant negative mutants induces mitotic arrest and apoptosis (Lu *et al* 1996; Rippmann *et al.* 2000), whereas overexpression of Pin1 can lead to a G2 phase arrest of the cell cycle (Lu *et al* 1996). The cell growth and apoptosis regulatory functions of Pin1, are thought to be mediated through the interaction with several important cell growth regulatory proteins including p53 (Zacchi *et al.* 2002; Zheng *et al.* 2002), Cdc25c (Zhou *et al.* 2000) and cyclin D1 (You *et al.* 2002).

XIAP is one of the more characterised members of the inhibitor of apoptosis family. XIAP, along with other IAPs, functions to prevent apoptosis, by inhibiting caspase activity. Accentuating its importance in cell growth regulation is the finding that XIAP is a transcriptional target of NF-κB (Stehlik *et al.* 1998), but more recently, XIAP has been shown to regulate NF-κB activation (Hofer-Warbinek *et al.* 2000). In addition to regulating apoptosis, various members of the IAP family have been implicated in the cell cycle. XIAP has been reported to exist in a ternary complex with caspase-3 and the cell cycle regulator $p21^{WAF1}$ (Suzuki *et al.* 1998).

The transcription factor p53, sensitive to cellular stress, has been implicated in the regulation of apoptosis (Yin *et al.* 1998; Burns *et al.* 1999; El-Diery *et al.* 1994). Additionally, p53 functions as a cell growth regulator by controlling progression of the cell cycle (mainly G1 progression). This response is thought to occur mainly through regulation of $p21^{WAF1}$ an inhibitor of CDK1 (El-Diery *et al.* 1994, 1998a, 1998b), but also by acting directly on CDK1 expression (Yun *et al.* 1999). Thompson *et al.* (1997) have shown that specific inhibition of p53 by human papilloma virus E6 protein, increased the number of normal lung fibroblasts entering mitosis after exposure to ionising radiation, suggesting that p53 is required for G2 arrest. Importantly, p53 interacts with NF-κB (Wu *et al.* 1994), which itself is a pivotal cell survival and cell death pathway, depending on cell type and inducing signal.

The above reports emphasize the importance of studying the cell cycle in relation to the development and progression of cardiovascular diseases such as atherosclerosis. When considering this evidence, it is feasible that attempting to regulate the cell cycle or apoptosis (through dietary intervention) could provide an alternate method in combating atherosclerosis instead of, or in conjunction with lipid-lowering treatments. Therefore the potential of EGCG to influence the growth of the B lymphoblast (hypertensive and normotensive) cell lines and HUVECs, specifically the affect on the cell cycle and induction of apoptosis, was explored.

4.2 Results

4.2.1 EGCG inhibits cell growth in B lymphoblast cell lines and HUVEC

The effect of EGCG on cell growth/proliferation in the B lymphoblast cell lines and HUVEC was investigated. For the purpose of this thesis, cell growth or proliferation was simply defined as an increase in cell number. For these initial experiments, cells were grown in complete medium in the presence or absence of EGCG or vehicle control at the indicated concentrations and time points. Relative cell densities were established using a coulter counter, which measured the number of cells of a particular size in a set volume of isoton solution (see chapter 2). Data were normalised to the untreated control, which was taken as 100 %.

Figure 4.1 shows the effect of EGCG on the C143 B lymphoblast cell line. Cell growth was not significantly changed following treatment with DMSO. After 24 hours of EGCG treatment there was no significant effect between the treated and control samples. EGCG inhibited growth in a concentration-dependent manner at 48, 72 and 96 hours treatment. At 48 and 72 hours, significant inhibition was seen with 6, 9, 12, and 15 μM EGCG ($p<0.05$) (figure 4.1 B, C), whereas significant inhibition was noted also at 3 μM, at 96 hours EGCG treatment ($p<0.05$). The IC_{50} value of EGCG for C143 was 10 μM.

Figure 4.2 represents the effect of EGCG on the normotensive, C149 B lymphoblast cell line. DMSO treatment did not significantly affect C149 cell growth. Data indicated that the C149 cell line behaved in a similar manner to the C143 cell line, although it was not as sensitive. Significant growth inhibition was seen at 12 μM at 48 hours and 12 and 15 μM at 72 and 96 hours EGCG treatment ($p<0.05$). The IC_{50} for C149 was 17 μM.

The effect of EGCG on the growth characteristics of the H308 B lymphoblast cell line was examined (figure 4.3). As in the normotensive C143 and C149 cell line, EGCG was found to cause a concentration-dependent reduction in cell numbers, demonstrating a growth inhibitory effect. The control DMSO treatments did not significantly alter the growth of H308. The EGCG-inhibitory effect was significant at 30 μM after 24 hours, 15 to 30 μM at 48 hours, 10 to 30 μM at 72 hours and 15 to 30 μM at 96 hours ($p<0.05$). The IC_{50} value for the H308 cell line was 18 μM indicating that the H308 cell line was less

sensitive to the growth inhibitory effect of EGCG, than the C143 cell line. EGCG had a similar growth-inhibitory effect in the H242 hypertensive cell line (figure 4.4). EGCG caused a concentration-dependent growth inhibition at 48, 72 and 96 hours treatment. Significant growth inhibition was noted at 12 and 15 μM at 48 hours and 9, 12, and 15 μM at 72 and 96 hours EGCG treatment ($p<0.05$). The IC_{50} value for H242 growth inhibition was 13 μM.

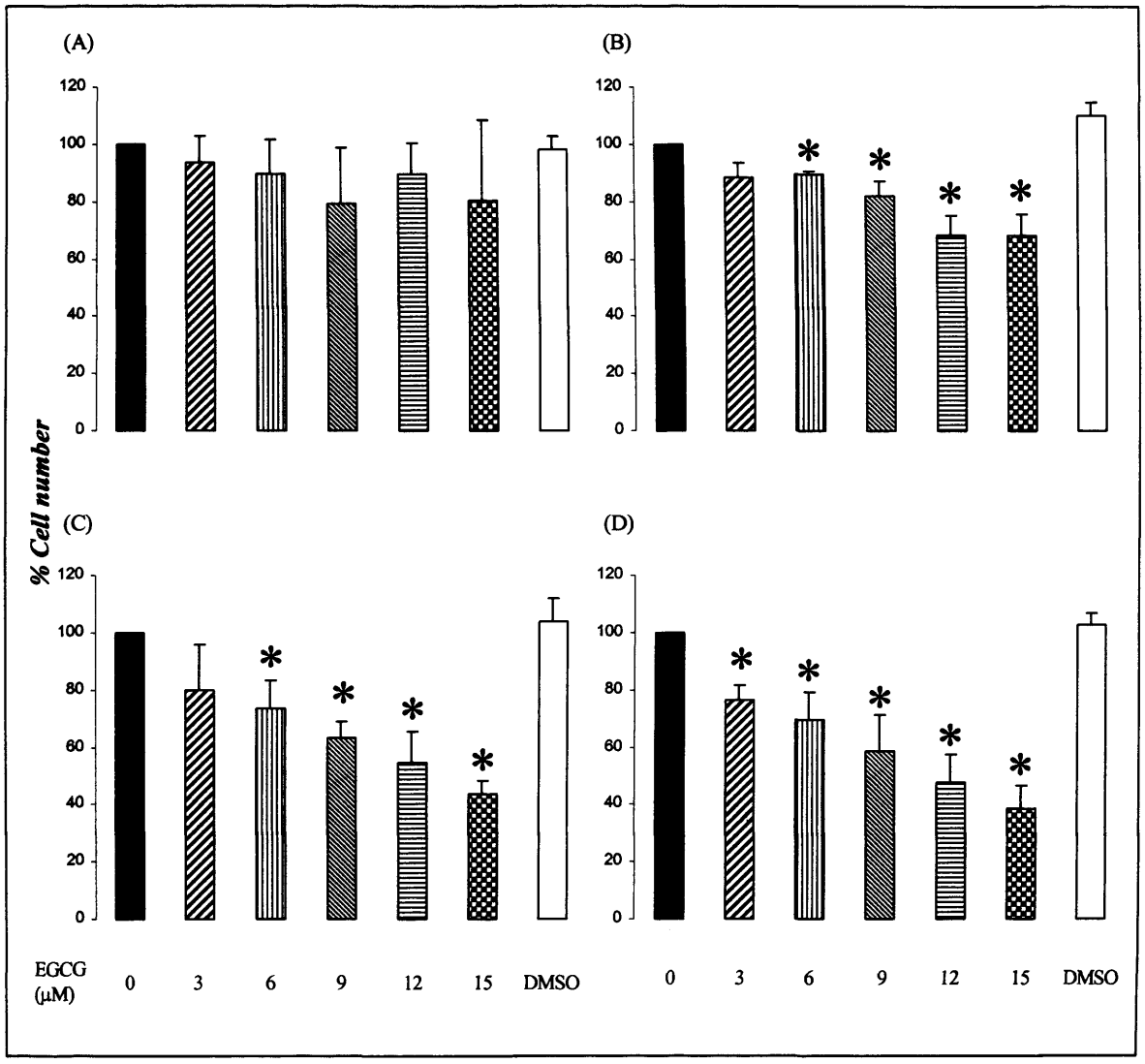

Figure 4.1 - Effect of EGCG on the growth of C143. *In full growth medium, cells were untreated, or exposed to DMSO (control), or to EGCG at the indicated concentrations (μM) for (A) 24 hours; (B) 48 hours; (C) 72 hours; (D) 96 hours. Data were normalised to the untreated control (100%) and are the mean of 3 independent experiments performed in duplicate, * indicates significance from controls ($p<0.05$) as determined by the ANOVA balanced model followed by Tukey's least significant difference post hoc test.*

Comparing the inhibitory effect of EGCG in the B lymphoblasts to the effect in the HUVEC (figure 4.5) it was evident that the HUVEC behaved in a similar manner. At earlier time points of 24 and 48 hours exposure, there was evidence of an EGCG-induced

growth inhibitory effect, although this inhibition was not dose-dependent. However at longer time points of 72 and 96 hours, it was evident that EGCG inhibited cell growth in a concentration-dependent manner. Significant growth inhibition was observed at 10, 15, and 50 μM after 24 hours, 30 and 50 μM after 48 hours, and 10, 15, 30 and 50 μM after 72 and 96 hours respectively ($p<0.05$). The IC_{50} for growth inhibition in HUVEC was 15 μM indicating that HUVEC were overall, as sensitive as the B lymphoblast cell lines.

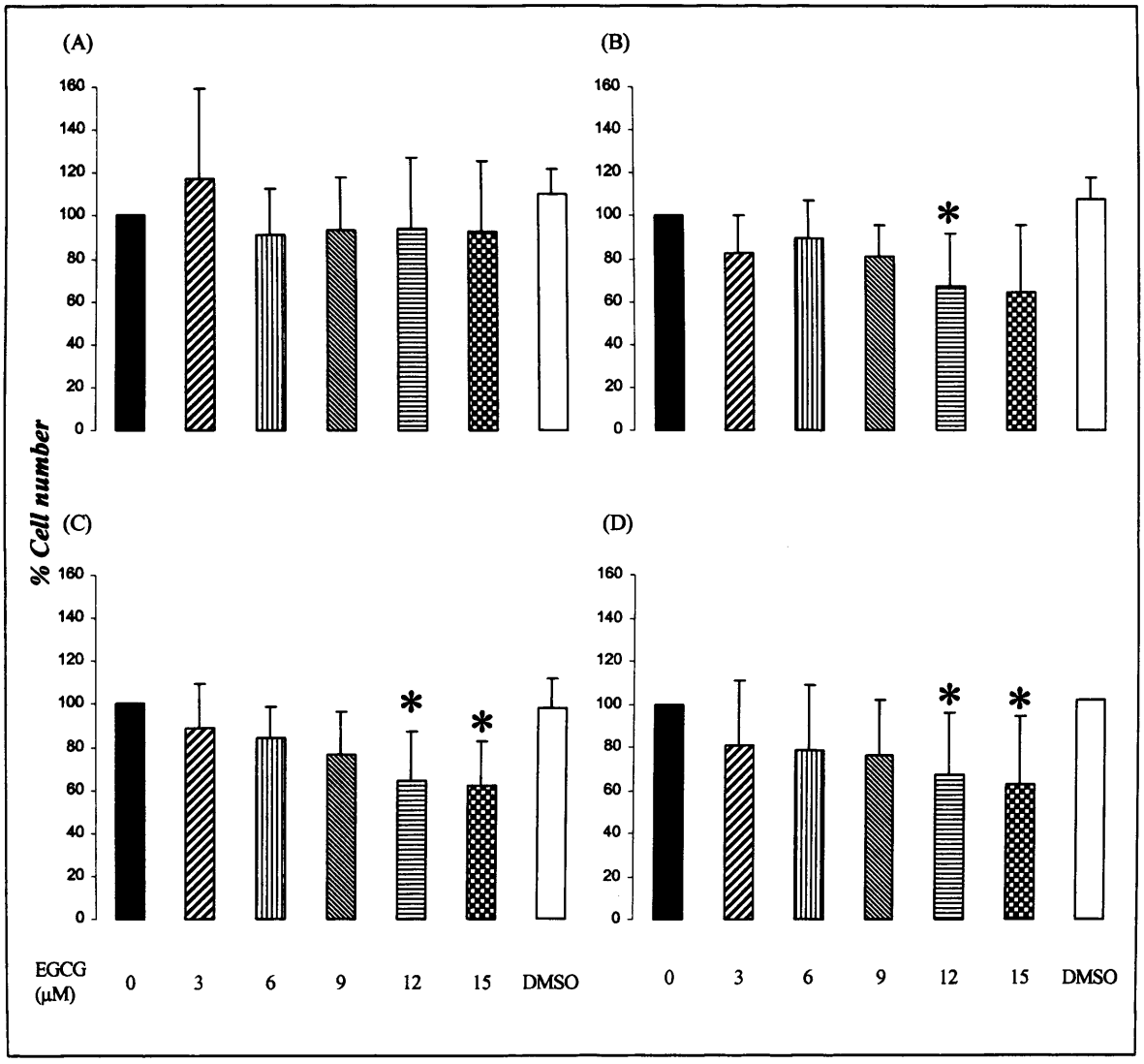

Figure 4.2 - Effect of EGCG on the growth of C149. *In full growth medium, cells were untreated, or exposed to DMSO (control), or to EGCG at the indicated concentrations (μM) for (A) 24 hours; (B) 48 hours; (C) 72 hours; (D) 96 hours. Data were normalised to the untreated control (100%) and are the mean of 3 independent experiments performed in duplicate, * indicates significance from controls ($p<0.05$) as determined by the ANOVA balanced model followed by Tukey's least significant difference post hoc test.*

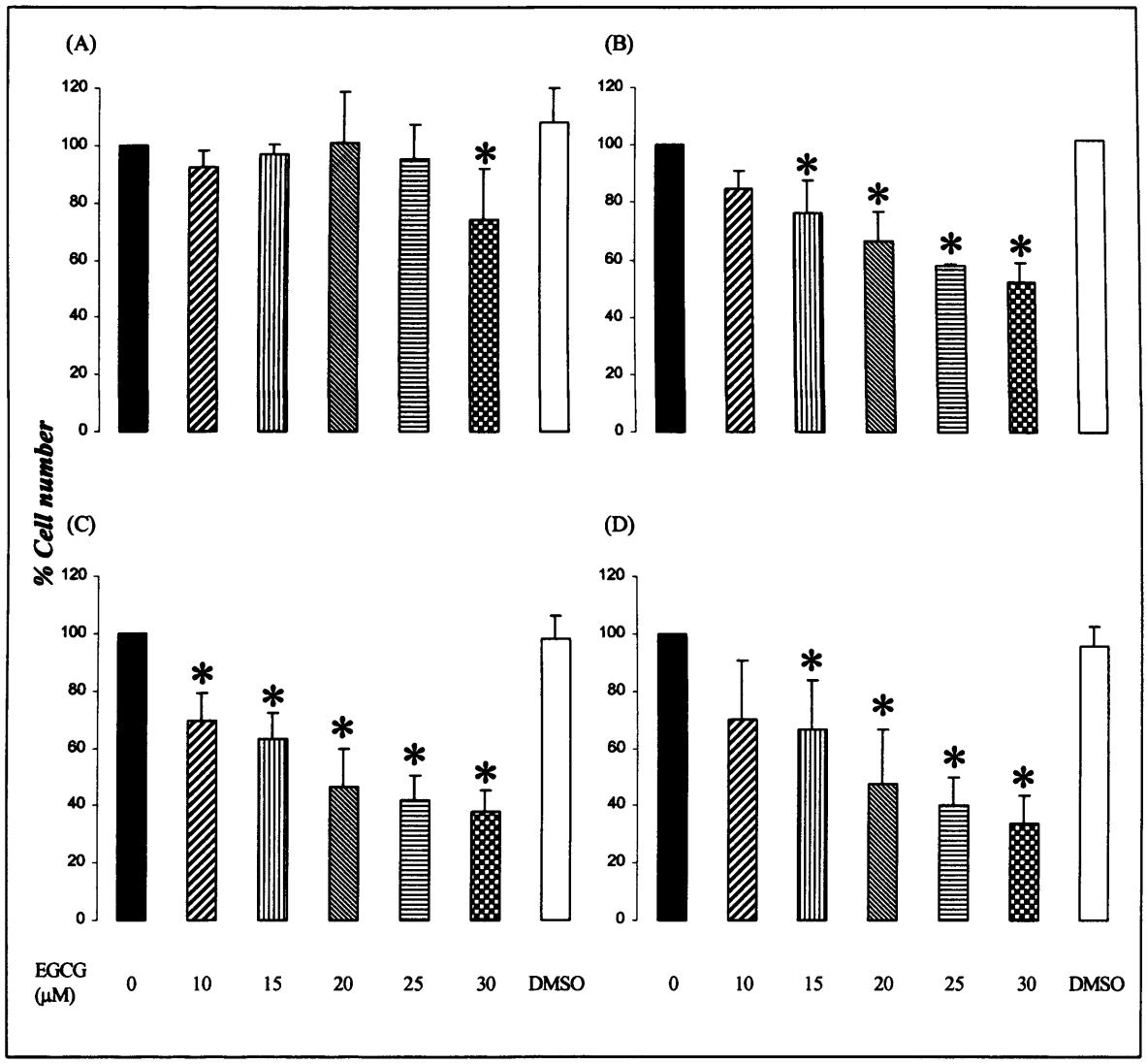

Figure 4.3 - Effect of EGCG on the growth of H308. *In full growth medium, cells were untreated, or exposed to DMSO (control), or to EGCG at the indicated concentrations (μM) for (A) 24 hours; (B) 48 hours; (C) 72 hours; (D) 96 hours. Data were normalised to the untreated control (100%) and are the mean of 3 independent experiments performed in duplicate, * indicates significance from controls ($p<0.05$) as determined by the ANOVA balanced model followed by Tukey's least significant difference post hoc test.*

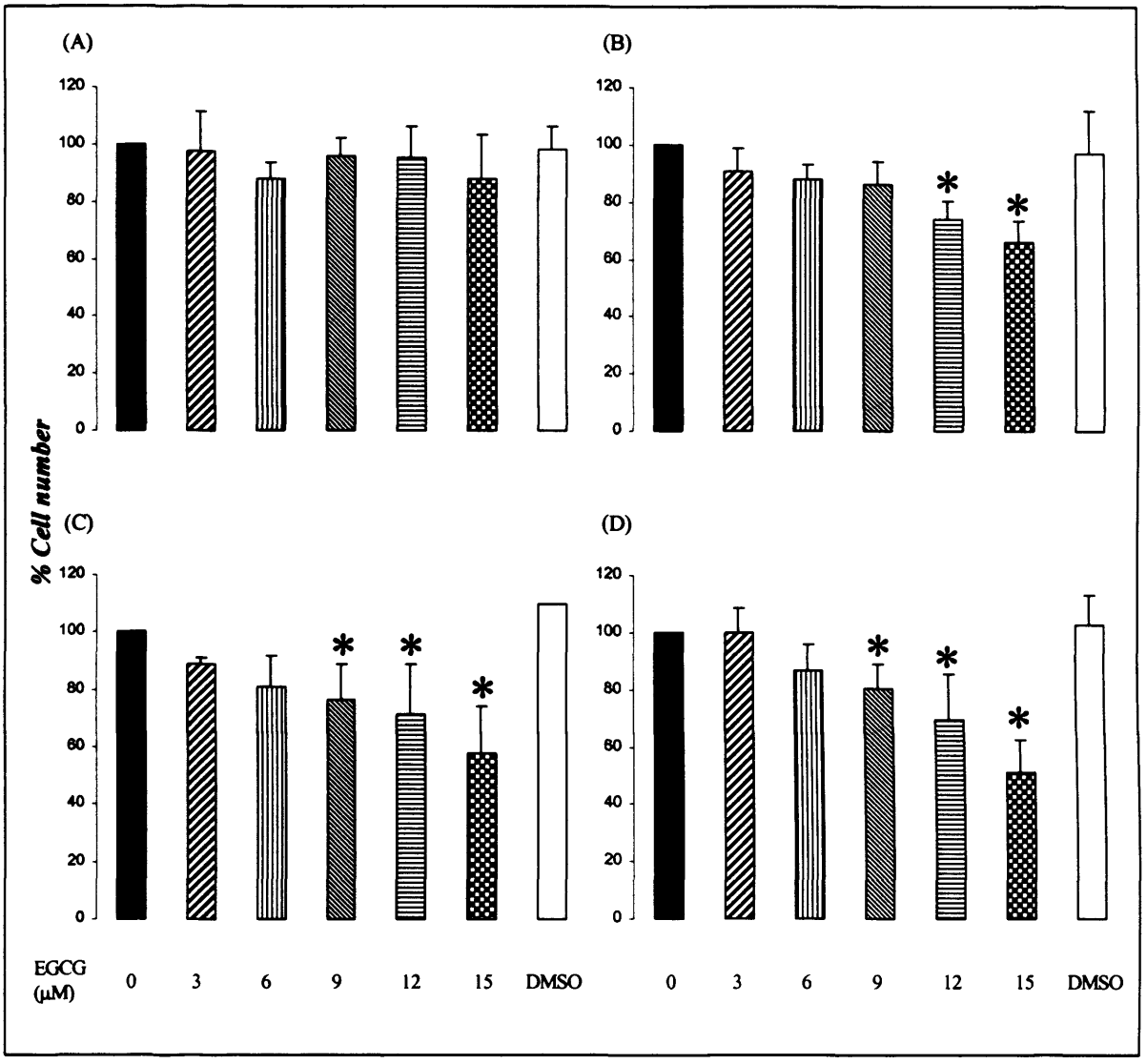

Figure 4.4 - Effect of EGCG on the growth of H242. *In full growth medium, cells were untreated, or exposed to DMSO (control) or to EGCG at the indicated concentrations (μM) for (A) 24 hours; (B) 48 hours; (C) 72 hours; (D) 96 hours. Data were normalised to the untreated control (100%) and are the mean of 3 independent experiments performed in duplicate, * indicates significance from controls ($p<0.05$) as determined by the ANOVA balanced model followed by Tukey's least significant difference post hoc test.*

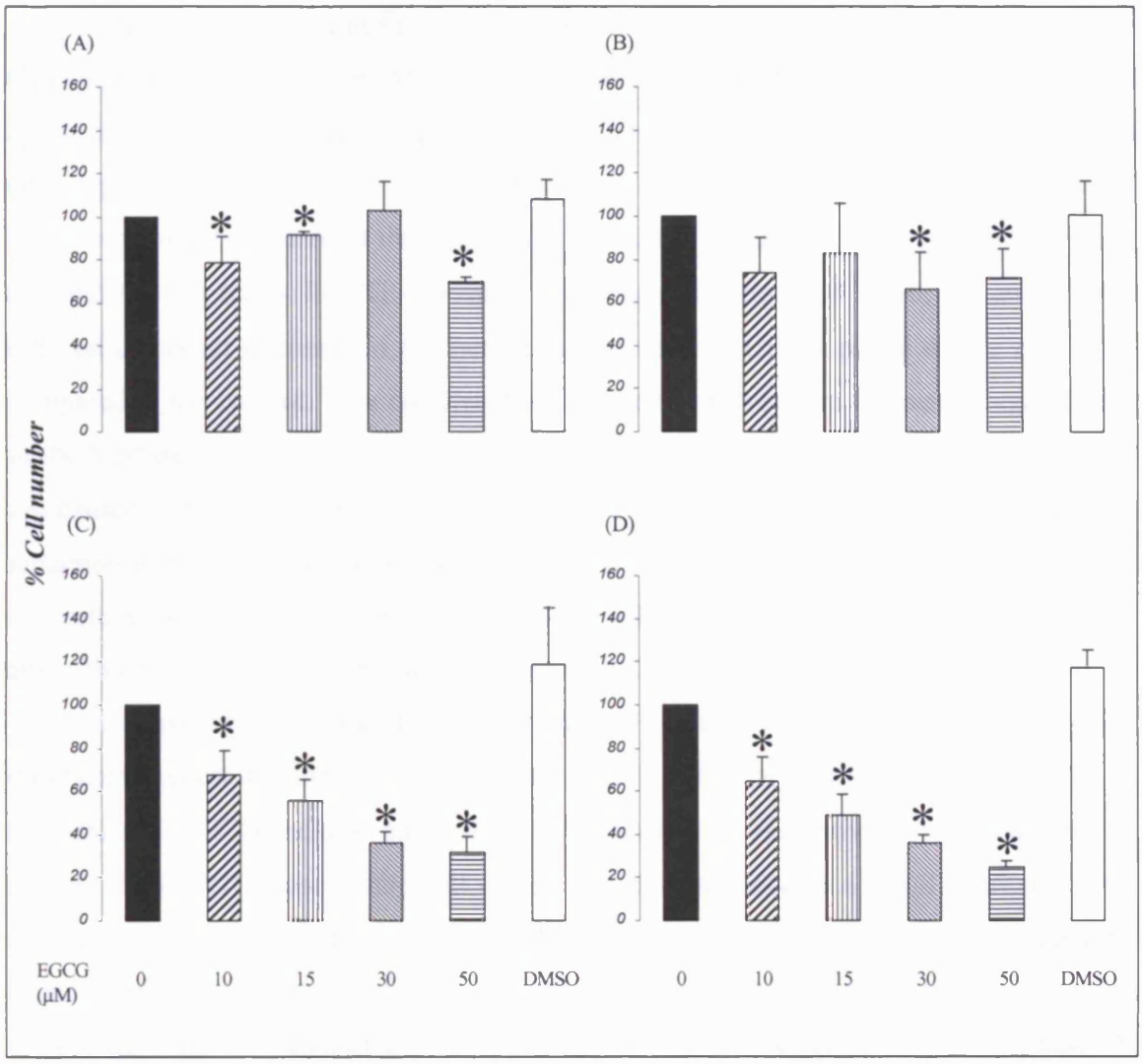

Figure 4.5 - Effect of EGCG on the growth of HUVEC. *In full growth medium, cells were untreated, or exposed to DMSO (control), or to EGCG at the indicated concentrations (μM) for (A) 24 hours; (B) 48 hours; (C) 72 hours; (D) 96 hours. Data were normalised to the untreated control (100%) and are the mean of 3 independent experiments performed in duplicate, * indicates significance from controls ($p<0.05$) as determined by the ANOVA balanced model followed by Tukey's least significant difference post hoc test.*

4.2.2 EGCG modulates the progression of the cell cycle

Having established that EGCG inhibits cellular proliferation in all cell types, to further characterise this response the effect of EGCG on cell cycle progression was considered. For this series of experiments, cells were incubated with concentrations and at times similar to those used in the cell proliferation studies. The content of cellular DNA was established using propidium iodide (PI) staining in conjunction with FACS analysis.

The data contained in Figure 4.6 show the effect of EGCG on the cell cycle in the C143 normotensive cell line. At earlier time points of 24 (A) and 48 (B) hours, EGCG appeared to induce a modest concentration-dependent increase in the percentage of cells in G0/G1 phase of the cell cycle, when compared to the control. This increase was reflected by a concomitant decrease in the percentage of cells in S phase. At later time points of 72 (C) and 96 (D) hours EGCG exposure, lower concentrations of 5, 10, 15 and 20 μm induced a modest decrease in the percentage of cells in G0/G1 phase of the cell cycle compared to the control. This correlated with a modest increase in the percentage of cells in the S phase of the cell cycle. Interestingly, EGCG at concentrations of 30, 50 and 100 μm caused a modest increase in the percentage of cells in G0/G1 phase, corresponding to a reduction of cells in S phase of the cell cycle. It was noted that there were no significant differences between the untreated control and the DMSO control treatment indicating that any effect on cell cycle progression was due to EGCG alone and not the carrier solvent.

Comparing C143 to another normotensive B lymphoblast cell line C149, figure 4.7, these data suggest that the cells behaved in a slightly different manner. At 24 hours (A), 50 and 100 μM EGCG induced an increase 10 % and 12 % respectively, in the number of cells in the G0/G1 phase of the cell cycle. This corresponded to a small decrease in the number of S phase cells of the cell cycle. At 48 hours (B), there was evidence to suggest a concentration-dependent increase in the number of cells in the G0/G1 phase of the cell cycle. Again this was consistent with a decrease in the number of cells in the S phase. At 72 hours (C), EGCG at lower concentrations did not significantly alter the number of cells in any phases of the cell cycle when compared to the control cells. However, at the higher concentrations of 50 and 100 μM there was evidence to suggest an accumulation of cells in the G0/G1 phase of the cell cycle, suggesting an arrest at this stage. At 96 hours (D) there may have been a modest increase in the number of cells in the G2/M phase of the cell cycle following treatment with 5 to 30 μM EGCG.

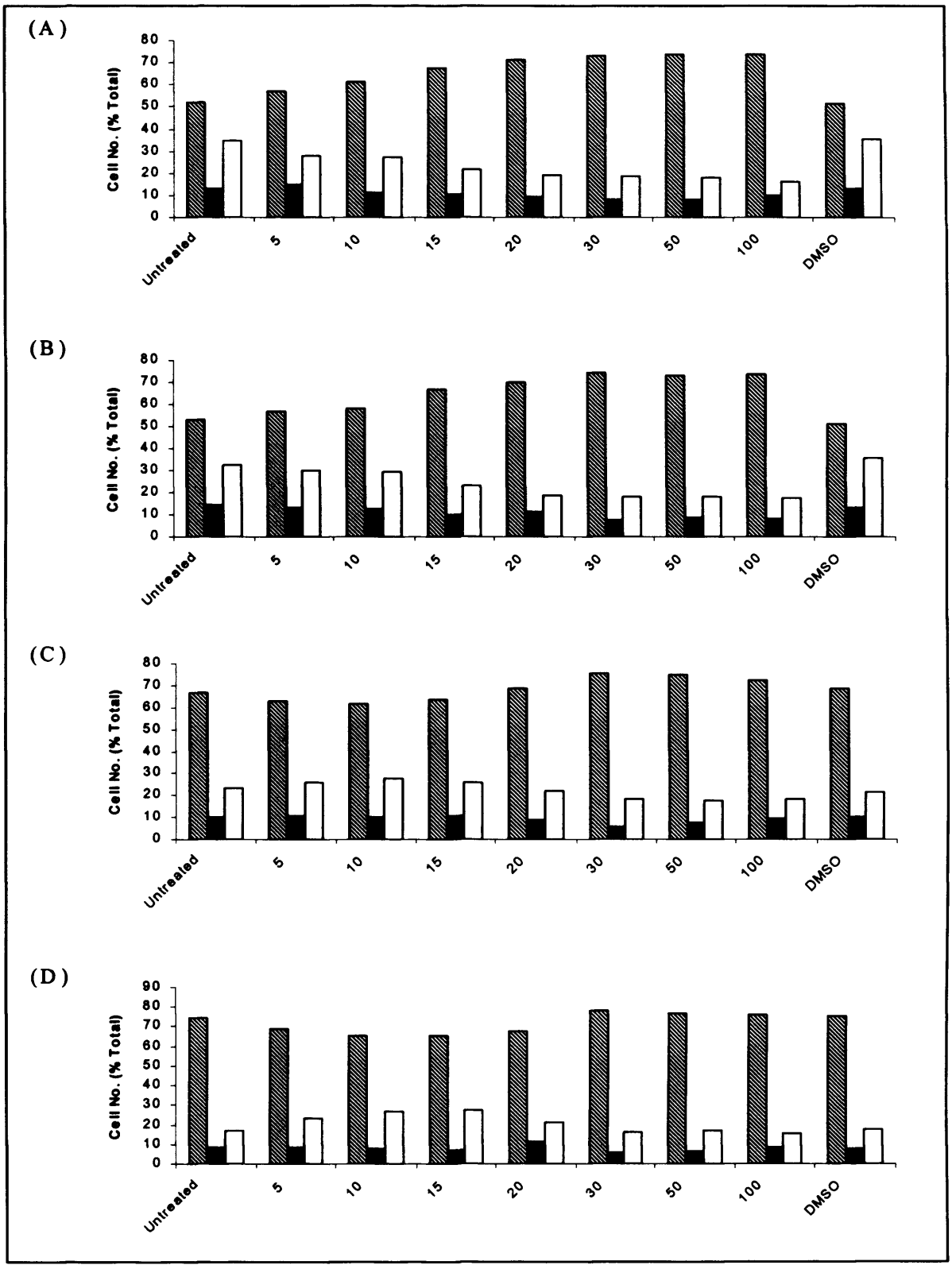

Figure 4.6 - Analysis of cell cycle in C143 following exposure to EGCG. *In full growth medium, asynchronous C143 cells were untreated, or exposed to DMSO (control) or EGCG at the indicated concentrations (μm) for (A) 24 hours; (B) 48 hours; (C) 72 hours; (D) 96 hours. Cells were stained with PI and analysed by flow cytometry. The percentage of cells in each phase of the cell cycle was estimated using cell quest and modfit software. The crossed bars represent G0/G1, the black bars G2/M and the clear bars S phase. Data shown were the mean of two independent experiments.*

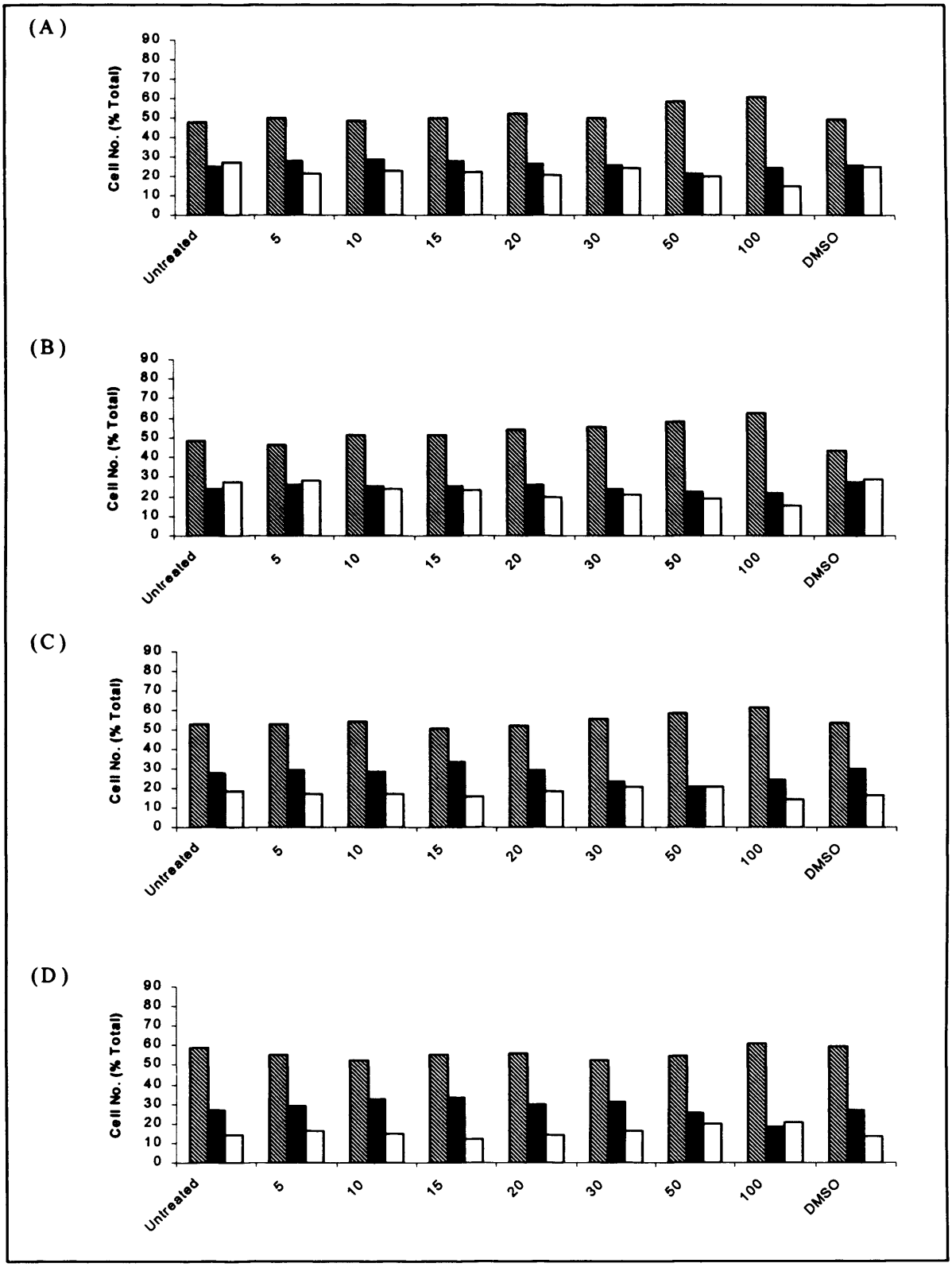

Figure 4.7 - Analysis of cell cycle in C149 following exposure to EGCG. *In full growth medium, asynchronous C149 cells were untreated, or exposed to DMSO (control) or EGCG at the indicated concentrations (μm) for (A) 24 hours; (B) 48 hours; (C) 72 hours; (D) 96 hours. Cells were stained with PI and analysed by flow cytometry. The percentage of cells in each phase of the cell cycle was estimated using cell quest and modfit software. The crossed bars represent G0/G1, the black bars G2/M and the clear bars S phase. Data shown was the mean of two independent experiments.*

Figure 4.8 exemplifies the effect of EGCG on one of the hypertensive B lymphoblast cell lines, H308. At 24 hours there was evidence of a modest increase in the number of cells in the G0/G1 (5%) phase of the cell cycle at 50 and 100 μM EGCG. This was matched by a decrease in the number of S phase cells. This modest increase was also evident at lower EGCG concentrations at 48, 72 and 96 hours treatment. However, these changes were not statistically significant ($p<0.05$). Intriguingly, when compared to both types of the normotensive cell line, on the whole there appeared to be substantially less cells in the G2/M and S phases of the cell cycle. That is to say there was a higher proportion of cells in the G0/G1 phase of the cell cycle. Furthermore the overall distribution profile of the number of cells at each phase of the cell cycle was different compared to the C149, but similar to C143 normotensive cell lines.

The effect of EGCG on cell cycle progression in the primary cell line HUVEC is represented in figure 4.9. There were no observed disparities between the untreated and DMSO controls. At 24 hours, EGCG (50 μM) caused a (6 %) decrease in the number of cells in the S phase of the cell cycle, corresponding to a modest increase in the number of cells in the G0/G1 and G2/M phase of the cycle. At 48, 72 and 96 hours there was a similar decrease in the number of cells in S phase. At 30 μM, there was an increase in the number of G2/M cells (8%, 10 % and 12 %) at 48, 72 and 96 hours respectively, reflected by a decrease in the number of cells in the G0/G1 phase of the cell cycle. The cell cycle profile, namely the proportion of cells at each phase of the cell cycle for HUVEC, was similar to the C149 normotensive B lymphoblast cell line. That is to say overall the majority of cells were in the G0/G1, followed by G2/M and then S phases of the cell cycle.

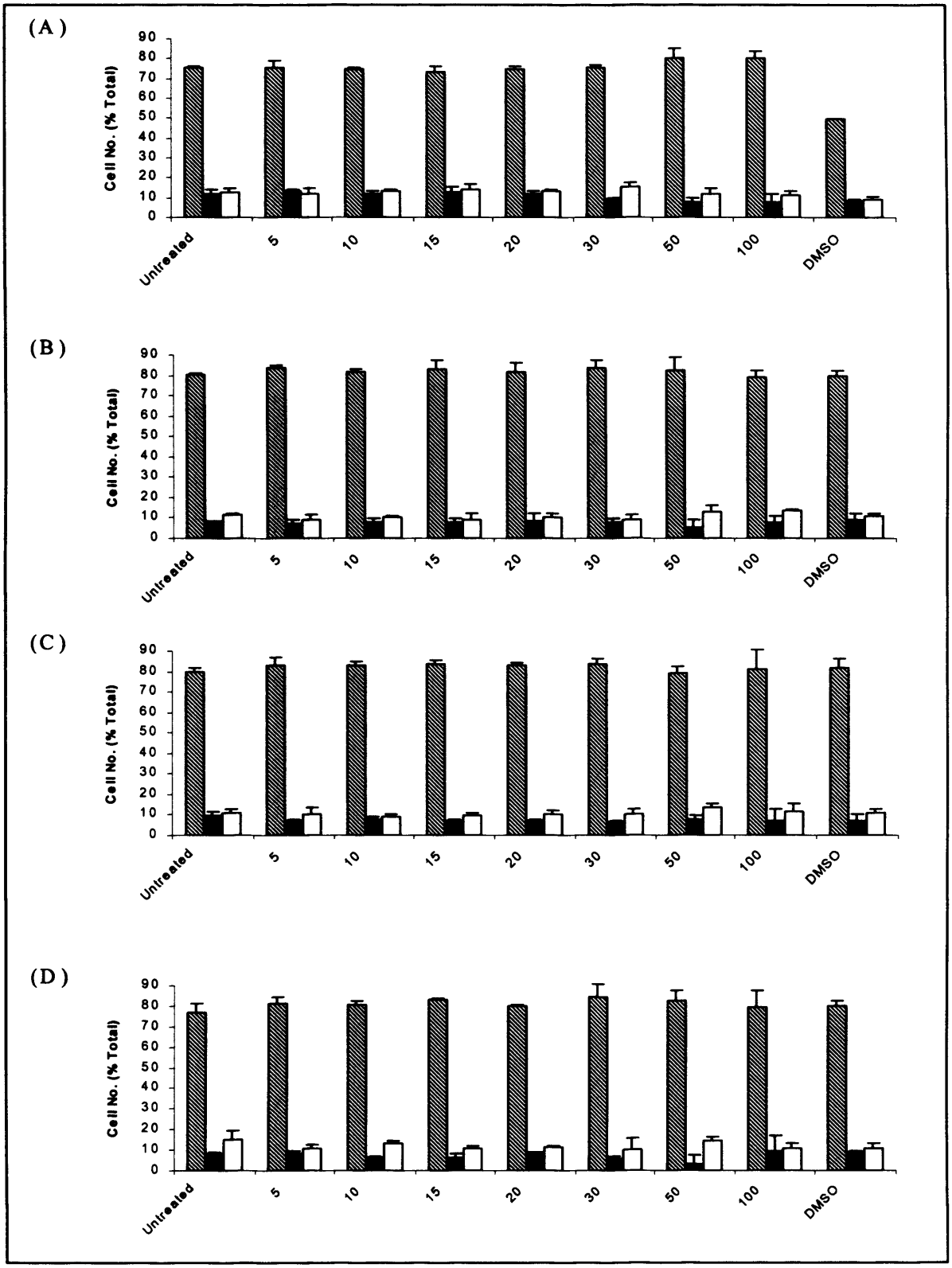

Figure 4.8 - Analysis of cell cycle in H308 following exposure to EGCG. *In full growth medium, asynchronous H308 cells were untreated, or exposed to DMSO (control) or EGCG at the indicated concentrations (μm) for (A) 24 hours; (B) 48 hours; (C) 72 hours; (D) 96 hours. Cells were stained with PI and analysed by flow cytometry. The percentage of cells in each phase of the cell cycle was estimated using cell quest and modfit software. The crossed bars represent G0/G1, the black bars G2/M and the clear bars S phase. Data shown were the mean of 3 independent experiments.*

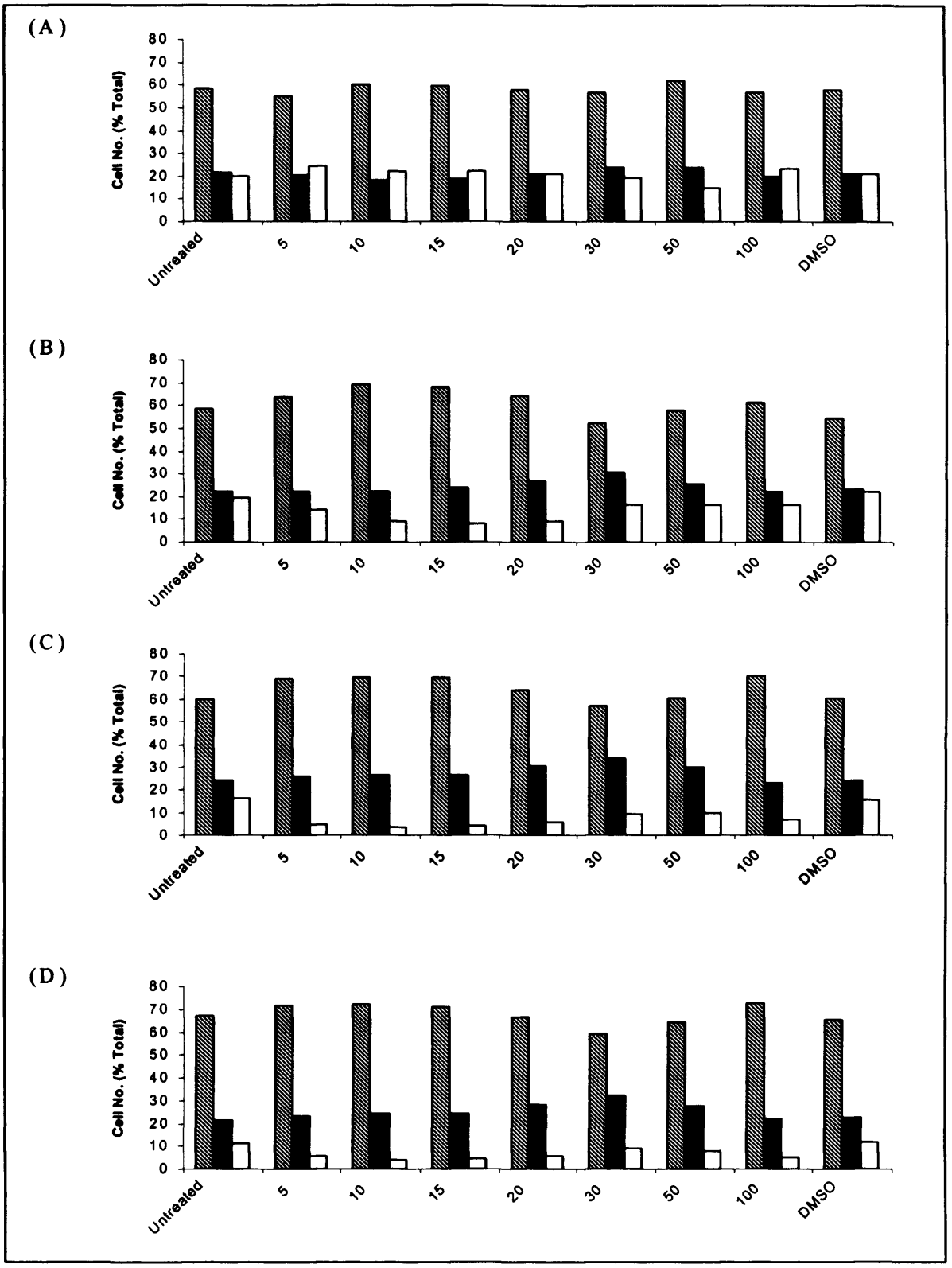

Figure 4.9 - Analysis of cell cycle in HUVEC following exposure to EGCG. *In full growth medium, asynchronous HUVEC were untreated, or exposed to DMSO (control) or EGCG at the indicated concentrations (μm) for (A) 24 hours; (B) 48 hours; (C) 72 hours; (D) 96 hours. Cells were stained with PI and analysed by flow cytometry. The percentage of cells in each phase of the cell cycle was estimated using cell quest and modfit software. The crossed bars represent G0/G1, the black bars G2/M and the clear bars S phase. Data shown were the mean of two independent experiments.*

4.2.3 EGCG induces apoptosis in the B lymphoblast cell lines and HUVEC

EGCG was shown to modulate cell growth in all cell lines under study, which in part may be due to an effect on the cell cycle. Further analysis was carried out to determine the contribution of apoptosis to the EGCG-induced growth inhibition. Incubation of cells in full growth medium, over similar EGCG concentrations (5 – 200 μM) and time range (24 – 96 hours) as those used in the cell proliferation studies was carried out. Induction of apoptosis was ascertained using an Annexin V-FITC conjugated peptide in conjunction with propidium iodide staining, which was detected using FACscan analysis.

The data contained in figures 4.10 and 4.11 show the apoptotic response evoked in the normotensive (control) cell lines C143 and C149, following EGCG exposure. There were no obvious changes in the number of viable, apoptotic and necrotic cells following treatment with DMSO. Data were the mean of 3 independent experiments in the C143 cell line (figure 4.10) and at 24 hours showed that EGCG induced a dose-dependent decrease in the percentage number of viable cells, which corresponded inversely with a concentration-dependent increase in the percentage number of necrotic cells at 10 and up to 200 μM EGCG. This pattern was noted at all time points under investigation. Significant apoptosis was seen to occur over the dose-range of 10 μM to 30 μM EGCG at 48 hours and 10 μM to 50 μM at 72 and 96 hours EGCG ($p<0.05$). This induction of apoptosis was found to increase in a time-dependent manner, with the greatest percentage of apoptotic cells occurring at 30 μm (68 %), EGCG at 96 hours.

Figure 4.11 demonstrates that C149 cell line responds in a similar manner to the C143 cell line, with a peak level of cells undergoing apoptosis occurring over 10 μm to 50 μm EGCG dose range. The DMSO control had no obvious effect on the percentage number of viable, apoptotic and necrotic cells. These data suggest that this cell line is not as sensitive to EGCG. This was concluded as the peak number of apoptotic cells was seen to occur at 50 μm EGCG (58 %) at 96 hours, compared to 30 μM in C143.

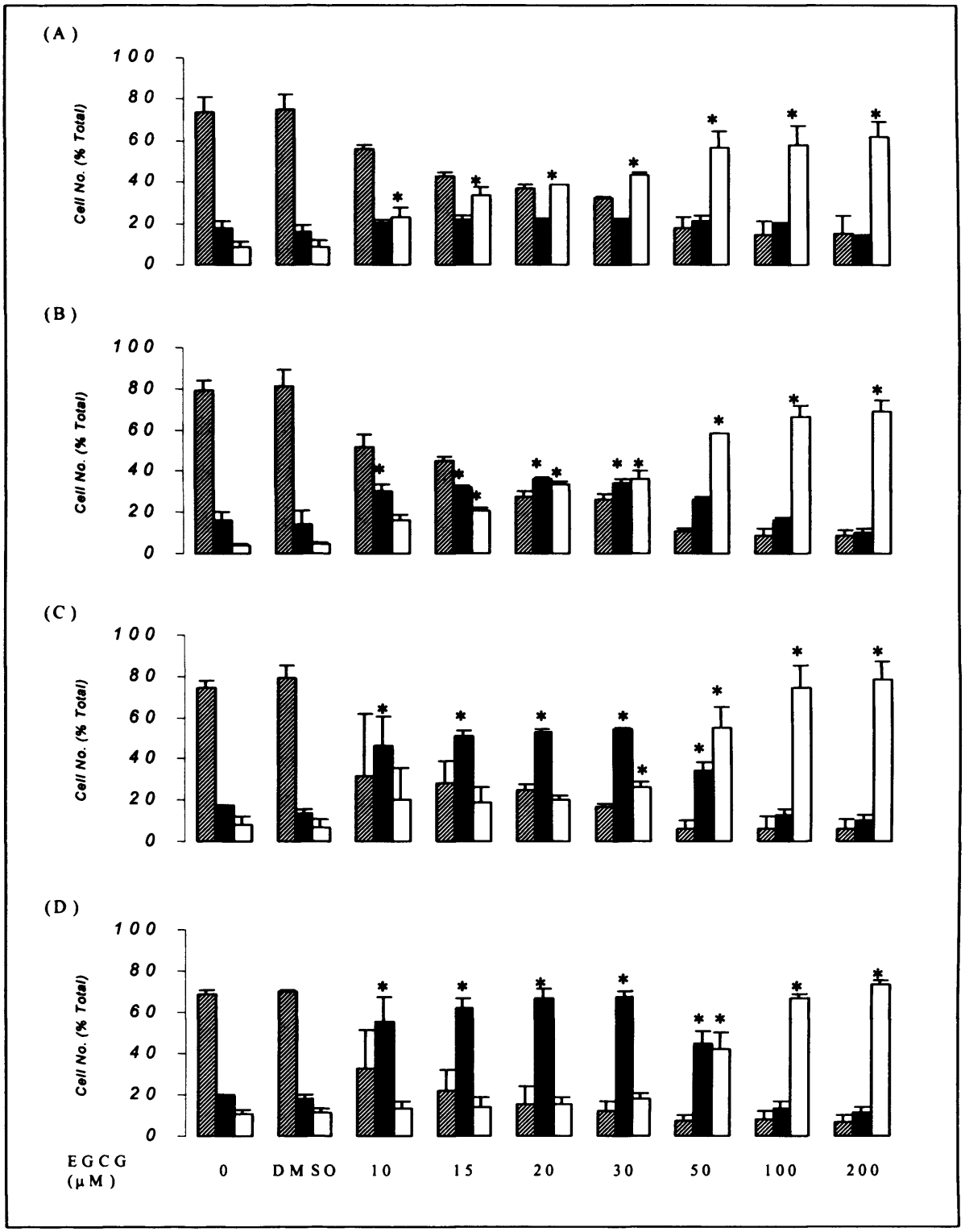

Figure 4.10 - Annexin and PI staining showing the effect of EGCG on the induction of apoptosis in C143. *C143 'normotensive' B lymphoblast cell lines were untreated, or exposed to DMSO (control) or EGCG at the indicated concentrations (μm) for (A) 24 hours; (B) 48 hours; (C) 72 hours; (D) 96 hours. The percentage of viable cells is represented by hatched bars, the percentage apoptotic by the black bars and the percentage of necrotic cells by the clear bars. Data shown are the mean of 3 independent experiments, * indicates significance from the untreated control ($p<0.05$) as determined by the ANOVA balanced model followed by Tukey's least significant difference post hoc test.*

The effect of EGCG on the induction of apoptosis in the two-hypertensive B lymphoblast cell lines can be represented as in figure 4.12 and 4.13. The responses in these two cell types mimic those observed in the two normotensive cell lines. That is, the number of cells undergoing apoptosis increased in a time dependent manner, with peak levels observed after 96 hours EGCG exposure. Furthermore, as the level of viable cells decreased there is a concomitant increase in the number of necrotic cells. Figure 4.12 shows the mean of 3 independent experiments on the number of H308 cells undergoing apoptosis following exposure to EGCG. In this cell line, there were no significant increases in the number of apoptotic cells at early time points (A). However, like the C143 cell lines, significant necrosis was noted at 20 and up to 200 μM EGCG ($p<0.05$). Significant apoptosis was observed at 15, 20, 30, 50 and 100 μM EGCG at 48 hours, and at 10, 15, 20, 30, 50 and 100 μM at 72 and 96 hours EGCG. The peak number of apoptotic cells (50 %) was observed at 50 μm EGCG for 96 hours exposure ($p<0.05$), compared to 60 % after 30μM EGCG for 96 hours, in H242 cell line (figure 4.13).

Figure 4.14 represents the effect of EGCG on the primary HUVEC line, with the data the mean of 3 independent experiments. Although there were no significant changes in the percentage number of cells undergoing apoptosis or necrosis at 24 or 48 hours, there was a suggestion of an increase in the number of apoptotic cells, at 10 μM peaking at 15 μM, and a dose-dependent increase in number of necrotic cells peaking at 100 μM. At 72 hours, there was a significant increase in the number of cells undergoing apoptosis at 10, 15 and 20 μM EGCG ($p<0.05$). This response was also evident at 96 hours where 15, 20, and 30 μM EGCG caused a significant increase in the number of apoptotic cells ($p<0.05$). This increase was matched by a decrease in the number of viable cells.

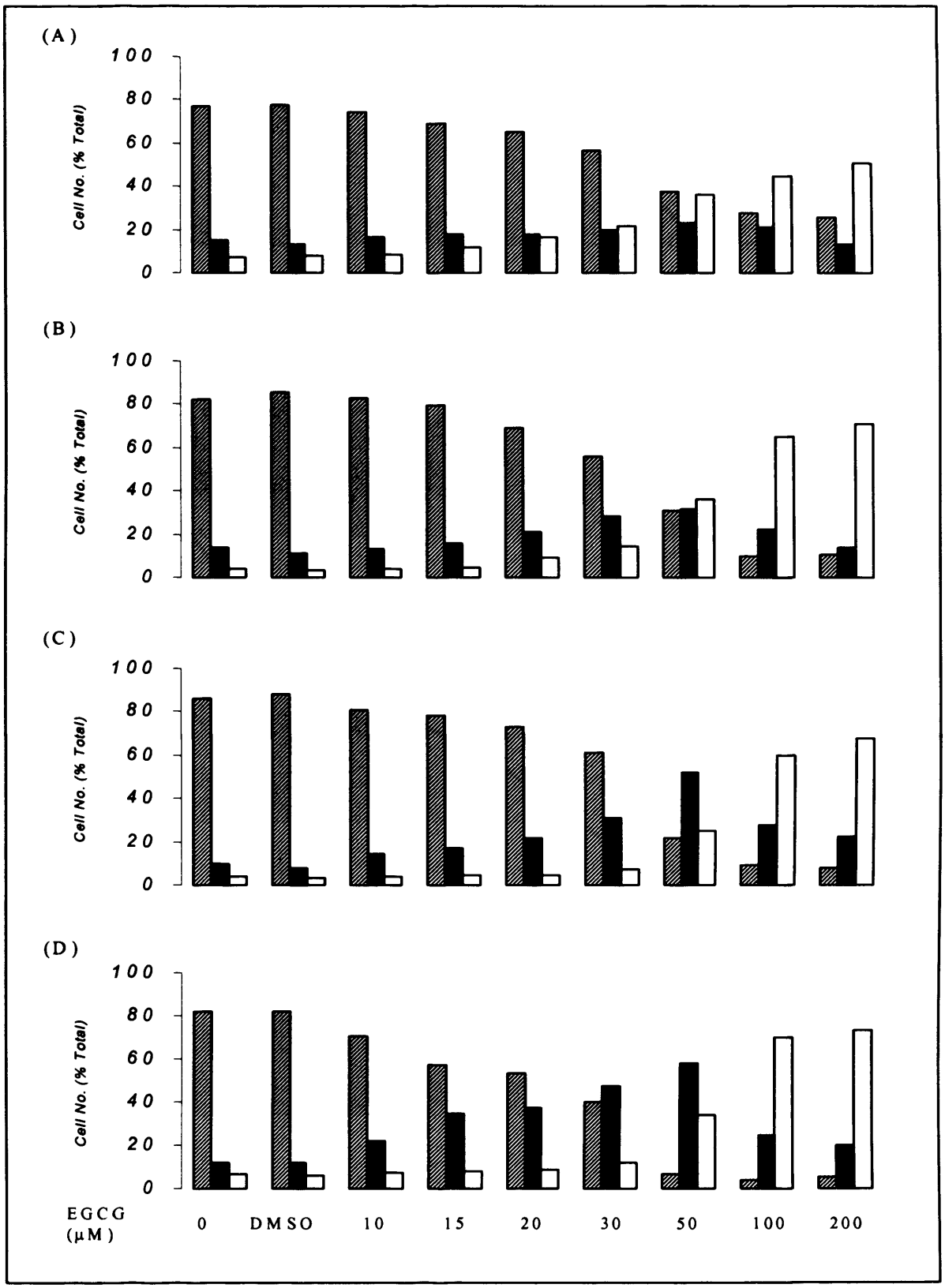

Figure 4.11 - Annexin and PI staining showing the effect of EGCG on the induction of apoptosis in C149. *C149 'normotensive' B lymphoblast cell lines were untreated, or exposed to DMSO (control) or EGCG at the indicated concentrations (μm) for (A) 24 hours; (B) 48 hours; (C) 72 hours; (D) 96 hours. The percentage of viable cells is represented by hatched bars, the percentage apoptotic by the black bars and the percentage of necrotic cells by the clear bars (N=2).*

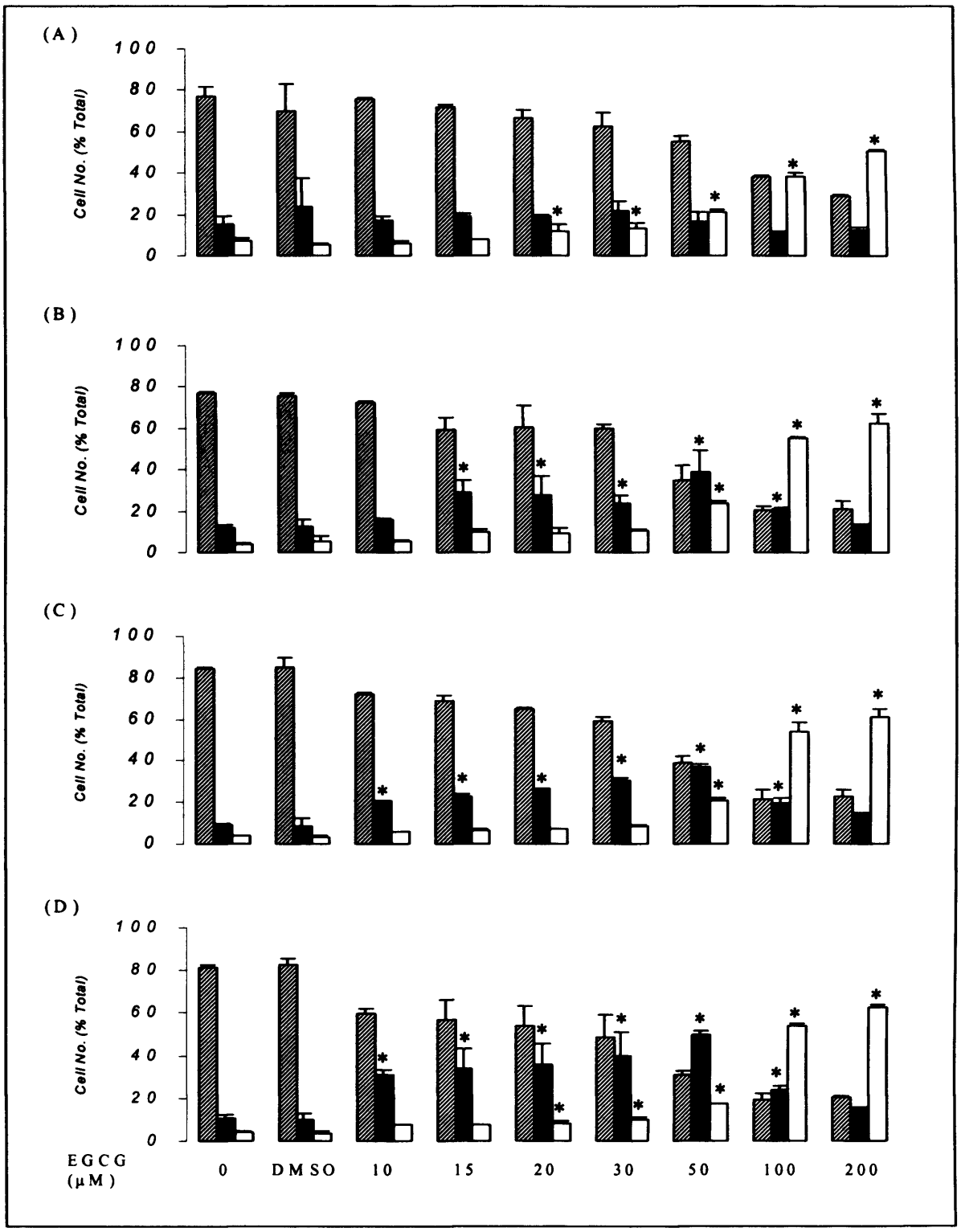

Figure 4.12 - Annexin and PI staining showing the effect of EGCG on the induction of apoptosis in H308. *H308 'hypertensive' B lymphoblast cell lines were untreated, or exposed to DMSO (control) or EGCG at the indicated concentrations (μm) for (A) 24 hours; (B) 48 hours; (C) 72 hours; (D) 96 hours. The percentage of viable cells is represented by hatched bars, the percentage apoptotic by the black bars and the percentage of necrotic cells by the clear bars. Data shown are the mean of 3 independent experiments, * indicates significance from the untreated control ($p<0.05$) as determined by the ANOVA balanced model followed by Tukey's least significant difference post hoc test.*

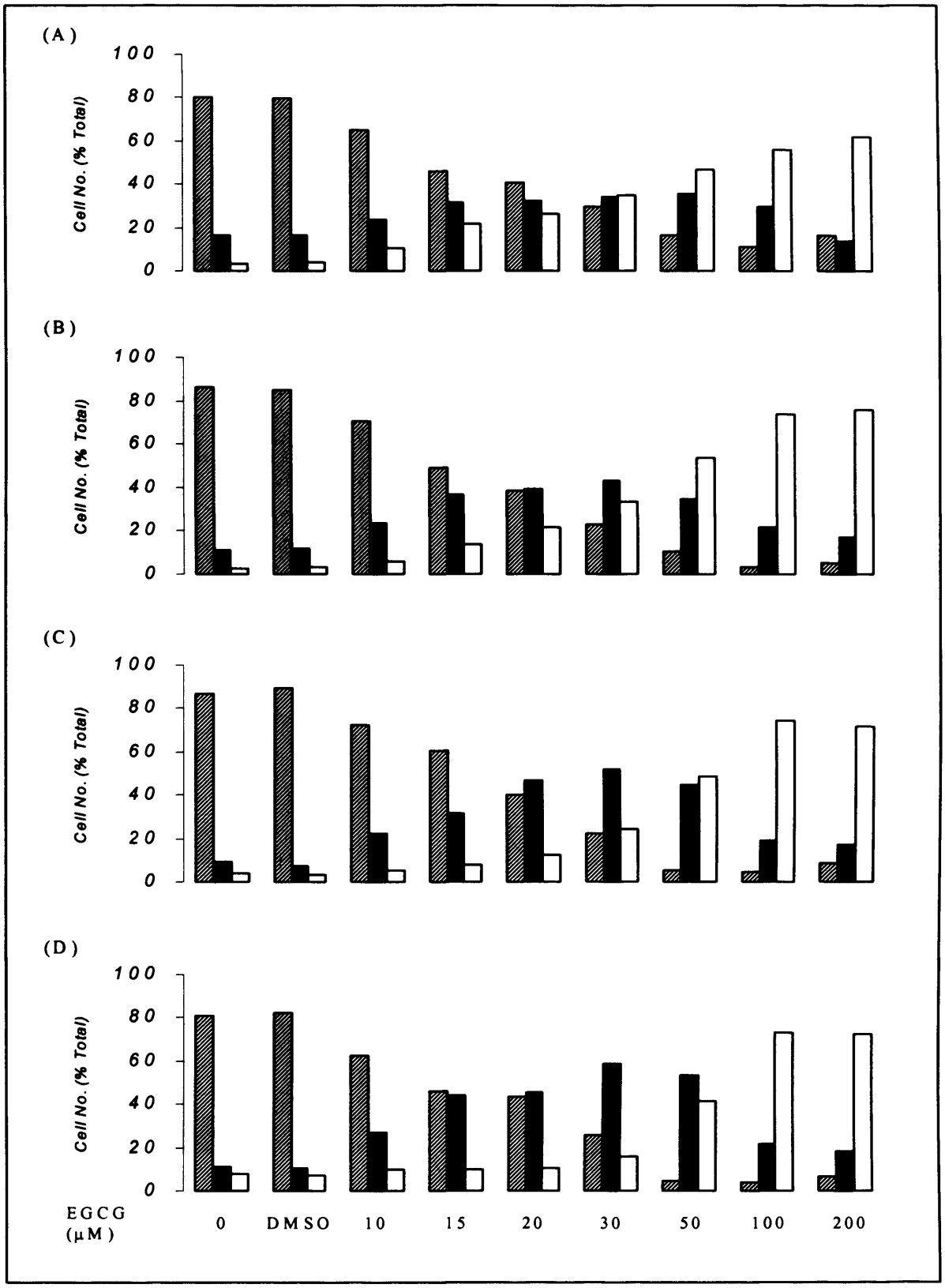

Figure 4.13 - Annexin and PI staining showing the effect of EGCG on the induction of apoptosis in H242. *H242 'hypertensive' B lymphoblast cell lines were untreated, or exposed to DMSO (control) or EGCG at the indicated concentrations (μm) for (A) 24 hours; (B) 48 hours; (C) 72 hours; (D) 96 hours. The percentage of viable cells is represented by hatched bars, the percentage apoptotic by the black bars and the percentage of necrotic cells by the clear bars (N=2).*

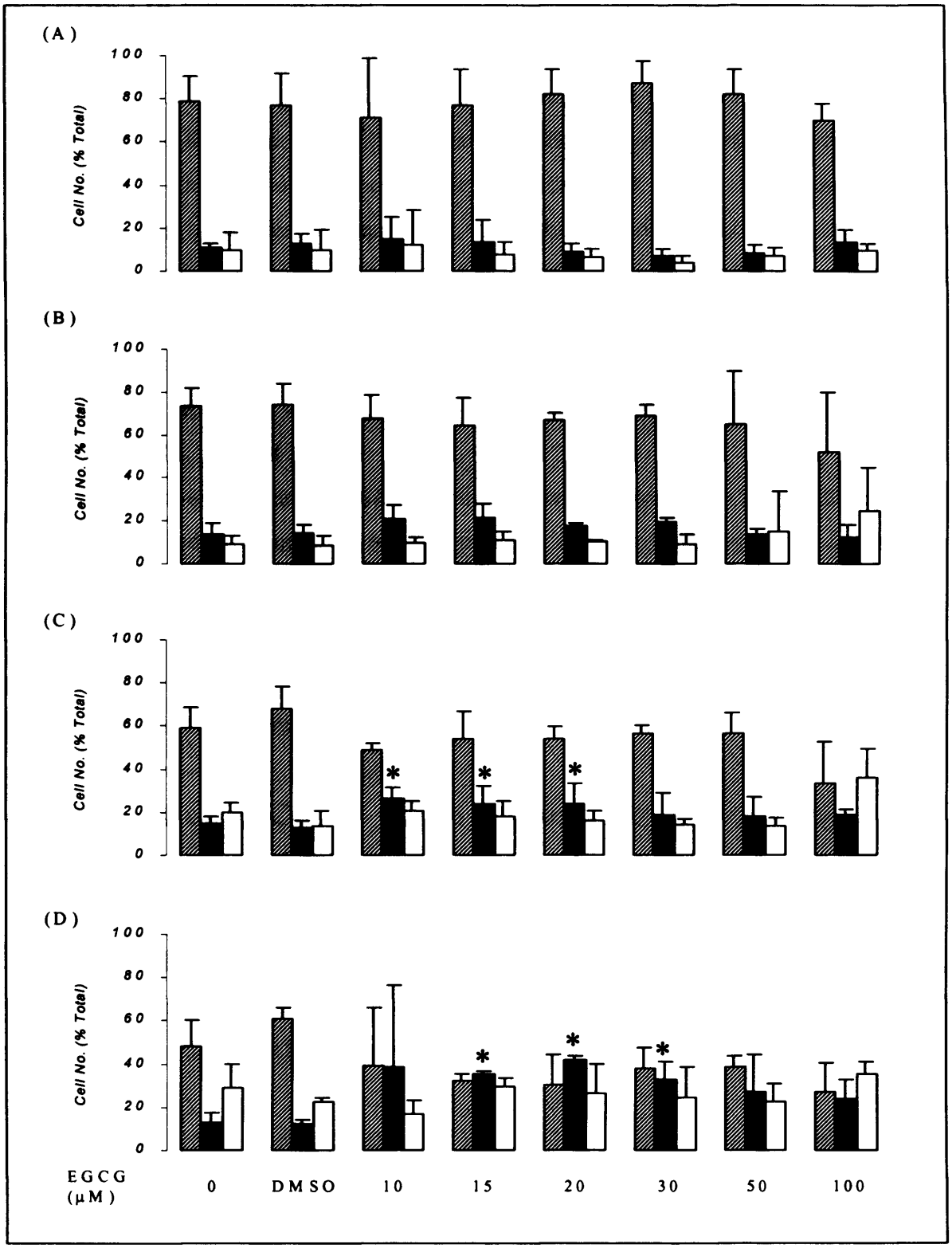

Figure 4.14 - Annexin and PI staining showing the effect of EGCG on the induction of apoptosis in HUVEC. *HUVEC were untreated, or exposed to DMSO (control) or EGCG at the indicated concentrations (μm) for (A) 24 hours; (B) 48 hours; (C) 72 hours; (D) 96 hours. The percentage of viable cells is represented by hatched bars, the percentage apoptotic by the black bars and the percentage of necrotic cells by the clear bars. Data shown are the mean of 3 independent experiments. * indicates significance from the untreated control ($p<0.05$) as determined by the ANOVA balanced model followed by Tukey's least significant difference post hoc test)*

4.3 Effect of EGCG on cell growth regulatory proteins

Having acknowledged that EGCG effects the growth characteristics of both the B lymphoblast cell lines and HUVEC, and that this outcome was in part due to an affect on the cell cycle and apoptotic response, the significance of several important regulator proteins in these processes was appraised. It has been reported that the proteins under examination are involved in regulating the phases of the cell cycle wherein an affect was perceived. Also to be considered were effectors of apoptosis that are recognised to be involved in the modulation of apoptosis by impinging on the NF-κB pathway, such as XIAP and p53. With this in mind, the cell cycle and apoptotic regulators included Pin1, cyclin D1, CDK1, p53 and XIAP.

For these experiments similar conditions were employed as in the initial cell cycle and apoptotic studies. That is, cells were grown in complete medium in the presence or absence of EGCG (30 μM) and whole cell lysates were prepared over the time range of 2 to 96 hours. Thirty μM EGCG was selected for this study because this concentration significantly inhibited cell growth and affected cell cycle progression and the apoptotic response without being directly cytotoxic.

4.3.1 Effect of EGCG on cyclin D1 levels in B lymphoblast and HUVEC

Firstly the role of the cyclin D1 was explored. Cyclin D1 is implicated in regulating cell growth through modulation of the cell cycle and induction of apoptosis. Data from the Western blots in figure 4.15 are representative of two independent experiments in each cell type.

Figure 4.15 represents the effect of EGCG (30 μM) in the two B lymphoblast cell lines, C143 (A) and H308 (B), respectively. When comparing these two cell lines it appeared that they responded in a similar manner following EGCG treatment. Taking into consideration the α-tubulin loading control, there were no obvious changes in cyclin D1 protein levels in either C143 or H308 B lymphoblasts following treatment with EGCG. As there was no change in cyclin D1, it was decided not to complete the data sets for these cell lines.

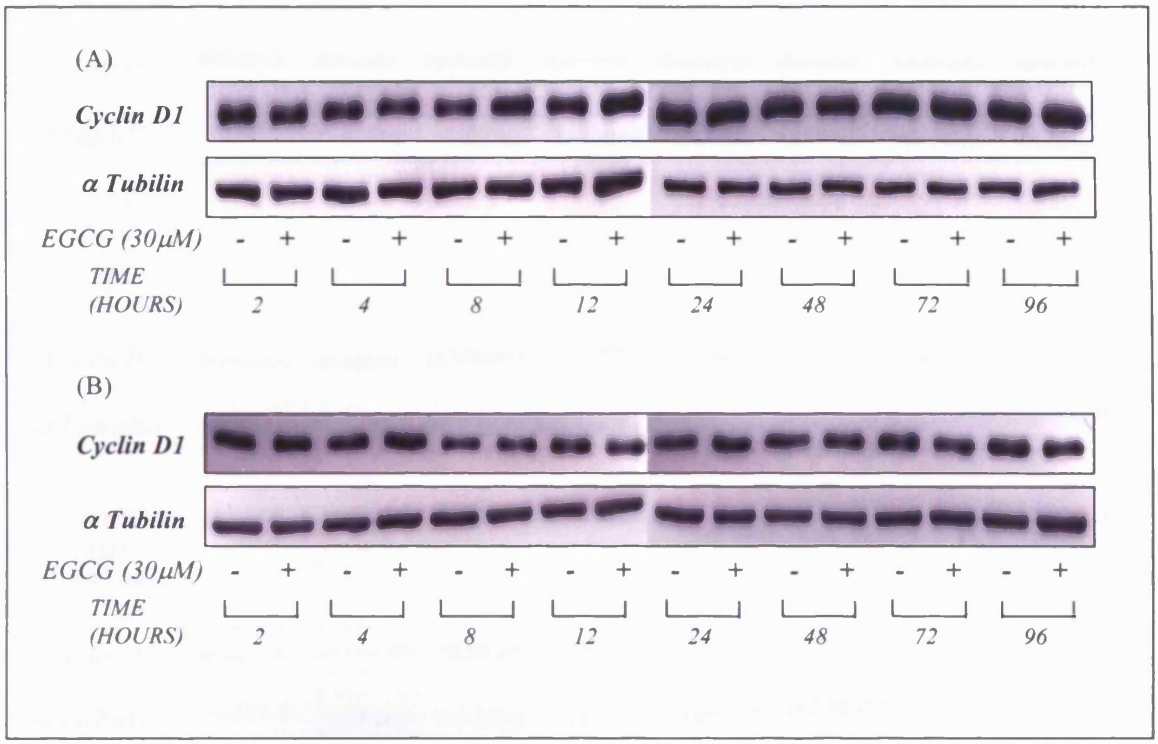

Figure 4.15 - Western analysis showing the effect of EGCG on cyclin D1 protein levels. *C143 (A) and H308 (B) B lymphoblast were untreated or exposed to EGCG (30μm) for the indicated times. Proteins were subjected to immunoblot analysis as described in chapter 2. Immunoblots were probed with an antibody directed against cyclin D1 and were quantified using a Syngene chemigenius multiimager. Blots were stripped and reprobed with α tubulin. Western blots shown are representative of two independent experiments.*

Data contained in figure 4.16 illustrate the effect of EGCG (30 μM) on cyclin D1 in HUVEC, and are representative of two independent experiments. Following treatment with EGCG, there was a reduction in cyclin D1 levels at 2 hours, which was more obvious at 24, 30, 48, 54, 72 and 96 hours. The decrease in cyclin D1 protein following EGCG (30 μM) correlates with the initial change in the number of cells in the G1 phase of the cell cycle (figure 4.9).

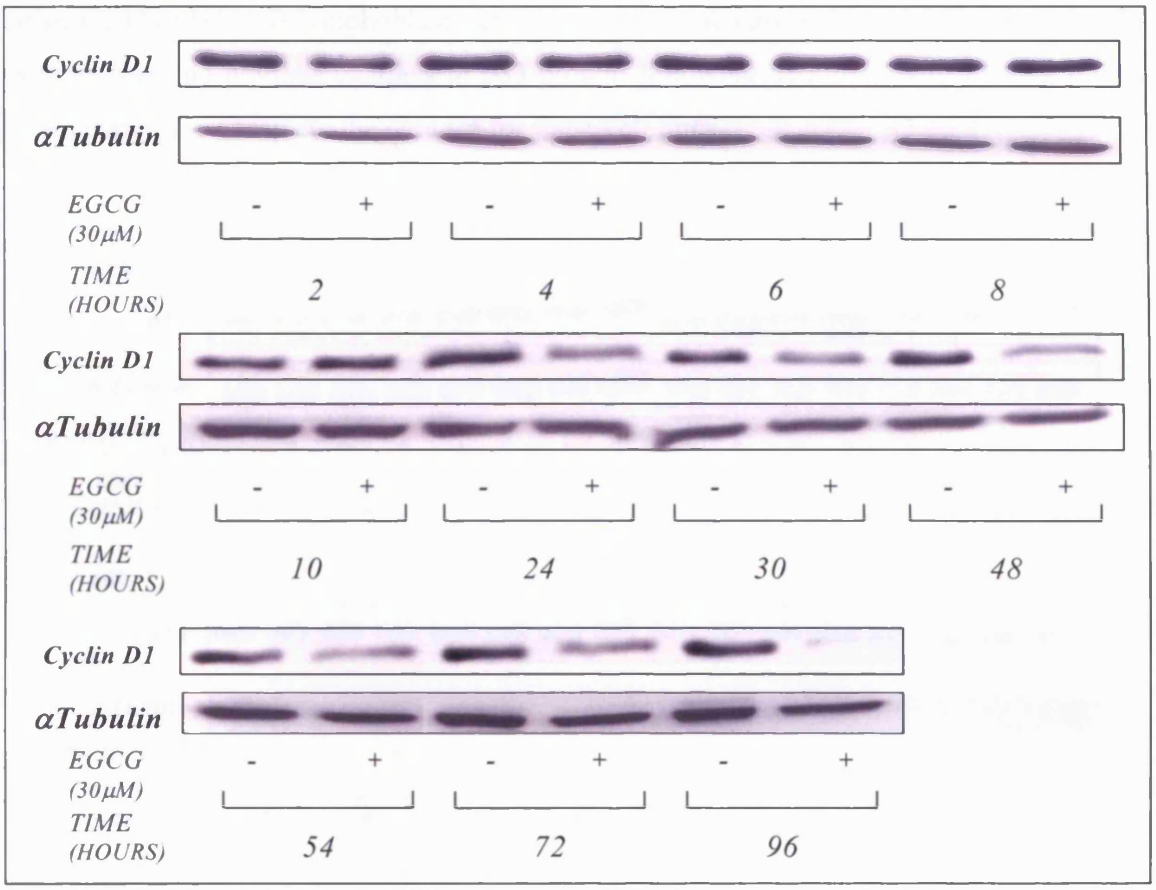

Figure 4.16 - Western analysis showing the effect of EGCG on cyclin D1 protein levels. *HUVEC were untreated or exposed to EGCG (30μm) for the indicated times. Proteins were subjected to immunoblot analysis as described in chapter 2. Immunoblots were probed with an antibody directed against cyclin D1 and were quantified using a Syngene chemigenius multiimager. Blots were stripped and reprobed with α tubulin. Western blots shown are representative of two independent experiments.*

4.3.2 Effect of EGCG on p53 levels in B lymphoblast and HUVEC

As previously mentioned p53 has been implicated as a pivotal regulator of cell growth, mainly by regulation of the apoptotic response. Therefore, it seemed rational to examine the effect of EGCG on p53 protein levels in B lymphoblasts and HUVEC. For this Western analyses were performed on whole cell lysates and immunoblotted with an antibody directed against wildtype and mutant forms p53 protein. The Western blots in figure 4.17, are representative of two independent experiments in each B lymphoblast cell line.

There was a similar trend in both C143 and H308 B lymphoblasts (figure 4.17 A, B). Basal levels of p53 increased from 2 hours to 12 hours and remained fairly constant thereafter, in both cell lines. EGCG treatment had no obvious effect on p53 protein in

either C143 or H308 B lymphoblasts, except a modest increase at 24 and 48 hours. Overall, as there were no obvious changes in p53 protein following treatment with EGCG, it was concluded not to complete the data set for these cell lines.

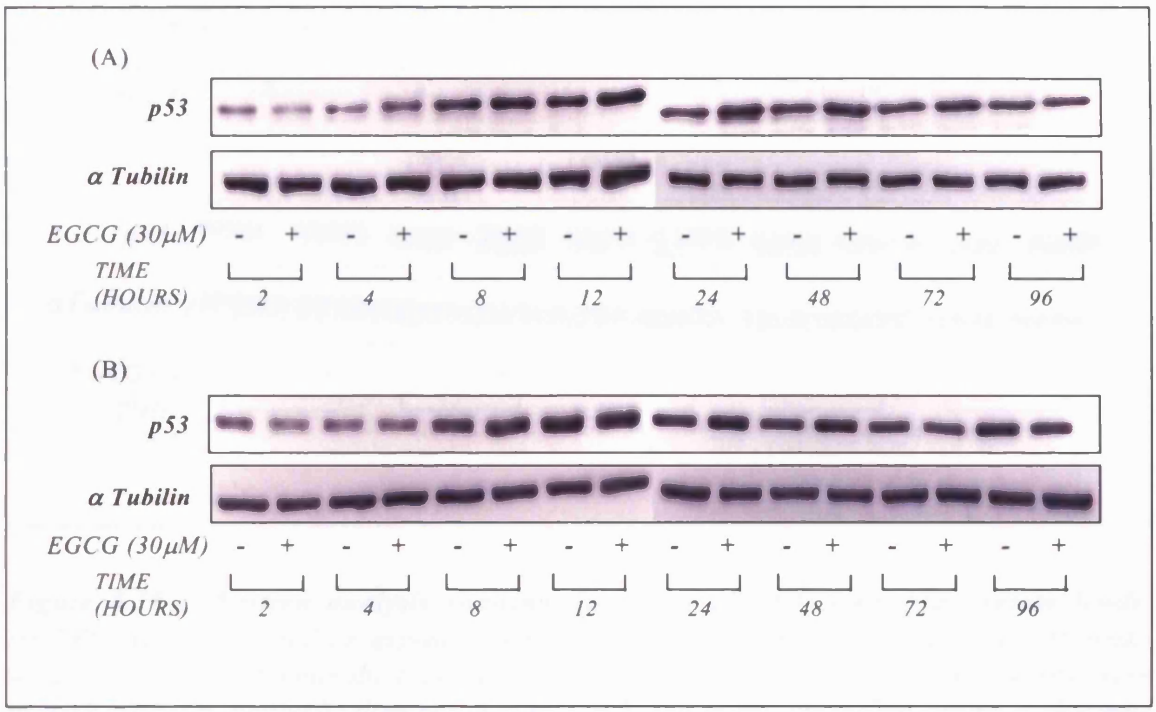

Figure 4.17 - Western analysis showing the effect of EGCG on p53 protein levels. *C143 (A) and H308 (B) B lymphoblast were untreated or exposed to EGCG (30μm) for the indicated times. Proteins were subjected to immunoblot analysis as described in chapter 2. Immunoblots were probed with an antibody directed against p53 and were quantified using Syngene chemigenius multiimager. Western blots shown are representative of two independent experiments.*

Figure 4.18 shows representative Western blots demonstrating the effect of EGCG on p53 protein levels in HUVEC. There was a significant increase in basal p53 protein levels at 6 hours compared to the levels at 2 hours ($p<0.05$). Interestingly, the data also showed that EGCG brought about an increase in the p53 protein compared to the respective untreated control samples. This EGCG-induced increase occurred from 30 hours onwards, and was significant at 54, 72 and 96 hours treatment ($p<0.05$), correlating with an increase in the induction of apoptosis as observed in figure 4.15.

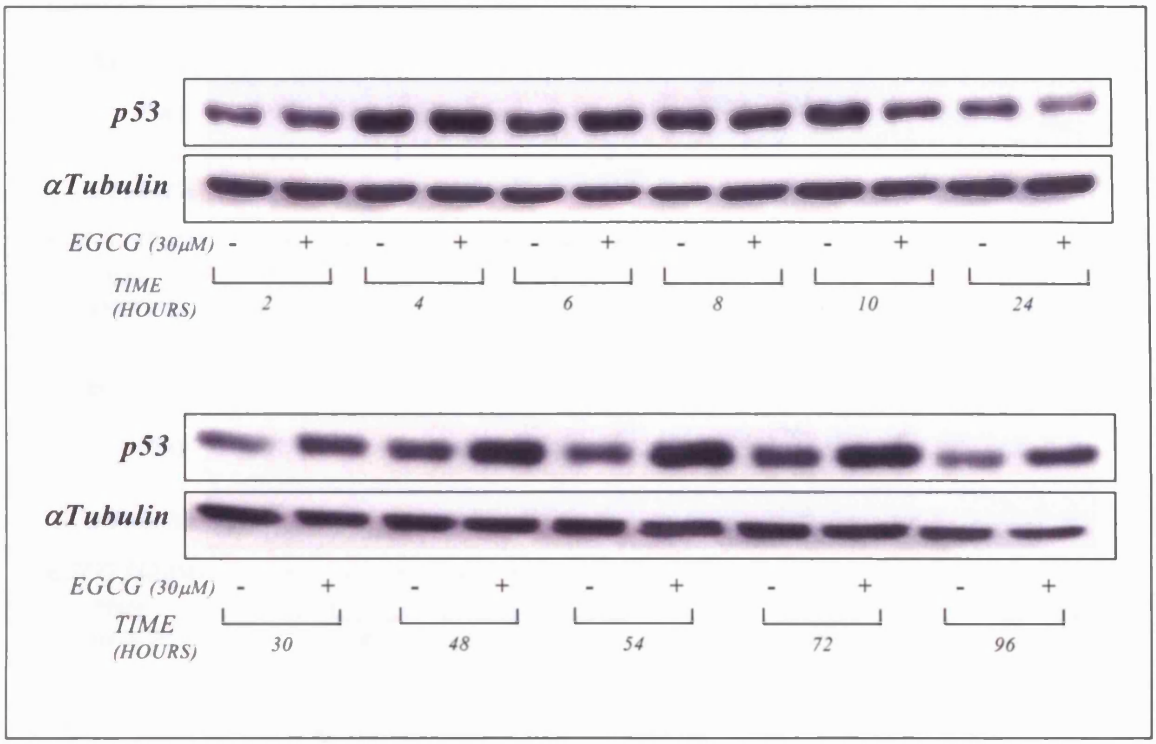

Figure 4.18 - Western analysis showing the effect of EGCG on p53 protein levels. *HUVEC were untreated or exposed to EGCG (30μm) for the indicated times. Proteins were subjected to immunoblot analysis as described in chapter 2. Immunoblots were probed with an antibody directed against p53 and were quantified using a Syngene chemigenius multiimager. Blots were stripped and reprobed with α tubulin. Western blots shown are representative of three independent experiments.*

4.3.3 Effect of EGCG on Pin1 levels in B lymphoblast and HUVEC

The effect of EGCG on Pin1 levels was examined in B lymphoblasts and HUVEC. Pin1 has recently been identified to be important in the regulation of cell growth, possibly through interaction with either p53, cyclin D1 or NF-κB. To examine the effect of EGCG on Pin1, Western analyses were performed on whole cell lysates and immunoblotted with an antibody directed against total Pin1. In B lymphoblasts and HUVEC, the Western blots illustrated are representative of two independent experiments for each cell type.

Figure 4.19 shows the effect of EGCG (30 μM) on the levels of Pin1 protein in both C143 (A) and H308 (B) B lymphoblasts. Considering the α-tubulin loading control, both the untreated (basal) and EGCG treated Pin1 protein levels appear to increase at later treatment times, between 24 to 96 hours EGCG. However, there were no obvious changes in Pin1 protein following treatment with 30 μm EGCG in either C143 (A) or H308 (B) B lymphoblasts, compared to each the control.

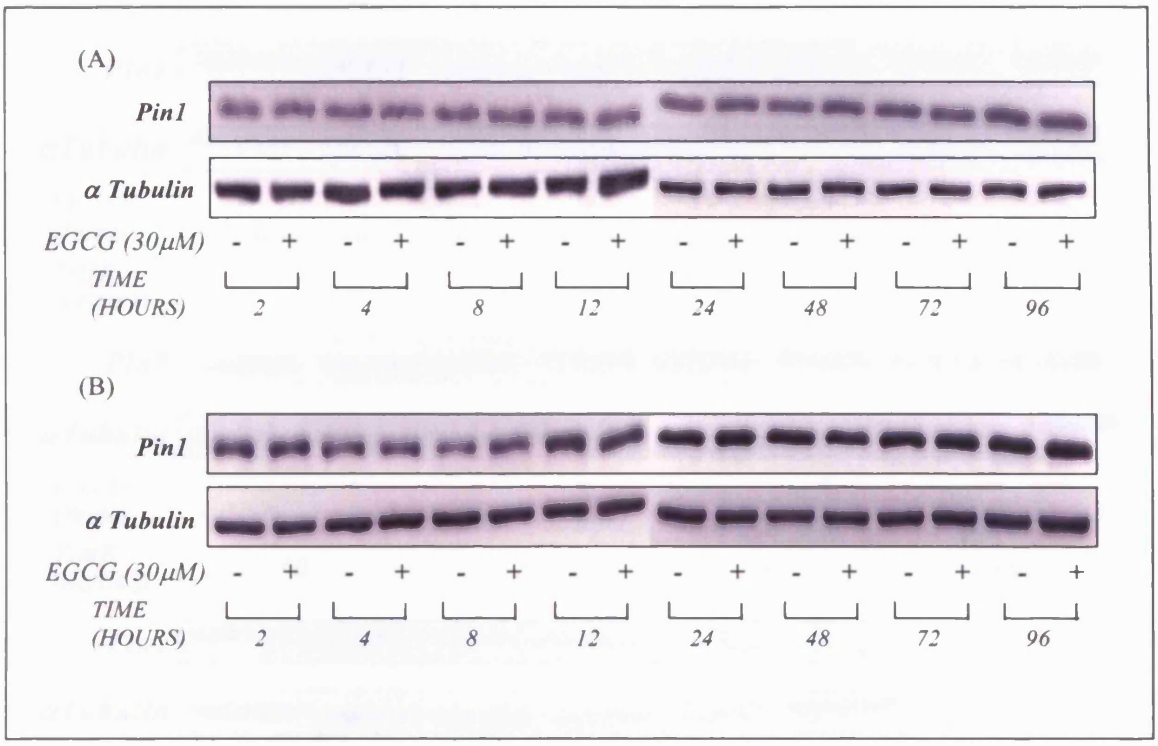

Figure 4.19 - Western analysis showing the effect of EGCG on Pin1 protein levels. *C143 (A) and H308 (B) B lymphoblast were untreated or exposed to EGCG (30μm) for the indicated times. Proteins were subjected to immunoblot analysis as described in chapter 2. Immunoblots were probed with an antibody directed against Pin1 and were quantified using a Syngene chemigenius multiimager. Western blots shown are representative of two independent experiments.*

In HUVEC (figure 4.20) there was a time-dependent increase in basal Pin1 protein from 2 hours up to 48 hours, which began to drop thereafter. There was no obvious change in Pin1 protein following treatment with EGCG (30 μM) when compared to the controls. As there was no obvious changes in protein levels between EGCG treated and untreated samples, it was decided not to pursue the Pin1 experiments further in any of the cell types.

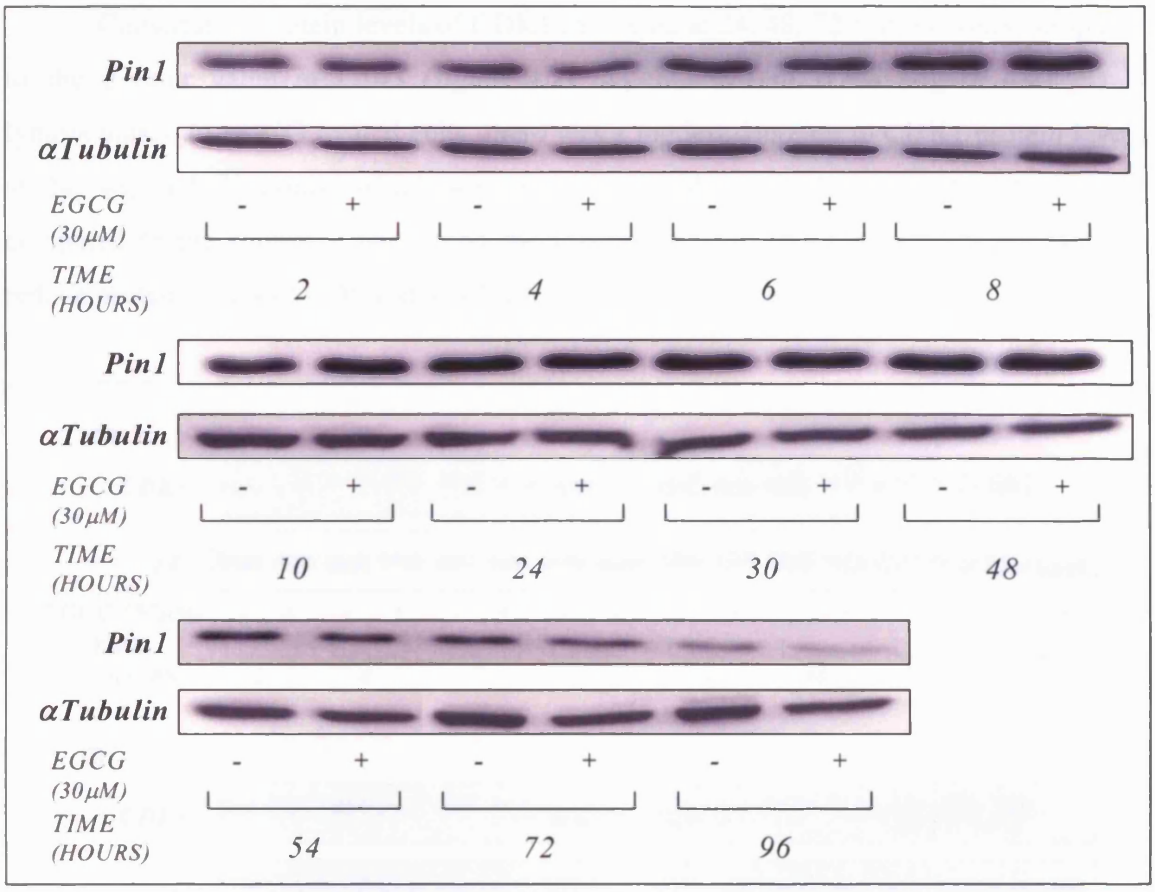

Figure 4.20 - Western analysis showing the effect of EGCG on Pin1 protein levels. *HUVEC were untreated or exposed to EGCG (30μm) for the indicated times. Proteins were subjected to immunoblot analysis as described in chapter 2. Immunoblots were probed with an antibody directed against Pin1 and were quantified using a Syngene chemigenius multiimager. Blots were then stripped and reprobed for α tubulin. Western blots shown are representative of two independent experiments.*

4.3.4 Effect of EGCG on CDK1/Cdc2 levels in B lymphoblast and HUVEC

As CDK1/Cdc2 is an important regulator of G2/M phase and maybe G0/G1 phase transition, the effect of EGCG on the modulation of CDK1 protein was examined. Cells were treated with EGCG (30μM) over the time range of 2 to 96 hours. Whole cell lysates were then subjected to Western analysis and immunoblotted with an antibody directed against human CDK1 (Cdc2). For B lymphoblasts, the Western blots shown are representative of two independent experiments performed in each cell line, whereas in HUVEC, the Western blots shown are representative of 3 independent experiments.

Constitutive protein levels of CDK1 increased at 24, 48, 72 and 96 hours compared to the 2 hour value in C143 (figure 4.21 A), but not in H308 (figure 4.21 B) B lymphoblasts. In EGCG treated cells, there was a modest decrease in CDK1 protein levels at 24, 48, and 72 hours, which was most noticeable at 96 hours treatment in C143 compared to the control. Considering the loading control, EGCG treatment appeared to reduce protein levels of CDK1 at 4, 12, 24 and 96 hours in the H308 cell line.

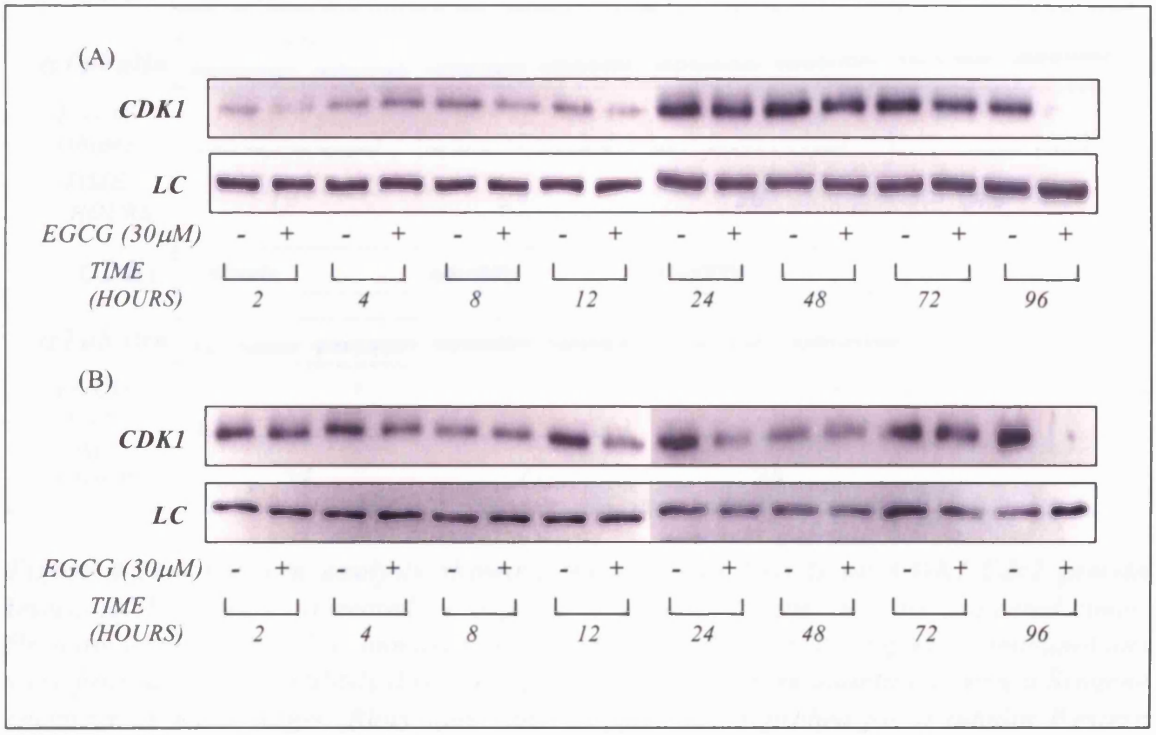

Figure 4.21 - Western analysis showing the effect of EGCG on CDK1/Cdc2 protein levels. *C143 (A) and H308 (B) B lymphoblast were untreated or exposed to EGCG (30μm) for the indicated times. Proteins were subjected to immunoblot analysis as described in chapter 2. Immunoblots were probed with an antibody directed against CDK1 and were quantified using a Syngene chemigenius multiimager. LC or loading control gives an indication to the loading. Western blots shown are representative of two independent experiments.*

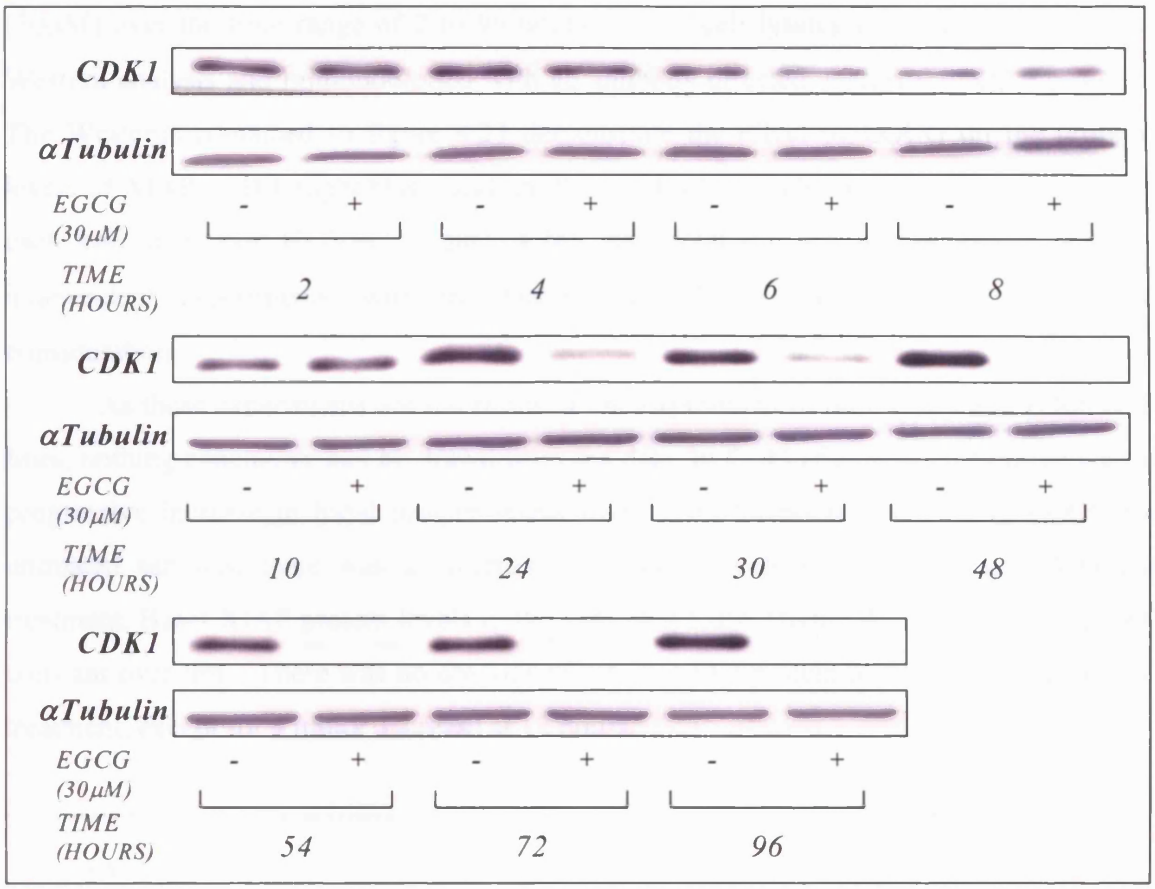

Figure 4.22 - Western analysis showing the effect of EGCG on CDK1/Cdc2 protein levels. *HUVEC were untreated or exposed to EGCG (30μm) for the indicated times. Proteins were subjected to immunoblot analysis as described in chapter 2. Immunoblots were probed with an antibody directed against CDK1 and were quantified using a Syngene chemigenius multiimager. Blots were then stripped and reprobed for α tubulin Western blots shown are representative of three independent experiments.*

Figure 4.22 is a representative Western blot demonstrating the effect of EGCG on CDK1 protein levels in HUVEC. Treatment with EGCG (30 μM) induced a decrease in CDK1 protein from 24 to 96 hours, which was significant at 54, 72 and 96 hours ($p<0.05$). Interestingly this decrease may have corresponded to a G2/M phase cell cycle arrest as noted in figure 4.9.

4.3.5 Effect of EGCG on XIAP protein levels in B lymphoblast and HUVEC

The protein X-linked inhibitor of apoptosis (XIAP), a target of NF-κB, impinges on the apoptotic pathway by inhibiting caspase-induced cell death. The involvement of XIAP in the EGCG-induced apoptotic response was investigated by exposing cells to EGCG

(30μM) over the time range of 2 to 96 hours. Whole cell lysates were then subjected to Western analysis and immunoblotted with an antibody directed against the XIAP protein. The Westerns contained in figure 4.23 demonstrate the effect of EGCG on the protein levels of XIAP in B lymphoblasts and are the result of a single experiment performed in each cell line. For HUVEC (figure 4.24), the Westerns are representative of two independent experiments, with the loading controls (α tubulin) being taken into consideration.

As these experiments are the result of one experiment in both C143 and H308 cell lines, nothing conclusive can be drawn from the data. In C143 (figure 4.23 A) there was a progressive increase in basal protein levels from 12 to 96 hours. Comparing EGCG to untreated samples, there was an increase in protein levels at 4, 8, 12, and 24 hours treatment. Basal XIAP protein levels in the untreated H308 (figure 4.23 B) remain almost constant over time. There was no obvious effect on XIAP protein levels following EGCG treatment, except for a minor decrease at 12 hours.

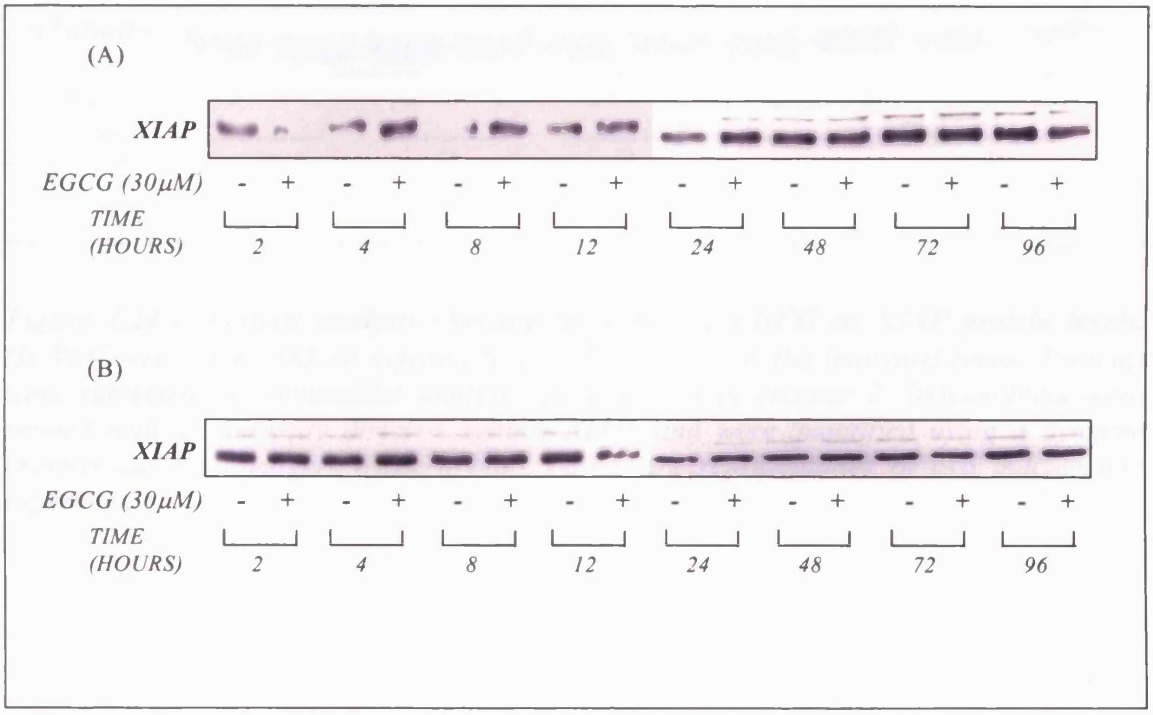

Figure 4.23 - Western analysis showing the effect of EGCG on XIAP protein levels. *C143 (A) and H308 (B) B lymphoblast were untreated or exposed to EGCG (30μm) for the indicated times. Proteins were subjected to immunoblot analysis as described in chapter 2. Immunoblots were probed with an antibody directed against XIAP and were quantified using a Syngene chemigenius multiimager. Western blots shown are the result of one single experiment performed in each B lymphoblast cell line.*

The Western blot illustrated in figure 4.24 shows the effect of EGCG on XIAP in HUVEC. Considering the loading control, EGCG had no obvious effect on XIAP at any treatment time, except for a modest decrease at 96 hours. As there was no obvious EGCG effect in any of the cell types, it was decided not to continue with these series of experiments.

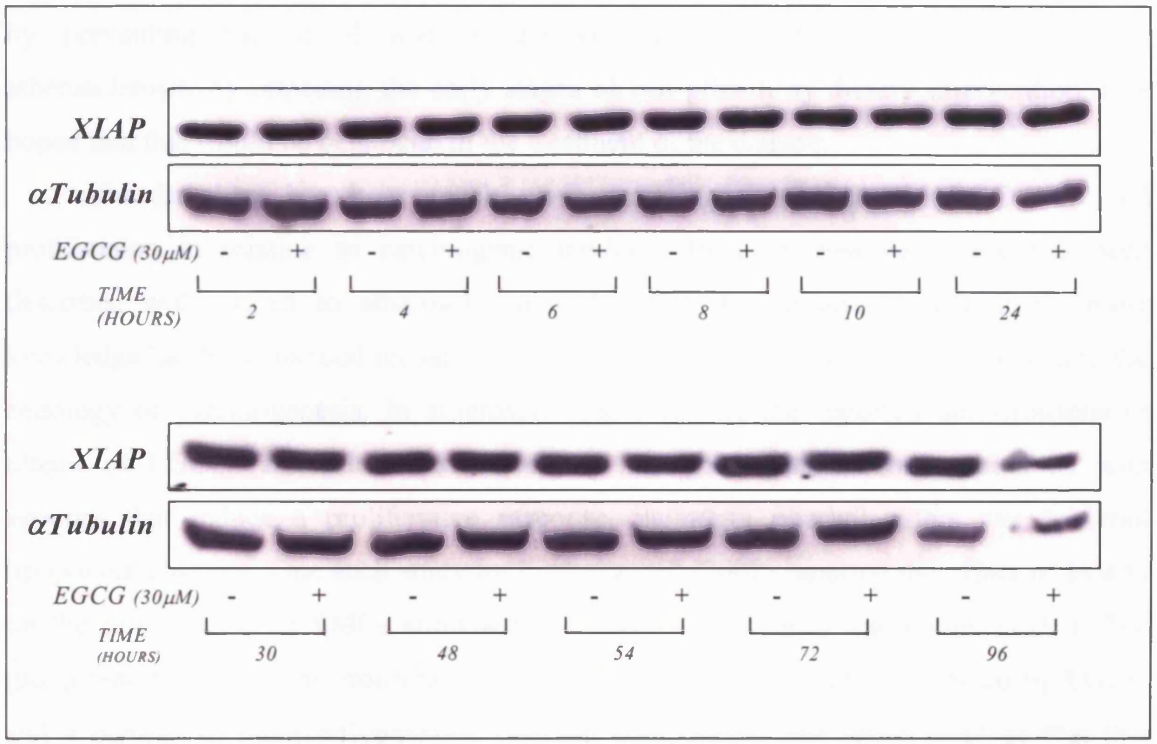

Figure 4.24 - Western analysis showing the effect of EGCG on XIAP protein levels. *HUVEC were untreated or exposed to EGCG (30μm) for the indicated times. Proteins were subjected to immunoblot analysis as described in chapter 2. Immunoblots were probed with an antibody directed against XIAP, and were quantified using a Syngene chemigenius multiimager. Western blots shown are representative of two independent experiments.*

4.4 Discussion

The effect EGCG has on cell growth regulation or proliferation has significant implications for all forms of cardiovascular disease such as atherosclerosis and restenosis. The concept of cardiovascular chemoprevention dictates that dietary agents should prevent the onset of the disease. One way in which chemopreventive agents attain this objective is by preventing the initial and progressive stages of disease development. For atherosclerosis, by impeding the early stages of cell growth by dietary intervention, it is hoped that this would be beneficial in the treatment of the disease.

So far there has been much work reporting the effects of EGCG on cell proliferation in relation to carcinogenic models. However, relatively little has been described with regard to atherosclerosis. This may be because comparatively more knowledge has been accrued recognising the significance of deregulated cell growth to the aetiology of carcinogenesis. In atherosclerosis, many of the reported anti-proliferative effects of EGCG (and related tea catechins) have been studied in combination with agonists that induce a proliferative response, including phorbol esters and bacterial lipopolysaccharides. One such study by Locher *et al.* (2002) reported the effect of EGCG on the proliferation of SMCs stimulated by native low-density lipoprotein (LDL). The group report that cellular proliferation was concentration-dependently inhibited by EGCG and a number of other polyphenolic catechin compounds. The group disclose that this effect may in part be due to inhibition of the ERK 1/2 pathway, which is recognised to be involved in aspects of cellular proliferation.

A similar report by Lu *et al.* (1998) again described the concentration-dependent inhibitory effect of EGCG and other constituent polyphenols of tea, on the proliferation of a range of vascular smooth muscle cells derived from dissimilar sources and also on human lymphoid type cells. Interestingly, this inhibitory effect was partly attributed to a decrease in protein tyrosine kinase activity, as well as a reduction in c-jun mRNA expression and inhibition of JNK1 activation, but not activation of ERK 1/2 or PKC.

The experiments described in section 4.2, were intended to examine the effect of EGCG on the growth characteristics of the B lymphoblast cell lines and HUVEC. All cell types studied showed a similar trend of growth inhibition by EGCG, that is, they exhibit comparable IC_{50} values in the low micromolar range (C143=10μM, C149=17μM, H308=18μM, H242=13μM, HUVEC=15μM). These IC_{50} values and hence growth inhibitory effects are supported by more recent studies on a number of different cells

(Liang *et al.* 1999; Yang GY *et al.* 2000). Conversely, other studies report slightly different findings. A report by Tan XH *et al.* (2000) in human colon carcinoma cells (LoVo) has shown that EGCG dose-dependently inhibited cell growth with an IC_{50} of 375 μM, thus at a significantly higher concentration to that observed here. Kondo *et al.* (2002) again showed that EGCG inhibited cellular growth with an IC_{50} in the 100 μM range. However, the difference in EGCG sensitivities may be explained when considering the disparate cell types used in these independent studies. Further discrepancies may be explained due to the diverse means used to evaluate cellular proliferation and hence growth inhibition. For example Lu *et al.* (1998), measured cellular proliferation by way of tritiated thymidine uptake into the cells.

Intriguingly, work in LoVo cells (Tan XH *et al.* 2000) found that (-)-epicatechin (EC), an EGCG related compound, at concentrations of between 10 and 250 μM, appeared to promote a minor proliferative response. This finding emphasises that chemopreventive agents such as EGCG can have differential effects depending on the working concentration and the system under study. This principle must be considered when appraising the overall affect of a specific chemopreventive agent such as EGCG.

The involvement of EGCG on the modulation of the cell cycle has important implications for the onset and progression of atherosclerosis. Interpretation of data demonstrated that EGCG had different effects on the cell cycle, dependent on the cells under scrutiny and also the concentration of treatment used. It was concluded that the growth inhibitory quality of EGCG was partly attributable to an effect on the cell cycle. For the C143 cell line there was evidence that EGCG induced both a modest G0/G1 phase arrest and also a G2/M phase cell cycle arrest, which was dependent on concentration and time point under scrutiny. For the other normotensive B lymphoblast cell line C149 there only appeared to be a modest G2/M phase cell cycle arrest following treatment with EGCG. Interestingly there was no significant affect on cell cycle progression in the H308 hypertensive cell line when treated with EGCG. In HUVEC, EGCG had different effects that were dependent on the concentration used. At the lower EGCG concentrations of 5, 10, 15 μM there was a modest increase in the number of cells in the G0/G1 phase of the cell cycle indicating a G0/G1 phase arrest. This was complemented by a decrease in the number of cells in the S phase of the cell cycle. Also, there was no change in the number of cells in the G2/M phase of the cell cycle at these concentrations. However at 30 μM EGCG, there was a modest decrease in the number of cells in the G0/G1 phase, corresponding to an increase in the number of cells in the G2/M phase of the cell cycle.

Some of the apparent differences between the cell types may be due to the cell cycle experiments being performed on a population of asynchronous cells. Also, EGCG may have a different effect depending on the cell type under study or the concentration used. However overall, these results are similar to reports in the literature, where the effect of EGCG and other related catechins on the cell cycle have been documented. One such study by Ahmad *et al.* (1997) in A431 cells has shown that EGCG caused apoptosis and cell cycle deregulation, inducing a G_0/G_1 phase arrest. The response induced by EGCG was also verified in a variety of other cell systems from several other laboratories (Li HC *et al.* 2000; Otsuka *et al.* 1998; Paschka *et al.* 1998; Yang GY *et al.* 1998; Gupta *et al.* 2000). However as observed in the C143 and HUVEC cells, EGCG has also been revealed to block a G2/M progression (Liang *et al.* 1999). These different responses infer that the effects of EGCG on cell cycle distribution are dependent on concentration used and possibly cell types.

The contribution of apoptosis to the growth inhibitory effect of EGCG was also assessed, since this plays a pivotal role in both the initial and late stages of vascular disease. Interpretation of the data contained in section 4.2.3, revealed that EGCG induces apoptosis in all cell types under investigation. All the B lymphoblast cell lines responded in a similar manner following treatment with EGCG. That is, there was evidence of a time and dose dependent increase in the number of cells undergoing apoptosis. This increase was evident up to 50 μM EGCG, after which the concentration was reasoned to be directly cytotoxic as documented by an increase in the population of necrotic cells, which corresponded to a concomitant decrease in viable and apoptotic cells. These findings were supported by morphological observations in which cells appeared irregular in shape at concentrations corresponding to increases in apoptotic and necrotic cells.

The HUVEC responded in a similar manner to the B lymphoblasts. There was a time dependent increase in the number of cells undergoing apoptosis, which was more evident at EGCG concentrations of 10, 15, and 20 μM. This induction of apoptosis corresponded to the observed growth inhibition

It is important to note EGCG concentrations of 10 to 30 μM i.e. levels which induced apoptosis, were not considered to be directly toxic to any of the cell lines under investigation, even though growth inhibition was observed. This supposition was based on the finding that apoptosis was observed at the later and not early times of EGCG treatment. The importance of this remark becomes clear when considering the propensity of EGCG to bind to and affect the biological properties of lipid bilayers. It has been described in the

literature that EGCG and related polyphenolic esters display an affinity for lipid bilayers (Nakayama *et al.* 1998, 2000; Ikigai *et al.* 1993; Tsuchiya *et al.* 1999). In the reports by Ikigai *et al.* (1993) and Tsuchiya *et al.* (1999) it was suggested that tea catechins (at concentrations of 10 μM and above) immediately damage the lipid bilayer resulting in cell death. This would infer that EGCG-induced cell death might occur simply by decreasing membrane stability. However, as significant apoptosis was only noted after 24 hours, this would suggest induction of a specific apoptotic-signalling pathway as oppose to immediate cell death due to membrane instability.

The biological activities of black and green tea polyphenol extracts on apoptotic responses have been documented in certain cell types. Theaflavin digallate (TfdiG) a polyphenol present in black tea, epigallocatechin (EGC) and EGCG have been shown to induce apoptosis in human bronchial epithelial cells, following a 24-hour exposure (Yang GY *et al.* 2000). In this study the authors suggest that H_2O_2 generated following EGC and EGCG exposure, was the cause of this type of cell death. The finding that exogenously added catalase significantly prevented EGC- and EGCG-induced cell apoptosis supported this conjecture. The possibility that EGCG-induced H_2O_2 could mediate the observed induction of apoptosis as noted here remains conceivable. Interestingly, work by other groups (Dashwood *et al.* 2002; Long *et al.* 2000) has supported this notion that EGCG appears to generate H_2O_2 in cell culture medium. Using a relatively crude colorimetric test it was confirmed that EGCG generated low micromolar concentrations of H_2O_2 in cell culture medium alone (refer to chapter 3). This production of H_2O_2 was attenuated following the addition of catalase. However by employing a considerably more sensitive method, no significant change in the levels of reactive oxygen intermediates (ROIs) could be detected, importantly in RPMI medium containing cells that had been treated with or without EGCG (refer to chapter 3). Therefore, even though unlikely, the possibility that EGCG-generated H_2O_2 actually induces apoptosis in B lymphoblast and HUVEC cannot be excluded without further analysis.

The ability of EGCG to exert an effect on proteins important to the cell cycle and apoptosis was examined. These included cyclin D1, CDK1, Pin1, XIAP and p53. It is important to realise that many of these regulatory proteins interact with each other, hence offer points of crosstalk, which may lead to cell growth regulation. They were selected as they have been shown to be involved in the stages of cell cycle where an arrest was observed. Moreover it is now recognised that many of the cell growth regulatory proteins play critical roles in the modulation of apoptosis. Furthermore in various cell systems,

several of these proteins are also regulated by NF-κB, which was inhibited by EGCG (refer to chapter 3).

When examining cyclin D1 levels in the two B lymphoblast cell lines, there were no obvious changes in protein levels following EGCG treatment (30 μM). However examining the effect in HUVEC, there was a decrease in protein levels in EGCG (30 μM) treated samples, which may explain the G1 phase arrest and induction of apoptosis. These findings indicate that the effect mediated by EGCG is again dependent on the cell type under investigation. This EGCG-induced decrease in cyclin D1 is supported by more recent evidence in the literature (Agarwal *et al.* 2000; Ahmad *et al.* 2000; Liang *et al.* 1999). In the report by Ahmad *et al.* (2000) down-regulation of cyclin D1 correlated with a G_0/G_1 phase cell cycle arrest in A431 cells. However, in HUVEC as well as a G1 arrest there was also a partial G2/M phase arrest following treatment with EGCG (30 μM). Even though cyclin D1 is accepted as a regulator of G1/S phase transition (as reviewed in Reed *et al.* 1997), there may be a plausible explanation for the decrease in cyclin D1 levels, which occurred prior to the observed G2/M arrest. As noted in chapter 3, EGCG brought about a decrease in the translocation and DNA binding capabilities of NF-κB. This reduction occurred at time-points preceding the observed decrease in cyclin D1 levels, implying that NF-κB may regulate cyclin D1 expression. This proposition is supported by evidence in the literature (Ahmad *et al.* 2000; Guttridge *et al.* 1999; Hinz *et al.* 1999; Joyce *et al.* 1999 Westerheide *et al.* 2001).

The protein p53 has been recognised to be involved in regulating cell proliferation, and apoptosis (Yin *et al.* 1998; Burns *et al.* 1999; Lakin *et al.* 1999; Woods *et al.* 2001). The possibility that EGCG-induced apoptosis in HUVEC occurred through modulation of p53 protein is feasible. This supposition is based on the finding that EGCG brought about an increase in the levels of p53 in HUVEC, but had no significant effect in B cells. This increase preceded the observed increase in the levels of apoptotic cells. In human breast carcinoma cells, Liang *et al.* (1999) have provided evidence that 30 μM EGCG brought about an increase in $p21^{WAF1}$ levels in a p53 dependent manner. This increase in p53, hence $p21^{WAF1}$ correlated with an overall reduction of cell growth through inhibition of cell cycle progression. However, the same group also reveal that in a related cell line $p21^{WAF1}$ levels could be induced independent of changes in p53 levels. It is important to point out that this related cell line expressed mutant p53 and hence was not deficient in p53 protein. Again this evidence supports the notion that the effect evoked by EGCG is dependent on cell type. Interestingly, p53 has been shown to modulate the progression of G1 and G2 phase of

the cell cycle (Bunz *et al.* 1998; Thompson *et al.*, 1997). In HUVEC, EGCG induces an increase in p53 levels, at times preceding the observed G2/M phase cell cycle arrest. Hence it is possible that the EGCG-mediated G2/M arrest observed in HUVEC, occurs through the induction in the levels of the p53 protein.

The mechanisms behind this increase in protein levels may be explained by a number of alternate proposals. The increase in p53 levels may not be due to direct modulation by EGCG, but could be a secondary effect, indirectly brought about by EGCG-directed damage at the DNA level. However, although the effect of EGCG on the integrity of DNA was not examined during this investigation, there is much evidence in the literature intimating a protective role of anti-oxidants (including EGCG) against DNA damage (Hsu *et al.* 2001; Tobi *et al.* 2002; Anderson *et al.* 2001), making this an unlikely explanation. The level of any protein is a balance between production and/or degradation. Thus one possibility may be that EGCG functions to enhance p53 protein by acting at the gene expression level. Previous studies have shown that p53 is a transcriptional target of NF-κB (Wu *et al.* 1994) and also expression is partially dependent on NF-κB activity (Kirch *et al.* 1999). Given that NF-κB and p53 were modulated by EGCG, this may imply that NF-κB and p53 interact in these cell types. However, NF-κB translocation and DNA binding activity were repressed following EGCG treatment at times preceding the observed increase in p53 levels. Therefore, unless NF-κB negatively regulates the expression of p53, which is unlikely, as it has not been reported elsewhere, the above proposition remains improbable.

An alternate explanation is that EGCG may protect the protein from degradation. There is strong evidence in the literature that dictates p53 levels are almost exclusively regulated by the rate of degradation. Wild-type p53 is degraded through proteosomes (Ciechanover *et al.* 1994) and by calpain (Kubbutat 1997; Zhang *et al.* 1997; Gonen *et al.* 1997) and inhibition of proteosomes leads to the accumulation of wild-type p53 (Maki *et al.* 1996, Blagosklonny *et al.* 1996; Dietrich *et al.* 1996). Nam *et al.* (2001) have reported that EGCG (among other ester bond containing tea polyphenols) potently and selectively inhibited the activity of purified 20S subunit of the proteosome, 26S subunit of the proteosome of tumour cell extracts, and 26S subunit of the proteosome in intact tumour cells at nannomolar and micromolar concentrations respectively. Considering the above evidence, it is likely that in HUVEC, EGCG inhibits proteosomal degradation, and thus stabilises and enhances the protein levels p53.

There is a possibility that increases in wild p53 protein may be beneficial in preventing the onset and progression of atherosclerosis. This thought is based on evidence in the literature that supports, but in some instances undermines, the benefit of enhancing p53. For example, Speir *et al.* (1994) and Zhou *et al.* (1996) have shown that post-angioplasty coronary restenosis, an aggressive form of atherosclerosis, is associated with the accumulation of wildtype (wt) p53 in this lesion. Therefore p53 inactivation may be an important factor in the development of atherosclerosis. Conversely, other reports in the literature support the concept that enhancement of wt p53 is essential in preventing lesion formation (Van Vlijmen *et al.* 2001 Guevara *et al.* 1999). The disparity between some of these findings is interesting but not unexpected. One factor is the type of cell that was examined in these studies. In certain cell types such as monocytes the importance of p53 augmentation could be considered beneficial. Up-regulation of p53 could lead to inhibition of monocytic cellular proliferation by impinging on the cell cycle or apoptotic response. A decrease in monocytic cell numbers would result in an associated-decrease in the thickness of the arterial lumen and also a decrease in the levels of pro-atherogenic inflammatory chemokines. This outcome would culminate in the amelioration of normal vascular function.

Also, differences in the methods used to measure apoptosis and cell proliferation between individual groups, can be subject to inherent inaccuracies. For example, in the study by Guevara *et al.* cellular proliferation was measured by means of bromodeoxyuridine (BrDU) incorporation. This method can be inappropriate in some instances, particularly because it is a static technique of assessing cellular proliferation (Gilchrist *et al.* 1999). Similarly, using the method of *in-situ* DNA end extension (TUNEL) assay for detecting apoptotic cells as in Guevara *et al.* (1999) can also be prone to artifactual error (Kockx M. 1998).

It is evident when reviewing the literature that p53 and NF-κB are central to the modulation of many signalling pathways that affect cellular growth. Mutual negative regulation of p53 and NF-κB, in which each transcription factor inhibits the activity of the other by competing for a limiting pool of the co-activator p300/CBP, has been described (Webster *et al.* 1999). It is possible that EGCG cell growth inhibition could occur either by the interaction of these two signalling pathways, or through these pathways independently. NF-κB can be both pro- and anti-apoptotic depending on cell type and conditions under scrutiny. Certainly in the lymphoblasts NF-κB is likely to be constitutively active due to the immortalisation process (McFarland *et al.* 1999; Hammarskjold *et al.* 1992; Huen *et al.*

1995, Cho *et al.* 1998; Kaye *et al.* 1996). In fact B lymphocytes transfected with EBV, have the potential to produce indefinitely proliferating cultures as evidenced by Henle *et al.* (1967). With this in mind, it is likely that NF-κB is involved in the cell growth regulation (by protecting from apoptosis) at least in the B lymphoblast cell lines, and maybe in HUVEC. This is a reasonable proposition given that EGCG inhibited NF-κB p65 translocation and DNA binding activity in all cell types investigated. In fact, several reports show that loss of constitutive and inducible NF-κB activity in EBV transformed B cells results in apoptosis (Feuillard *et al.* 2000; Frost *et al.* 2001; Cahir-McFarland *et al.* 2000). Therefore, although inhibition of NF-κB activity occurred at significantly earlier time points than the observed increase in apoptosis, it is likely that NF-κB plays a role in the apoptotic response. Future experiments may employ specific inhibitors to the NF-κB pathway to examine this hypothesis.

To date there have been no reports describing the effects of EGCG on the protein Pin1. Various studies have identified Pin1 as a regulator of mitotic events and apoptosis, mainly through its specific interaction with proteins that are phosphorylated at serine/threonine-proline motifs (Lu *et al.* 1996; Yaffe *et al.* 1997; Wulf *et al.* 2001, Liou *et al.*, 2002). Pin1 has been found to modulate the activity of Cdc25c (Crenshaw *et al.* 1998; Patra *et al.* 1999), which regulates progression of the G2/M phase of the cell cycle. Pin1 also regulates the expression of cyclin D1 supporting the notion that it is important for G0/G1 cycle progression (Wulf *et al.* 2001, Liou *et al.*, 2002). Intriguingly, Pin1 has been found to interact with the p53 pathway and thus facilitate in genotoxic responses leading to apoptosis (Zacchi *et al.* 2002; Zheng *et al.* 2002). Given that in certain conditions, p53, NF-κB and also cyclin D1 levels were affected following EGCG treatment, it seemed prudent to examine the effect of EGCG on Pin1 protein levels. There were no obvious changes in Pin1 protein levels in either the B lymphoblast cell lines or HUVEC, following treatment with EGCG (30 μM). These results would intimate the cell growth inhibitory actions induced by EGCG are independent of any significant changes in Pin1 protein levels. It has been reported that Pin1 levels remain constant during the cell cycle in transformed cells compared to non-transformed cells (Shen *et al.* 1998; Winkler *et al.* 2000), which may explain why no changes were observed in the EBV-transformed B lymphoblast cell lines.

Although no changes in protein levels was detected, this does not exclude changes in the phosphorylation status of Pin1. It can be phosphorylated on a number of amino-acid residues providing an alternate level to the complexity of its regulation and thus its

downstream consequences. These include serine 16, argnine 17 and tyrosine 23, with serine 16 being the most important as this is located at the centre of the phospho-ser/thr-pro-binding pocket. Lu *et al.* (2002) demonstrated that phosphorylation of this serine16 residue completely abolished the ability of Pin1 to interact with its target substrates and hence abrogated its function. Also by mutating the serine 16 residue to alanine, these authors demonstrated Pin1 was able to bind constitutively to its substrate and act in a dominant-negative manner to block mitosis and apoptosis. Considering this evidence, it remains possible that EGCG may affect the phosphorylation status of Pin1 rather than at the protein level.

The effect of EGCG on the protein levels of CDK1 in the B lymphoblast cell lines and also HUVEC was investigated. In the B lymphoblast cell lines, there were no significant changes in the protein levels or phosphorylation status of CDK1. Conversely in HUVEC, CDK1 protein levels were found to be down-regulated at 24, and up to 96 hours EGCG exposure. These findings are substantiated by more recent reports in the literature that show a decrease in CDK1 following EGCG treatment (Bhatia *et al.* 2001; Agarwal *et al.* 2000). This outcome is not surprising, given that 30 μM EGCG brought about a G2/M phase cell cycle arrest in HUVEC, and CDK1 is important in the regulation of G2/M phase cell cycle progression. Interestingly, there are a number of reports in the literature that also suggest a role for CDK1 in the transition from G1 to S phase (Furukawa *et al.* 1990; Krek *et al.* 1991), although this is still a matter of contention. This may explain the increase in the number of cells in the G1/S phase of the cell cycle in HUVEC.

The mechanisms behind the EGCG-induced down-regulation of CDK1 in HUVEC have not been examined. However, it is plausible that the observed decrease in CDK1 levels was due to the increase in p53 following treatment with EGCG. There are numerous reports demonstrating that p53 represses or negatively regulates CDK1 expression (Taylor *et al.* 1999; Azzam *et al.* 1997; Passalaris *et al.* 1999). In addition, it has been reported by several groups the involvement of $p21^{WAF1}$ or cyclin B1 in the p53-mediated repression of CDK1 gene transcription, demonstrating a further complexity to CDK1 regulation by p53 (Chang *et al.* 2000; Taylor *et al.* 1999, 2001; Passalaris *et al.* 1999).

An attempt to ascertain the significance of both $p21^{WAF1}$ and cyclin B in the observed G2/M cell cycle arrest was made. Unfortunately, the results were inconclusive due to technical difficulties. It thus remains feasible that CDK1 levels may be regulated in a p53-dependent manner through the modulation of some of the above-mentioned regulatory mechanisms.

Pivotal to many forms of agonist-induced cell death is the recruitment of various caspases in the apoptotic response. Caspases are activated in a sequential cascade of cleavage events from their inactive forms (as reviewed in Cohen *et al.* 2002). Once activated, caspases can subsequently cleave their cognate substrate at a specific site leading to signal propagation and eventual death of the individual cell. Pan *et al.* (2000a,b) demonstrated in U937 monocytes, the induction of apoptosis by oolong tea polyphenols was through cytochrome C release and activation of caspase-9 and caspase-3. In this study the contribution of XIAP, an inhibitor of caspase-induced cell death and more recently a modulator of various cell cycle regulators was investigated (Levkau *et al.* 2001).

XIAP expression is accentuated by a NF-κB positive feedback loop, whereby NF-κB regulates expression of XIAP, which in turn regulates expression of NF-κB. Although EGCG was found to attenuate the nuclear translocation and DNA binding activities of NF-κB, there were no significant changes in levels of XIAP following EGCG exposure in the cell lines under study. This would suggest that EGCG-induced apoptosis occurred independently of XIAP.

Worthy of mention is the evidence from chapter five demonstrating an increase in both the expression and protein levels of heme oxygenase-1 (HMOX-1), following EGCG treatment. This increase was evident in both the B lymphoblasts and also HUVEC, although at slightly different treatment times. A report by Duckers *et al.* (2001), demonstrate that HMOX-1 expression inhibits the growth of vascular smooth muscle cells (VSMCs) both *in vitro* and *in vivo*. In addition expression of this protein lead to a G1/S phase arrest of the cell cycle, which was attributed to the up-regulation of $p21^{WAF1}$. Induction of *HMOX-1* in HUVEC and B lymphoblasts was observed at 4 hours and 24 hours EGCG treatment time. Levels of HMOX-1 protein increased at 8 hours and 30 hours in B lymphoblasts and HUVECs. Importantly these increases in both HMOX-1 expression and protein levels preceded any significant observed effect on cell growth. Moreover, the increases also occur at times before the observed effect on the cell cycle, that is the G_0/G_1 phase arrest in both the B lymphoblasts and HUVEC. Collectively these data suggest a possible role of HMOX-1 in cell growth regulation in B lymphoblasts and HUVECs.

One of the intentions of this thesis was to examine whether the B lymphoblast cell lines would make a suitable surrogate for the less readily obtainable vascular endothelial cells by comparing them to the endothelial cell model HUVEC. Certainly, when reviewing the effect of EGCG on the growth regulation/cell cycle progression and the induction of apoptosis, it is clear that many of the effects of EGCG are cell-type specific and in some

instances concentration-dependent. The data presented in this chapter would indicate that the B lymphoblast would not make a suitable surrogate biomarker cell for vascular endothelial cells, as their responses were different for most of the parameters examined. In addition there were no obvious differences in responses between the normotensive and the hypertensive cell lines.

The B lymphoblasts are established cell lines, transformed with EBV. This immortalisation process may result in an altered phenotype and thus affect the growth characteristics, when compared to a primary B-lymphocyte. It would be interesting to examine the growth characteristics of a primary B-lymphocyte and then to compare this to the primary HUVEC, before drawing any final conclusions.

To summarise, it is apparent that the growth-inhibitory actions of EGCG are mediated in part by the deregulation of the cell cycle and also more significantly by affecting the apoptotic response. Further investigation suggested that EGCG attains these effects through modulation of p53, cyclin D1 and CDK1 (in HUVEC), and possibly through the loss of NF-κB activity.

Chapter Five

Use of microarray to investigate the effect of EGCG on gene expression profiles in B lymphoblasts and HUVEC

5.0 Introduction

5.1 Application of the technique of microarray to cardiovascular disease

One of the aims of experiments described in this chapter was to examine the possible downstream consequences of NF-κB modulation by examining the mRNA profile using the technique of microarray. A second aim was to compare the phenotype of normotensive and hypertenisve cells. Despite intensive examination, the precise mechanisms behind common cardiovascular diseases such as hypertension and atherosclerosis have not been clearly dissected. The interplay between environmental and genetic factors responsible for these disorders may vary between patients, producing phenotypically similar outcomes, which may require different corrective treatment strategies. A more detailed characterisation of pathological processes at the molecular and cellular level will enhance comprehension of the underlying mechanisms. Cellular phenotype is determined by the combination of proteins expressed in the cell. Preferably, a description of phenotype would give an account of the level of expression, the spatial orientation and activity status of the proteins present within a cell. This undertaking is beyond the reach of current single experimental techniques (although it can be argued that it is not beyond the scope of combined autonomous techniques). However the technique of microarray has provided the opportunity to analyse and quantify thousands of cellular mRNA transcripts simultaneously. The advantage microarray has over Northern blotting is that relatively smaller amounts of RNA are required for the analysis of a far superior number of genes, without a big loss of sensitivity as demonstrated in a report by Taniguchi *et al.* (2001).

Microarray has been used effectively to analyse both *in vitro* and *in vivo* systems. The influence of mechanical stimuli has been studied on vascular smooth muscle cells grown on fibronectin-coated supports (Sompayrac *et al.* 1995). In this study only a handful of 5000 genes, scrutinised at two time points after the onset of mechanical stretch, varied more than 2.5 fold in expression, the arbitrary cut off set by the investigators. Applying this threshold may abrogate some of the inherent reproducibility problems associated with the technique, although small but functionally important changes in expression may have been overlooked. Plasminogen activator inhibitor-1 (PAI-1) and Tenascin-C, were found to be induced following stretch. Both these proteins are important for tissue remodelling and hence may be important in the disease process. These findings were confirmed with

Northern blotting, ELISA and Western blotting for corresponding changes in mRNA and protein production. Consequently, such studies indicate the validity and functional reliability of the microarray technique.

Oxidised low-density lipoprotein (Ox-LDL) is another pivotal pro-atherogenic factor involved in the plaque development. As stated previously (chapter one), monocytes become engorged with cholesterol within the plaque, forming foam cells. This process has been modelled by incubating the monocytic THP-1 cell line with Ox-LDL and a comparison of expression profiles with those of untreated cells has been made (Shiffman *et al.* 2000). The group disclose that 268 out of 6805 genes arrayed, had altered expression (a minimum of 2 fold), at one or more time points ranging up to 4 days after exposure to Ox-LDL. Importantly genes previously demonstrated to be responsive to Ox-LDL loading of macrophages, were induced in the THP-1 cells, again validating the technique.

The complexity of analysing patient samples is compounded by the additional variables of heterogeneity of the patient background i.e. polymorphisms, aetiology of underlying disease and the preceding drug treatments. Profiling of human atherosclerotic plaques is limited by the availability of tissue. In some instances this problem can be circumvented by applying novel methods of cDNA amplification (as described in Zohlnhofer *et al.* 2001). Human carotid endarterectomy samples have been used in one study to identify elevated activity of the transcriptional pathway involving early growth response element-1 (Egr-1), when compared to media layers of non-diseased arteries (McCaffrey *et al.* 2000). The transcription factor Egr-1 modulates a group of stress-responsive genes, including platelet-derived growth factor (PDGF) and transforming growth factor β (TGFβ) that may contribute to overall disease process through enhancement of smooth muscle and endothelial cell growth.

The knowledge that can be accrued from the technique of microarray can be overwhelming. Profiling information provides a rich source of additional questions and hypotheses. It is important, however, to repeat experiments to minimise the risk of false positives, which may arise through cross-hybridisation of homologous or conserved repeat sequences. Moreover, attaching a functional significance to changes in gene expression should be addressed, that is, identification of the types of post-translational modifications and protein synthesis and stability that may occur after a change in expression has been established. With this in mind it is possible to perform such techniques including Northern and Western analyses in conjunction with enzyme activity assays to satisfy these criteria. In addition further experiments are often required to examine whether expression profiles

translate from *in vitro* to *in vivo* models or from transgenic mice models to human disease tissue.

5.1.2 Problems associated with microarray

Microarray experiments are notoriously variable. For the results described in this chapter, each experiment was performed on 3 separate occasions, and the combined results viewed in Treeview programme. From Treeview, genes that were modulated substantially in at least 2 of the 3 experiments were considered for further analysis. A pooled average was then taken from these genes to give an overall estimate to the fold change in gene transcription. A report by Lee *et al.* (2000) examined the importance of replication in reducing the inherent variability in microarray gene expression studies. This report suggested that pooling data from replicates provided a more reliable classification of gene expression. Interestingly from this study the maximum attainable precision in classifying gene changes was reached after 3 replicates, assuming constant experimental conditions, such as hybridisation temperatures, in each individual experiment. Hence with the above information in mind, it was encouraging that the validity of data acquired in the present study may not significantly increase if further replicates were performed. It must be noted, however, that in some instances spurious results were obtained, which may be due to non-constant or non-uniformed experimental conditions between individual experiments. Such things as partial degradation of mRNA or maybe even poor quality hybridisation could give rise to variability. Poor hybridisation could be caused by disparities in the reagents used, as not all experiments were carried out using the same batch of reagents. In fact, when using older batches of the fluorophores, visually, the cDNA did not appear to label as efficiently. These points are certainly worth considering when evaluating the reliability of the data.

It is perceived that the technique of cDNA microarray analysis will benefit every corner of disease research. The human genome is expected to contain an estimated 60,000 to 100,000 genes. Ultimately, every open reading frame will be deposited on a microarray slide, representing the expression profile on a genome wide scale. In the experiments described in this chapter about 5800 genes were deposited on the single microarray slide and some of these were duplications. There is also considerable interest in specialised microarray chips that focus on genes or gene clusters specific for a particular biological function or a given cell type. Considering this it would be of interest to perform

experiments similar to the ones described in this chapter on gene chips specific for either NF-κB or targets perhaps a chip containing genes known to be proatherogenic.

Another possible drawback to this technique, which is certainly pertinent to the current investigation, results from the heterogeneity of individual subjects. An attempt was made to compare the normotensive to the hypertensive B lymphoblast cell lines, and evaluate whether there were any potential differentially expressed genes, that may give rise to the hypertensive disease state. This type of comparison can be difficult and is open to criticism due to the potential heterogeneity of individuals and not simply any differences due to the hypertensive disease status of the cells. This remains a problem with all microarray experiments, which compare two genetically non-identical individuals or dissimilar cell types. As of yet there is no ideal solution to circumvent this problem, except by screening a large number of individuals.

One of the principal problems of the microarray experiments was due to quality of data generated. Each hybridisation experiment would generate data for approximately 5800 different genes. Given that each experiment was repeated 3 times and this was performed on 3 independent cell types, this culminated in a substantial quantity of data. The analysis of the data was performed using a gene-clustering programme and Treeview imaging analysis software. The gene-clustering software clustered the data hierarchically using a complete linkage algorithm. This culminated in a linkage map whereby one cluster is successively split into further clusters and so on, according to similarities in the data. This allowed the identification of patterns of altered expression, but still required a considerable amount of computational/number-crunching time. There are now alternate methods to analysing microarray data that have become available since completion of this analysis. Such methods include the use of software like SIMCA-P (Umetrics, UK) which analyses clustered data on the basis of principle components algorithm. Therefore, in the future it may be possible to employ this technique, thus reducing the total time spent analysing data.

As previously stated expression data alone are not sufficient to establish firm functional associations among proteins. With this in mind, information, which establishes whether the change in gene expression translates through to the level of the protein, would have to be considered. Also worthy of attention is the type of post-translational modification such as protein phosphorylation, which can obviously not be determined from microarray information. Microarray should therefore be used in conjunction with other techniques, possibly with proteomics. This would allow the analysis of global patterns of gene expression at the level of the protein. Proteomics would circumvent some of the

existing problems mentioned above. In addition it would also provide information on a protein's subcellular localisation, turnover, and any protein:protein interactions.

Despite the problems, when evaluating the overall effectiveness of these experiments it was encouraging to note some patterns, which revealed biological processes and were also found in independent studies. The overall aim of this chapter was to investigate gene expression profiles of a number of different cell types, to identify novel and potentially beneficial genes regulated by EGCG. This study revealed some novel genes, which were regulated by EGCG such as HMOX-1, and MMP-1 that could be beneficial to cardiovascular chemoprevention.

5.2 Heme oxygenase System

One of the more significant, consistent gene changes that occurred following exposure of B lymphoblasts and HUVEC to EGCG, was the gene encoding heme oxygenase 1 (*HMOX-1*). Hence further studies using Western analysis were performed to verify a functional change of HMOX-1 at the level of the protein. HMOX is the rate limiting enzyme, responsible for the degradation of heme, catalysing the cleavage of the heme ring to form ferrous iron, carbon monoxide and biliverdin (figure 5.1). To date three isoforms have been identified, the oxidative stress and inflammatory-inducible protein HMOX-1 or HO-1 or HSP32, and the constitutive isoenzymes HMOX-2, and HMOX-3. HMOX-2 and HMOX-3 expression is mainly located in the brain, with HMOX-2 also being expressed in the testes. In the brain HMOX-2 enzymatic activity is higher than the HMOX-3, and hence accounts for the majority of enzymatic activity.

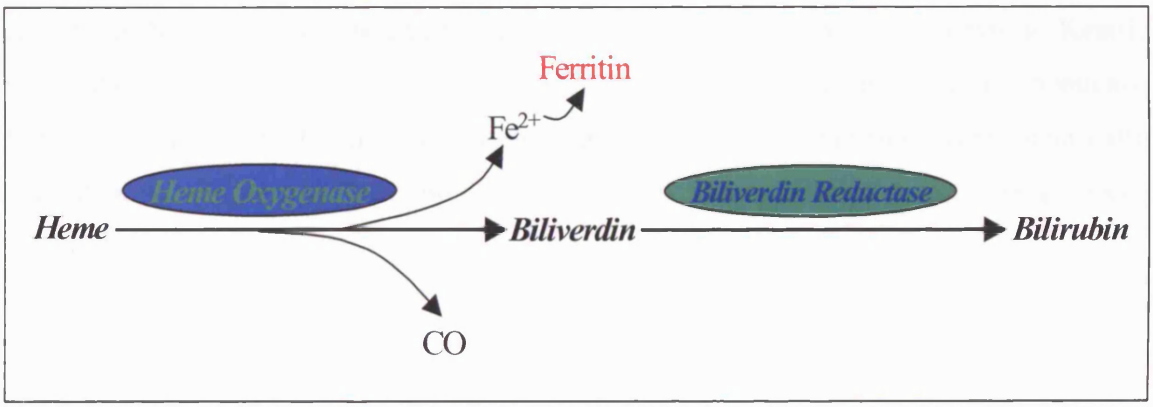

Figure 5.1 Enzymatic reaction of heme oxygenase to produce biliverdin, CO and free iron.

The HMOX-1 isoform has been found to be induced following exposure to various cytokines (Rizzardini *et al.* 1993; Terry *et al.* 1998) heavy metals, stress related factors such as heat shock (Taketani *et al.* 1998), hypoxia (Christou *et al.* 2000) and UV irradiation (Reeve and Tyrell 1999). HMOX-1 has also been found more recently to be up-regulated by some antioxidants, such as curcumin (Hill-Kapturczak *et al.* 2001; Motterlini *et al.* 2000) and the synthetic antioxidant tert-butylhydroquinone (tHBQ) (Ma *et al.* 2002). The actions of HMOX-1 attempt to rid the cell of pro-oxidants, allowing cells to withstand further exposure to the above stimuli.

Typically many of these agents that bring about this elevation, tend to be associated with conditions in which the cell can become stressed. Hence pathways that have been found to impinge on the HMOX-1 system tend to be modulated by various types of stress as well. These include the p38 SAPK (Kacimi *et al.* 2000; Chen K *et al.* 2000) the ERK 1/2 (Chen K *et al.* 2000), but interestingly not the JNK pathway (although AP1 binding sites have been found in the HMOX-1 promoter (Alam *et al.* 1995; Choi *et al.* 1996). Also thought to contribute the regulation of HMOX-1 gene expression is the NF-κB pathway (Lavrovosky *et al.* 1994, 2000; Bauer *et al.* 2000; Hill-kapturczak *et al.* 2001) and the PI3K pathway (Li *et al.* 2002).

Current understanding of the mechanisms of HMOX-1 gene modulation by antioxidants predominantly comes from studies on the induction of phase II detoxification enzymes, of which HMOX-1 is an example. Induction of phase II metabolising enzymes requires an antioxidant response element (ARE) located in the enhancer region of the particular gene (Favreau *et al.* 1991; Rushmore *et al.* 1990). Transcriptional regulation occurs through the binding of Nrf2, a member of the NF-E2 family of nuclear basic leucine zipper (bZIP) transcription factors, to the ARE elements (figure 5.2). Typically under basal conditions Nrf2 is largely bound to the cytoplasmic inhibitor protein known as Keap1, which itself is anchored to the actin cytoskeleton. Upon exposure to phenolic chemicals, Nrf2 dissociates from Keap1, translocates into the nucleus, dimerises heterodimerically with other transcription factors, and accelerates transcription of target genes through ARE-dependent expression.

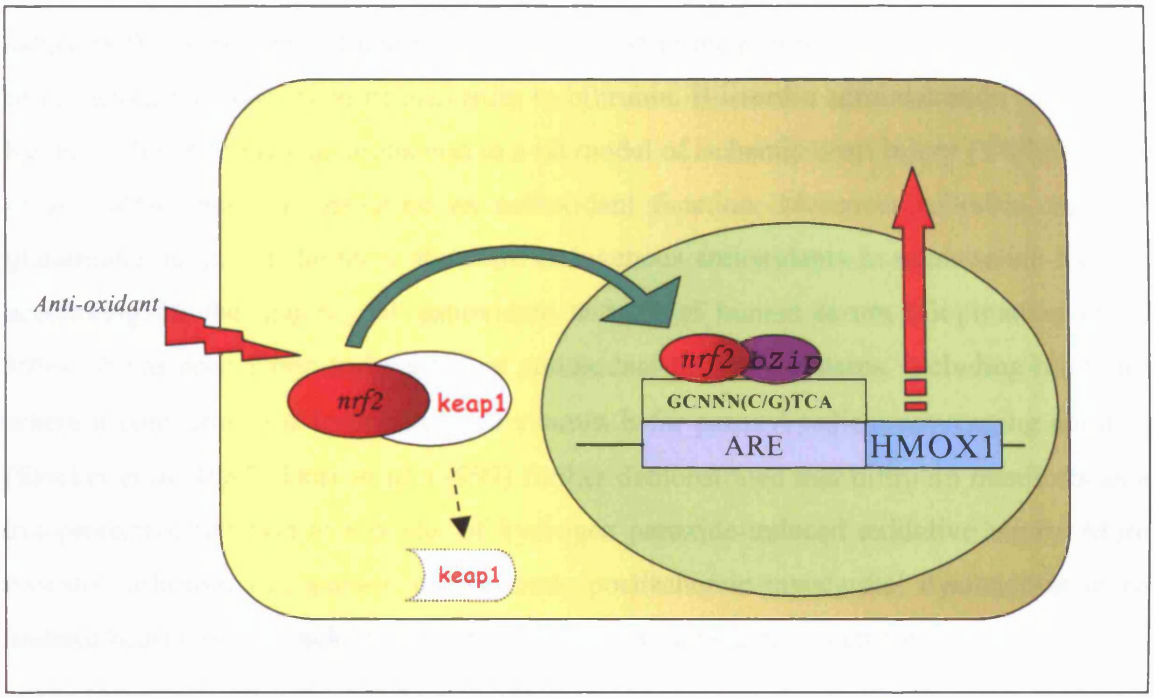

Figure 5.2 Mechanism of phase II-response regulation. *Nrf2 is sequestered in the cytoplasm by the binding of Keap1. Inducers disrupt the Keap1/Nrf2 complex, allowing Nrf2 to translocate to the nucleus, associate with other transcription factors and bind to ARE. Transcription of phase II enzymes is then enhanced.*

5.2.1 HMOX-1 and atherosclerosis

There have been several reports in the literature offering support to the concept that induction of HMOX-1 would be beneficial in preventing the onset and progression of various cardiovascular disorders (Platt *et al.* 1998; Schwartz *et al.* 2001; Luss *et al.* 2002). Atherosclerosis manifests as an inflammatory proliferative condition involving a complex series of events, including endothelial damage/dysfunction, platelet hyperactivity, oxidative modification of LDL and cellular hypertrophy. Reactive oxygen intermediates (ROIs) play a central mechanistic role in the development of atherosclerosis. They have also been implicated in vasospasm and the loss of endothelial-dependent vasodilation, which all contributes to the pathogenesis of the disease. Although the function of this enzyme is not completely understood, theoretically by increasing the expression or stability of HMOX-1 protein there are a number of possible cumulative mechanisms of action, leading to an enhanced cardioprotective prognosis (figure 5.3). Due to the complexity of the disease process, some of these modes of action may interact and thus enhance the overall protective effect.

HMOX-1 can rid the cell of pro-oxidants by a number of different actions. It catalyses the conversion of heme to biliverdin and in the presence of biliverdin reductase brings about the conversion of biliverdin to bilirubin. Biliverdin administration to rodents has been shown to provide protection in a rat model of ischemic heart injury (Vachharajani *et al.* 2000), thus demonstrating an antioxidant function. Moreover bilirubin, next to glutathione, is one of the most abundant endogenous antioxidants in mammalian tissues, accounting for the majority of antioxidant activity of human serum (Gopinathan *et al.* 1994). It has been found to be a potent antioxidant in many systems, including the brain where it compares to α-tocopherol and vitamin E for peroxyl radical scavenging abilities (Stocker *et al.* 1987). Dore *et al.* (1999) further demonstrated that bilirubin manifests as a cytoprotective function in a model of hydrogen peroxide-induced oxidative injury. More recently, bilirubin was shown to ameliorate postischemic myocardial dysfunction in an isolated heart model (Clark *et al.* 2000) thus endorsing its antioxidant role.

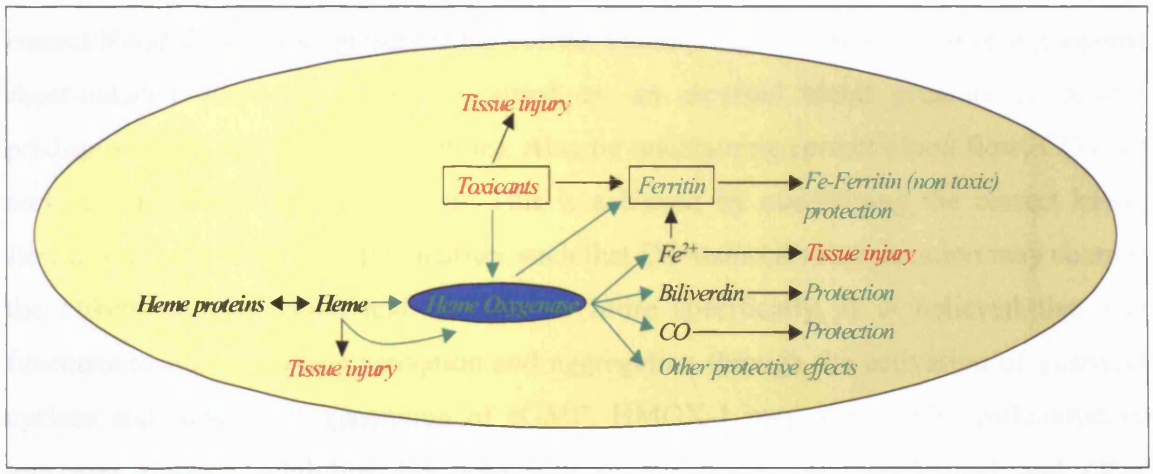

Figure 5.3 – The possible mechanisms of HMOX-1 cytoprotection. *Red indicates possible sources of tissue injury, and green indicates possible sources of cell protection. Adapted from Platt et al. (1998).*

Another mode of action that exemplifies its antioxidant/cytoprotective property is the ability to recruit ferritin, which acts to sequester free iron (Fe^{2+}) released during the formation of biliverdin. Free iron via its two free electrons can produce highly reactive hydroxyl radicals that can further augment the disease process. By sequestering this free iron, HMOX-1 preserves, if not enhances, the antioxidant status of the cell (Balla *et al.* 1992). HMOX-1-dependent release of iron has also been found to up regulate the expression of ferritin (Vile and Tyrell, 1993). Potentially this could lower the pro-oxidant

status of the cell further. Additional evidence by Balla *et al.* (1992) also showed that induction of ferritin was cytoprotective in a model of oxidant stress.

An alternate anti-atherogenic property of HMOX-1 is the release of carbon monoxide (CO) as a by-product, following conversion of heme to biliverdin. CO in high concentrations has always been thought of as toxic to the body. However, at lower concentrations, CO has been found to suppress the production of chemokines such as platelet-derived growth factor (PDGF) and endothelin (Morita *et al.* 1995), which contribute to the disease process. Moreover, enhanced CO production following HMOX-1 induction in vascular tissue effectively contributes to the suppression of both aortic vasoconstriction *in vitro* (Sammut *et al.* 1998) and acute hypertensive responses *in vivo* (Motterlini *et al.* 1998; Yet *et al.* 1997). Arterial plasticity remains an important predisposing determinant in the development of a number of cardiovascular diseases. After all vasorelaxation of endothelial and smooth muscle cells allows for the maintenance of the correct blood flow. This regulatory function is useful for two reasons. By allowing for correct blood flow it also preserves the correct blood pressure, possibly protecting against shear-induced blood vessel injury caused by an elevated blood pressure (a pivotal predisposing pro-atherogenic condition). Also by maintaining correct blood flow, CO may serve an anti-inflammatory function. This is achieved by maintaining the correct blood flow at sites of damage or inflammation, such that CO-induced vasorelaxation may counter the effects of coagulation and thrombosis. More specifically, it is believed that CO functions to inhibit platelet activation and aggregation through the activation of guanylyl cyclase and subsequent generation of cGMP. HMOX-1 may also inhibit inflammatory processes through inhibiting the activation of leukocytes by complement and other inflammatory factors (Willis *et al.* 1996).

5.3 Experimental Design and Aims

One of the aims of the array experiments conducted in this study was to compare differences in the global gene expression profiles between B lymphoblast cell lines derived from normotensive and hypertensive patients. This was carried out to identify or highlight potential novel genes that contribute to the 'hypertensive' disease state.

Further to this aim, was an investigation of the potential effects of EGCG on gene transcription profiles of both the B lymphoblast cell lines and also HUVEC. These experiments could help to identify potential novel target genes (and consequently their

respective proteins) for the chemopreventive agent, which may be useful in the prevention or reversal of the atherosclerotic disease state. For example, the level of the HMOX-1 protein was examined as it was significantly altered at the mRNA level following exposure to EGCG.

The data might allow tentative comparisons to be made between the B lymphoblast cell lines and HUVEC, thus highlighting any differences or similarities in these cells lines following EGCG exposure. It would then be feasible to draw conclusions as to whether the B lymphoblast cells make a suitable surrogate cell line to the less obtainable endothelial cells lining the arterial vessels.

The initial experiment comparing normotensive and hypertensive B lymphoblast cells was carried out using 'untreated' cells. The latter experiments involving EGCG, were carried out using a dose equivalent to 25 μM at time points of 4 and 24 hours respectively. Four hours was selected, as this was an appropriate time point in order to observe any 'early' changes in gene transcription. Conversely, 24 hours was the time point at which 'later' changes in gene transcription could be monitored.

Gene expression in HUVEC (a model for endothelial cells) following exposure to EGCG, may show a pattern of characteristic changes which can be used as predictive guide to the types of changes that occur in endothelial cells lining arterial vessels. It is conceivable that gene expression patterns in peripheral lymphocytes or immortalised lymphocytes (B-lymphoblast cell lines) may be useful surrogates, for those in endothelial cells displaying markers of disease processes such as inflammation, hypertension and malignancy.

5.4 Results

Due to the large number of genes whose expression was modified, it was resolved to focus on gene changes that were either relevant to the disease process or consistently changed at 4 or 24 hours for each cell type. Also included in this section are genes that were consistently modified between cell types. Information concerning the gene function was taken in the first instance from National Cancer Institute (NCI) Genecards website (http://nciarray.nci.nih.gov/cards), unless stated otherwise. Further information was collated from other databases such as ISI Web of Science and OVID journals, if there was a possibility that the genes were germane to cardiovascular disorders.

5.4.1 Gene expression profile associated with a hypertensive disease phenotype

The gene expression profiles of the two-hypertensive cell lines H308 and H242 were compared against the two-normotensive cell lines C143 and C149 respectively. For these experiments, normally dividing (untreated) cells were lysed, mRNA isolated and microarray analyses performed as described in chapter two. The analysis was performed on 3 individual mRNA samples for each pairing, making a total of 6 experiments (i.e. C143 Vs H308 x 3; C149 Vs H242 x 3). Figure 5.4 shows a typical array slide from these experiments, indicating red, green and yellow spots, which depict up-, down-regulated or unchanged genes respectively.

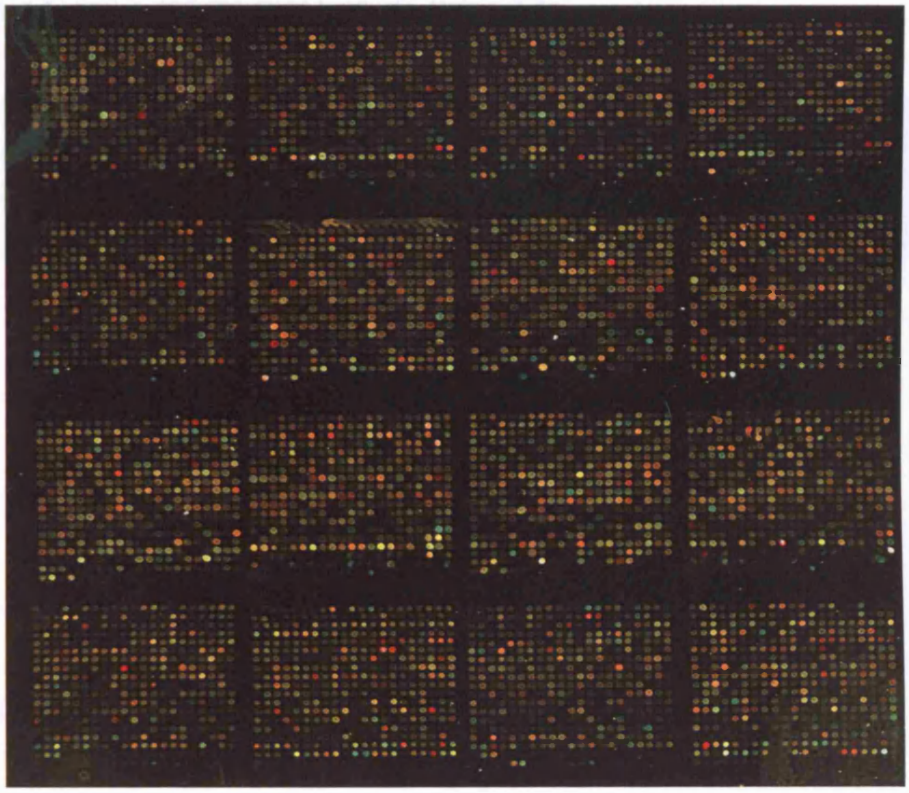

Figure 5.4 – A representative microarray slide following hybridisation and scanning as described in materials and methods. *Red, green and yellow spots represent genes whose expression was up-, down-regulated or unchanged.*

Table 5.1 lists the genes that were at least two fold differentially expressed in the hypertensive cell lines. The fold-change in gene expression was an average of the 3

individual experiments. An arbitrary two-fold threshold was recognised, as this was deemed significant and not simply due to background noise of the experiment.

Using these criteria, 100 genes were identified as differentially regulated in the hypertensive cell lines compared to the normotensive cell lines. The expression of 50 genes was found to be elevated, whereas the expression of 50 genes was down regulated in the hypertensive cell lines. Of these 100 genes only 4 genes were overexpressed in both pairings. The genes that were consistently found to be overexpressed in both hypertensive compared to the normotensive cell lines were *IGHG3* (an average of 18.4 fold), *NR5A2* (an average of 3.85 fold), *PLOD2* (an average of 3.8 fold), and *TARBP1* (an average of 2.7 fold). There were no genes whose expression was repressed consistently in both hypertensive compared to the normotensive cell lines following EGCG. However, there were genes that may encode proteins with similar functions, such as T cell receptor alpha (TCRA) (-2.8) and T cell receptor beta (TCRB) (-2.4).

Gene *C143 vs H308*	***Description***	***Fold change***
BASP1	brain abundant, membrane attached signal protein 1	2.0
IGHG3	immunoglobulin heavy constant gamma 3	14.5
IGL	immunoglobulin lambda chain	2.2
NR5A2	nuclear receptor subfamily 5, group A, member 2	3.6
PLOD2	procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2	4.6
SCYA3	small inducible cytokine A3	2.4
SCYA3L1	small inducible cytokine A3-like 1	2.1
SPARC	secreted protein, acidic, cysteine-rich	3.0
TARBP1	TAR (HIV) RNA binding protein 1	2.4
UBD	ubiquitin D	4.1
VPS41	vacuolar protein sorting 41	2.0
AARS	alanyl-tRNA synthetase	-2.1
ARP	ADP-ribosylation factor related protein	-2.0
CDR2	cerebellar degeneration-related protein 2	-2.1
CYP1A1	cytochrome P450, subfamily I A, polypeptide 1	-2.6
ERP70	N-ethylmaleimide-sensitive factor attachment protein, beta	-2.0
NAGR1	N-acetylglucosamine receptor 1	-2.0

NK4	natural killer cell transcript 4	-3.4
PBX3	pre-B-cell leukemia transcription factor 3	-2.2
PTPRC	protein tyrosine phosphatase, receptor type, C	-2.4
SLC7A5	solute carrier family 7, member 5	-2.5
SLU7	step II splicing factor	-3.8
TCRA	T cell receptor alpha locus	-2.8
C149 Vs H242		
ADD3	adducin 3 (gamma)	2.0
AGXT	alanine-glyoxylate aminotransferase	2.4
SIAT8A	alpha2-8-sialyltransferase	5.6
ATP6H	ATPase, H+ transporting, lysosomal 9kDa, V0 subunit e	2.2
BCHE	Butyrylcholinesterase	2.1
Cap	adenylyl cyclase-associated protein	3.8
CD9	CD9 antigen	4.0
CLN3	ceroid-lipofuscinosis, neuronal 3, juvenile	2.1
DPYD	dihydropyrimidine dehydrogenase	3.5
EPS8	epidermal growth factor receptor pathway substrate 8	2.8
ES1	chromosome 21 open reading frame 33	2.3
F13A1	coagulation factor XIII, A1 polypeptide	4.2
FYN	FYN oncogene related to SRC, FGR, YES	2.1
GPM6A	glycoprotein M6A	2.9
GF-2A9	Growthfactor-inducible 2A9 gene	3.7
HBG2	hemoglobin, gamma G	5.0
Homosapiens incomplete cDNA for a mutated	Homosapiens incomplete cDNA for a mutated	3.2
HSST	N-deacetylase/N-sulfotransferase(heparan glucosaminyl) 1	2.1
Humanmetallothionein I-B gene	Human metallothionein I-B	2.0
IGHG3	immunoglobulin heavy constant gamma 3	22.3
KRT18	keratin 18	3.4
KSR	kinase suppressor of ras	2.0
LNPEP	leucyl/cystinyl aminopeptidase	4.1
MT1B	metallothionein I-B gene	2.2

MFNG	manic fringe homologue	2.2
MGST1	microsomal glutathione S-transferase 1	3.7
MT1E	metallothionein 1E	2.5
MT1L	metallothionein 1L	2.3
N33	Putative prostate cancer tumour suppressor	3.0
NDUFC1	NADH dehydrogenase (ubiquinone) 1, subcomplex, 1	2.0
NR5A2	nuclear receptor subfamily 5, group A, member 2	4.1
PDCD10	programmed cell death 10	2.0
PFN2	profilin 2	5.8
PLOD2	procollagen-lysine, 2-oxoglutarate 5-dioxygenase	3.0
S100A6	S100 calcium binding protein A6 (calcyclin)	5.5
TAB1	TAK1-binding protein, 1	3.1
TARBP1	TAR (HIV) RNA binding protein 1	3.0
VELI1	LIN7A (lin-7 homolog A)	2.2
ACBP	diazepam binding inhibitor (GABA receptor modulator, acyl-Coenzyme A binding protein)	-2.2
APP	amyloid beta precursor	-3.7
BMP4	bone morphogenetic protein 4	-8.1
CD38	CD38 antigen	-3.6
CHIT1	chitinase 1 (chitotriosidase)	-4.5
CMKBR1	chemokine (C-C motif) receptor 1	-2.4
CSF2RB	colony stimulating factor 2 receptor, beta	-2.3
CSPG2	chondroitin sulphate proteoglycan 2	-4.3
CYP2C8	cytochrome P450, subfamily IIC, polypeptide 8	-2.2
CYP2J2	cytochrome P450, subfamily IIJ polypeptide 2	-2.3
D6S49E	leukocyte specific transcript 1	-3.2
GBP1	guanylate binding protein 1, interferon-inducible	-2.1
HLA-DQA1	major histocompatibility complex, class II, DQ alpha 1	-3.7
HSD17B3	hydroxysteroid (17-beta) dehydrogenase 3	-4.2
IGL	immunoglobulin lambda chain	-11.6
IL1R2	interleukin 1 receptor, type II	-2.4
ISG15	ubiquitin specific protease 18	-2.5
ITGB1	integrin, beta 1	-2.1
MAOB	monoamine oxidase B	-2.0

MHC2TA	MHC class II transactivator	-3.2
MRF-1	modulator recognition factor I	-2.2
MTM1	myotubular myopathy 1	-2.9
NGP-1	myelin associated glycoprotein	-3.7
NR1H4	nuclear receptor subfamily 1, group H, member 4	-2.3
Orphan G protein-coupled receptor (RDC1)	Orphan G protein-coupled receptor	-2.0
PSPHL	phosphoserine phosphatase-like	-2.6
PTH	parathyroid hormone	-2.1
RAB31	RAB31, member RAS oncogene family	-3.2
RARB	retinoic acid receptor, beta	-2.6
RBPMS	tumour rejection antigen (gp96) pseudogene 1	-2.3
SCA1	spinocerebellar ataxia 1	-2.0
SFRS10	splicing factor, arginine/serine-rich 10	-2.1
Smoothmuscle myosin-heavy chain isoform	myosin, heavy polypeptide 11, smooth muscle	-7.3
SMPD1	sphingomyelin phosphodiesterase 1, acid lysosomal	-2.0
SPRR2C	small proline-rich protein 2C	-2.0
SSR1	signal sequence receptor, alpha	-2.0
TCRB	T cell receptor beta locus	-2.4

Table 5.1 - Genes that were differentially modulated in hypertensive compared to normotensive B lymphoblast cell lines. *The red and green correspond to genes two fold or greater up-regulated or (-) down-regulated in H308vsC143 and H242vsC149 respectively. Data were the result of three independent experiments on each cell pairing (n=3), and were analysed using ConvertData (version 3.1.2) and Genecluster 3 software.*

5.4.2 Effect of EGCG on C143 'normotensive' B lymphoblast gene expression

The gene expression profiles examining the effect of EGCG were performed on three individual mRNA samples, isolated from normal dividing C143 B lymphoblast cells that had been treated identically on three separate occasions. Table 5.2 lists two-fold or greater changes in gene expression in C143 following treatment with 25 μM EGCG, for 4 or 24 hours.

Again using the two-fold criterion, the expression of 77 genes out of 5800 showed a significant response to EGCG. Out of these 77 genes, 2 were up-regulated at 4 hours (*HMOX1* by 3.3 fold and *PPIF* 2.0 fold) and 7 were up-regulated at 24 hours EGCG treatment. Among the 7 genes elevated at 24 hours EGCG exposure, *HMOX1* (2.4 fold) was enhanced the most. Table 5.2 also shows the fold-decrease in the expression of 49 genes following a 4-hour exposure to EGCG, and 18 genes at 24 hour EGCG exposure. Among the genes at 4 hours, *CDKN1A* mRNA levels decreased the most (4.4 fold) followed by *MGST2* (4.2 fold) and *MMP-1* (4.2 fold). At 24 hours *MMP1* (4.7 fold) expression was repressed the most followed by ABCC2 (4.2 fold).

Only 8 genes were consistently altered at both 4 and 24 hours EGCG treatment. Of these the gene encoding HMOX-1 was found to be overexpressed at both 4 and 24 hour EGCG treatment. The remaining 7 genes that were found to be down-regulated at 4 and 24 hour EGCG included *RPL10*, *ABCC2*, *cDNADKFZp434G0118 (from clone DKFZp434G)*, *H4FG*, *MMP-1*, *TCRA* and *GAPD*.

Gene	***Description***	***Fold change***
4 HOURS		
HMOX1	heme oxygenase 1	3.3
PPIF	peptidylprolyl isomerase F	2.0
AARS	alanyl-tRNA synthetase	-2.5
ABCB7	ATP-binding cassette, sub-family B, member 7	-2.5
ABCC2	ATP-binding cassette, sub-family C, member 2	-2.4
ADCYAP1	adenylate cyclase activating polypeptide 1	-2.2
APEG1	aortic preferentially expressed protein 1	-3.0

ATP1B1	ATPase, Na+/K+ transporting, beta 1 polypeptide	-3.4
CC1.3	RNA-binding region (RNP1, RRM) containing 2	-2.3
CD22	CD22 antigen	-2.2
CDC10	CDC10 cell division cycle 10 homolog	-2.2
CDKN1A	cyclin-dependent kinase inhibitor 1A	-4.4
DKFZp434G0118	deafness, autosomal recessive 27	-2.1
CSF1R	colony stimulating factor 1 receptor	-2.0
DB1-complete cds	zinc finger protein 161	-2.5
DEDD	death effector domain containing	-2.2
EGFL5	EGF-like-domain, multiple 5	-2.0
EPAS1	endothelial PAS domain protein 1	-2.5
GAPD	glyceraldehyde-3-phosphate dehydrogenase	-2.8
GATA6	GATA binding protein 6	-2.3
H4FG	H4 histone family, member G	-3.2
HSF1	heat shock transcription factor 1	-2.1
ILK	integrin-linked kinase	-2.4
JUND	jun D proto-oncogene	-2.7
LRP1	low density lipoprotein-related protein 1	-2.9
LRMP	lymphoid-restricted membrane protein	-3.1
LSP1	lymphocyte-specific protein 1	-2.2
MGST2	microsomal glutathione S-transferase 2	-4.2
MICA	MHC class I polypeptide-related sequence A	-2.2
MMP-1	matrix metalloproteinase 1	-4.2
P100	NFKB2	-2.4
PFKL	phosphofructokinase, liver	-2.1
PKD2	polycystic kidney disease 2	-3.4
PLK	polo-like kinase	-2.5
PMVK	phosphomevalonate kinase	-2.2
PPP2R1A	protein phosphatase 2, regulatory subunit A, α isoform	-2.5
PRKAG1	protein kinase, AMP-activated, gamma 1 subunit	-3.2
RCN1	reticulocalbin 1, EF-hand calcium binding domain	-2.2
RGS3	regulator of G-protein signalling 3	-2.4
RPL10	ribosomal protein L10	-2.4
RPS24	ribosomal protein S24	-2.5

SNRPA	small nuclear ribonucleoprotein polypeptide A	-2.4
SNX1	sorting nexin 1	-2.6
STX5A	syntaxin 5A	-2.4
SULT1C	sulfotransferase family, cytosolic, 1C, member 2	-3.2
TAGLN2	transgelin 2	-3.0
TARS	threonyl-tRNA synthetase	-3.0
TCRA	T cell receptor alpha locus	-2.0
TGFB1	transforming growth factor, beta 1	-2.1
U5-100K	N-ethylmaleimide-sensitive factor attachment protein, beta	-3.3
VASP	Vasodilator-stimulated phosphoprotein	-2.4
24 HOURS		
DLC1	deleted in lung and oesophageal cancer 1	2.0
HMOX	heme oxygenase 1	2.4
HSPA10	heat shock 70kDa protein 8	2.1
Mitochondrial ATP synthase subunit 9	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit c (subunit 9) isoform 3	2.1
PDCD5	programmed cell death 5	2.0
SNRPE	small nuclear ribonucleoprotein polypeptide E	2.1
UCHL3	ubiquitin carboxyl-terminal esterase L3	2.2
ABCC2	ATP-binding cassette, sub-family C, member 2	-4.2
ALDOA	aldolase A, fructose-bisphosphate	-2.2
ATP1A3	ATPase, Na+/K+ transporting, alpha 3 polypeptide	-2.5
CDKL1	cyclin-dependent kinase-like 1	-2.7
DKFZp434G0118	deafness, autosomal recessive 27	-2.2
GAPD	glyceraldehyde-3-phosphate dehydrogenase	-3.85
GSTP1	glutathione S-transferase pi	-3.0
H4FG	H4 histone family, member G	-2.2
HLA-DQA1	major histocompatibility complex, class II, DQ alpha 1	-2.7
IFIT1	interferon-induced protein with tetratricopeptide repeats 1	-4.0
MAX	MAX protein	-2.1
MGAT1	mannosyl-glycoprotein-β-1,2-N-acetylglucosaminyltransferase	-2.3
MMP-1	matrix metalloproteinase 1	-4.7

PAI1	serine (or cysteine) proteinase inhibitor, clade E, member 1	-2.6
PLAB	N-ethylmaleimide-sensitive factor attachment protein, beta	-3.8
RALA	v-ral simian leukemia viral oncogene homolog A	-2.2
RPL10	ribosomal protein L10	-3.3
TCRA	T cell receptor alpha locus	-2.4

Table 5.2 - Genes that were modulated in C143 B lymphoblast cell line, following exposure to EGCG (25 μM). *Genes at least 2 fold up- or down-regulated are represented in red or (-) green respectively. Data were the result of three independent experiments (n=3), and were analysed using ConvertData (version 3.1.2) and Genecluster 3 software.*

5.4.3 Effect of EGCG on H308 'hypertensive' B lymphoblast gene expression

The gene expression profile examining the effect of EGCG on H308, was preformed on mRNA isolated from three individually treated cell cultures of normally dividing H308 B lymphoblast cells. Table 5.3 lists the two-fold or greater gene changes in H308 following exposure to EGCG (25 μM) for 4 or 24 hours.

In total the expression of 69 out of 5800 genes was significantly altered following treatment with EGCG. The expression of 5 genes at 4 hours and 24 genes at 24 hours was enhanced. At 4 hours, expression of the gene metallothionein I-B protein was enhanced the most by EGCG, and was found to be 2.4 fold up-regulated. Not one of these genes was elevated at both treatment intervals of 4 and 24 hours. The expression of 15 genes was decreased at 4 hours EGCG treatment, with *MGST2* (4.4 fold decrease) found to be most effected. At 24 hours EGCG exposure, the expression of 25 genes were effected, with *ATP1B1* expression being reduced by 3.7 fold. However there were 6 genes that were down-regulated at 4 and 24 hours, including *MMP-1*, *LGALS9*, *MGST2*, *IFIT1*, *HMGCL* and *TDPX1*. As mentioned previously MMP-1 is involved in tissue remodelling and hence is pertinent to atherosclerosis (see 5.6.5).

Gene	Description	Fold change
4 HOURs		
CD69	CD69 antigen	2.1
KSR	kinase suppressor of ras	2.1
MT1B	Metallothionein I-B gene	2.3
NMI	N-myc (and STAT) interactor	2.0
RCN2	reticulocalbin 2, EF-hand calcium binding domain	2.0
GATM	glycine amidinotransferase	-2.1
GSTP1	glutathione S-transferase pi	-2.1
HMGCL	3-hydroxymethyl-3-methylglutaryl-Coenzyme A lyase	-2.0
IFIT1	interferon-induced protein with tetratricopeptide repeats1	-3.1
LGALS9	lectin, galactoside-binding, soluble, 9	-2.5
MGST2	microsomal glutathione S-transferase 2	-4.4
MMP-1	matrix metalloproteinase 1	-3.5
MOX2	antigen identified by monoclonal antibody MRC OX-2	-2.1
MTHFD1	methylenetetrahydrofolate dehydrogenase-methenyltetrahydrofolate cyclohydrolase, formyltetrahydrofolate synthetase	-2.6
MYBPC3	myosin binding protein C, cardiac	-2.0
NGFB	nerve growth factor, beta polypeptide	-2.0
PLAB	N-ethylmaleimide-sensitive factor attachment protein, beta	-3.3
SHMT1	serine hydroxymethyltransferase 1	-2.1
TDPX1	peroxiredoxin 2	-2.0
TUBG	tubulin, gamma 1	-2.0
24 HOURS		
ACRV1	acrosomal vesicle protein 1	2.0
ABCB9	ATP-binding cassette, sub-family B, member 9	2.0
ADAM11	a disintegrin and metalloproteinase domain 11	2.0
AMPH	amphiphysin	2.0
BMP6	bone morphogenetic protein 6	2.0
BTF3	basic transcription factor 3	2.0
CDC7L1	CDC7 cell division cycle 7-like 1	2.1
CKS1	CDC28 protein kinase regulatory subunit 1B	2.0

DNAH17L	dynein, axonemal, heavy polypeptide 9	2.0
DLAT	dihydrolipoamide S-acetyltransferase	2.0
ERBB2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog	2.0
EXTL3	exostoses (multiple)-like 3	2.0
GABPA	GA binding protein transcription factor, alpha subunit	2.0
HXB	tenascin C (hexabrachion)	2.0
IL13RA1	interleukin 13 receptor, alpha 1	2.2
LAP18	stathmin 1/oncoprotein 18	2.0
LRP2	low density lipoprotein-related protein 2	2.0
RPL5	ribosomal protein L5	2.0
RPS6KB1	ribosomal protein S6 kinase, 70kDa, polypeptide 1	2.0
SCYD1	chemokine (C-X3-C motif) ligand 1	2.3
SLC16A1	solute carrier family 16 (monocarboxylic acid transporters), 1	2.1
SMT3H2	SMT3 suppressor of mif two 3 homolog 2	2.0
TBR1	T-box, brain, 1	2.0
TLOC1	translocation protein 1	2.1
ANK2	ankyrin 2, neuronal	-2.0
ATP1B1	ATPase, Na+/K+ transporting, beta 1 polypeptide	-3.7
ADSS	adenylosuccinate synthase	-2.4
CD30L	tumour necrosis factor (ligand) superfamily, member 8	-2.0
CETP	cholesteryl ester transfer protein, plasma	-2.4
CTSB	cathepsin B	-2.0
EDG4	endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor, 4	-2.0
GSTA4	glutathione S-transferase A4	-2.4
HMGCL	3-hydroxymethyl-3-methylglutaryl-Coenzyme A lyase	-2.4
IFIT1	interferon-induced protein with tetratricopeptide repeats1	-2.0
ITGB3	integrin, beta 3	-2.0
LGALS9	lectin, galactoside-binding, soluble, 9	-2.3
MGST2	microsomal glutathione S-transferase 2	-2.0
MMP-1	matrix metalloproteinase 1	-2.2
MMP-14	matrix metalloproteinase 14	-2.4
NAPG	N-ethylmaleimide-sensitive factor attachment protein, gamma	-2.0

NME1	non-metastatic cells 1, protein (NM23A)	-2.0
PEPD	peptidase D	-2.0
PIR121	cytoplasmic FMR1 interacting protein 2	-2.0
SCYA4	chemokine (C-C motif) ligand 4	-2.2
SCYA18	chemokine (C-C motif) ligand 18	-2.4
TDPX1	peroxiredoxin 2	-2.1
TK1	thymidine kinase 1, soluble	-2.1
TLE4	transducin-like enhancer of split 4	-2.1
TNNT1	troponin T1, skeletal, slow	-2.0

Table 5.3 - Genes that were modulated in H308 B lymphoblast cell line, following exposure to EGCG (25 μM). *Genes at least 2 fold up- or down-regulated are represented in red or (-) green respectively. Data were the result of three independent experiments (n=3), and were analysed using ConvertData (version 3.1.2) and Genecluster 3 software.*

5.4.4 Effect of EGCG on HUVEC gene expression

HUVEC are a potential model for less readily obtainable endothelial cells that line the arterial lumen. The gene expression profiles examining the effect of EGCG, were obtained from mRNA samples isolated from normally dividing HUVEC (passage 5-7) that had been treated identically on separate occasions. The HUVEC used were isolated from three different individuals, to limit subject heterogeneity. Table 5.4 represents the two-fold or greater changes in gene expression in HUVEC following treatment with 25 μM EGCG, for 4 or 24 hours.

Treatment with EGCG induced the up-regulation of 4 genes, and repression of 6 genes. Of these, 2 genes *PAI2* (2 fold) and *RGS2* (2 fold) were found elevated after 4 hours, and *HMOX1* (2.4 fold) and *ALDH1* (2 fold) after 24 hours EGCG exposure. The 6 down-regulated genes included 5 that were down at 4 hours (*BHMT, ABCA6, ITPKB, SCYA2, KIT*) and just 1 that was down at 24 hours (*LMO7*). Among the 5 down-regulated at 4 hours, *BHMT* gene was the most affected at 2.3 fold, followed by *ABCA6* (2.1 fold), *ITKB* (2 fold), *SCYA2* (2 fold) and *KIT* (2 fold). LMO7 mRNA was reduced by 2.2 fold, and was the only gene down-regulated at 24 hours EGCG exposure.

Gene	Description	Fold change
4 HOURS		
PAI2	serine (or cysteine) proteinase inhibitor, clade B, member2	2.0
RGS2	regulator of G-protein signalling 2	2.0
ABCA6	ATP-binding cassette, sub-family A (ABC1), member 6	-2.1
BHMT	betaine-homocysteine methyltransferase	-2.3
ITPKB	inositol 1,4,5-trisphosphate 3-kinase B	-2.0
KIT	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	-2.0
SCYA2	chemokine (C-C motif) ligand 2	-2.0
24 HOURS		
ALDH1	aldehyde dehydrogenase 1 family, member A1	2.0
HMOX1	heme oxygenase 1	2.4
LMO7	LIM domain only 7	-2.2

Table 5.4 - Genes that were modulated in HUVEC, following exposure to (25 μM) EGCG. *Genes at least 2 fold up- or down-regulated are represented in red or (-) green respectively. Data were the result of three independent experiments (n=3), and were analysed using ConvertData (version 3.1.2) and Genecluster 3 software.*

There were no genes consistently modified at both treatment times, using the two-fold or above criterion. However if a lower threshold for fold change was considered, one gene that was consistently overexpressed following EGCG treatment was *HMOX-1* (see 5.6.5).

5.5 EGCG induces HMOX-1 protein in B lymphoblasts and HUVEC

Based on the microarray data, EGCG (25 μM) induced an increase in the mRNA of *HMOX-1* gene in HUVEC and C143 B lymphoblast cells at 24 or 4 and 24 hours respectively. To examine whether this increase in mRNA was reflected in the level of HMOX-1 protein, all cell types were exposed to EGCG (30 μM) over a time course of 2 to 96 hours. Whole cell lysates were prepared from treated cells and subjected to Western analysis as described in the materials and methods chapter.

As shown in representative Western blots (figure 5.5, A, B) the C143 and H308 B lymphoblast cell lines responded in a similar manner when exposed to EGCG. In both

lines, EGCG induced an increase in a HMOX-1 immunoreactive protein, which was evident at 8, 12, 24, and 48 hours respectively. In some of the untreated samples there was evidence of HMOX-1 immunoreactive protein, indicating constitutive HMOX-1 expression.

For HUVEC (figure 5.5 C) similar conditions were applied, except for a few minor changes in EGCG exposure times. Figure 5.5 C, is a representative Western blot exemplifying the effect of EGCG in HUVEC. There was an increase in HMOX-1 protein at 8 hours following treatment with EGCG (30 μM). This increase peaked over the EGCG treatment times of 30, 48, 54, 72 and 96 hours. Again it was evident in the untreated samples the presence of a HMOX-1 immunoreactive protein, indicating that HMOX-1 protein was expressed at basal levels. This may have been because the HUVEC were stressed to some extent during treatment, as they are a primary cell.

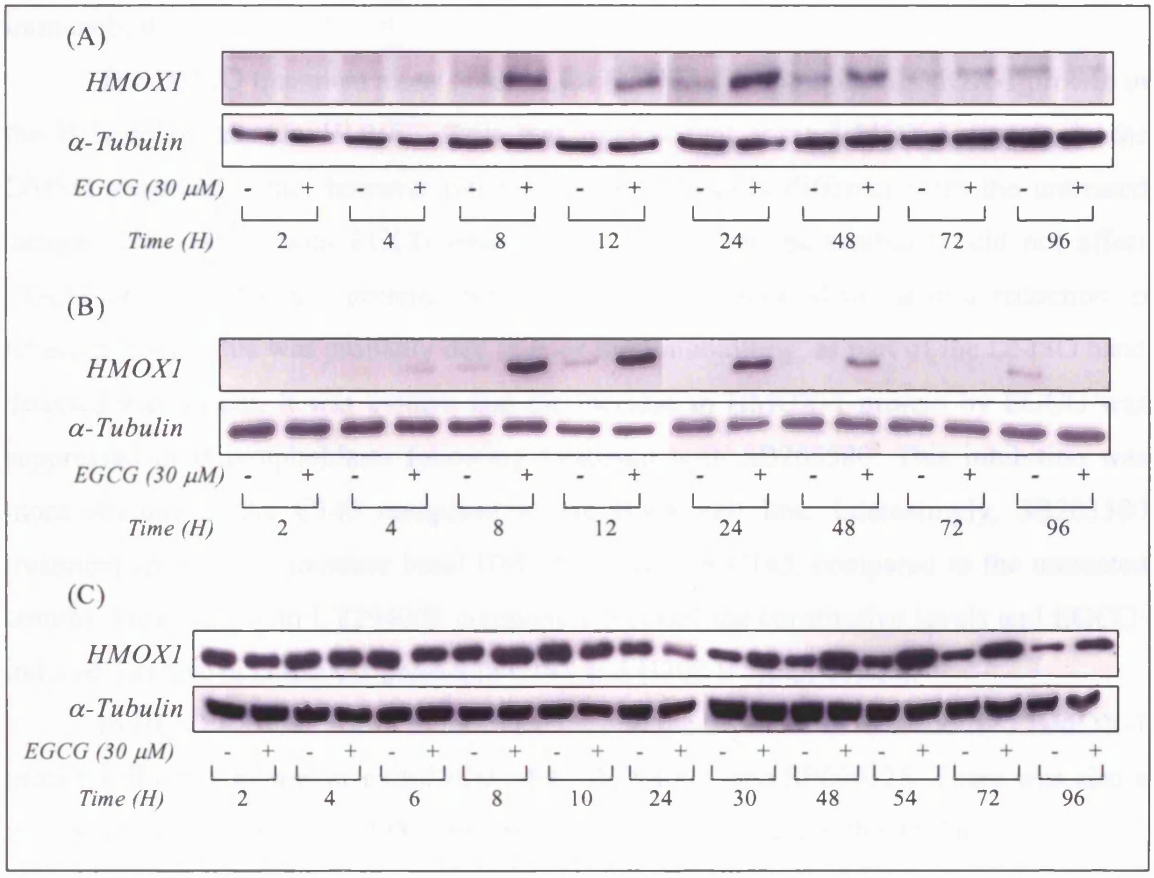

Figure 5.5 – EGCG-induced HMOX-1 expression in B lymphoblast and HUVEC. *C143 normotensive (A), H308 hypertensive (B) B lymphoblasts and HUVEC (C) were untreated or exposed to EGCG (30μm) for the indicated times. Proteins were subjected to immunoblot analysis as described in chapter 2. Immunoblots were probed with an antibody directed against HMOX-1. Membranes were stripped (as described in chapter 2), and reprobed with an antibody directed against α-tubulin to confirm equal protein loading. Data shown are representative of at least two independent experiments.*

5.5.1 Effect of inhibitors of cell signalling pathways on HMOX-1 protein levels

To characterise the increase in HMOX-1 protein following EGCG treatment, cells were exposed to EGCG (30 μM) in the presence or absence of specific signalling inhibitors (Figure 5.6). These inhibitors included 1 μM SP600125 (an inhibitor of the JNK MAPK pathway), 50 μM LY294002 (an inhibitor of the PI3K pathway), 1 μM SB203580 (an inhibitor of the p38 MAPK pathway) and 30 μM U0126 (an inhibitor of the ERK 1/2 MAPK pathway). Figure 5.6 contains representative Western blots indicating the effect of the inhibitors HMOX-1 protein induction, in normotensive C143 (A) hypertensive H308 (B) B lymphoblasts and HUVEC (C). Data were normalised to the EGCG positive control (which was 100 %). Also the HUVEC data were adjusted for loading using the α-tubulin immunoblot.

The DMSO treatment alone (control for EGCG) did not induce HMOX-1 protein in the B lymphoblasts. In HUVEC there was evidence of some HMOX-1 protein in the DMSO treatment alone, however this was not significantly different from the untreated sample. The DMSO with EGCG treatment or control for the inhibitors, did not affect EGCG-induced HMOX-1 protein levels in C143, but appeared to cause a reduction in levels in H308. This was probably due to poor immunoblotting, as part of the DMSO band detected was absent. It was evident that the increase in HMOX-1 protein by EGCG was suppressed in B lymphoblasts following treatment with SB203580. This inhibition was more obvious in the C143 compared to the H308 cell line. Interestingly, SB203580 treatment appeared to increase basal HMOX-1 levels in C143, compared to the untreated control. Treatment with LY294002 completely blocked the constitutive levels and EGCG-induced increase in HMOX-1 protein in C143 and H308 B lymphoblasts.

In HUVEC, there was a modest decrease in the levels of EGCG-induced HMOX-1 protein following treatment with SB203580, LY294002, and SP600125. There was also a modest decrease in basal HMOX-1 protein following treatement with UO126.

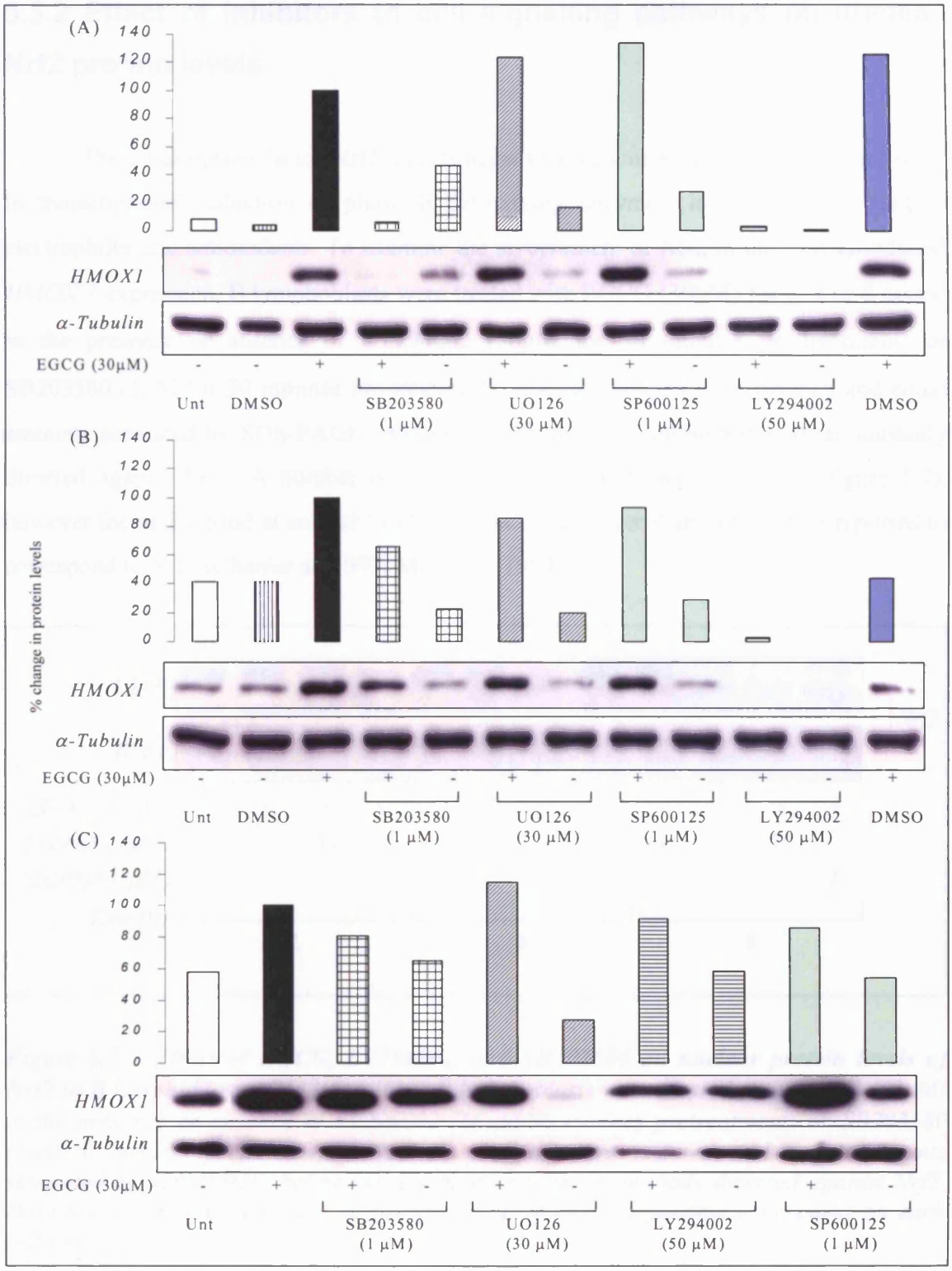

Figure 5.6 – Effect of inhibitors on EGCG-induced HMOX-1 expression in B lymphoblasts and HUVECs. *Normotensive C143 (A), and hypertensive H308 (B) B lymphoblast cells (N=1), and HUVEC (C) (N=2) were exposed to EGCG (30μm) in the presence or absence of specific signalling inhibitors (for 30 minutes pre-treatment). Proteins were subjected to immunoblot analysis as described in chapter 2. Immunoblots were probed with an antibody directed against HMOX-1 and were quantified using a Syngene chemigenius multiimager.*

5.5.2 Effect of inhibitors to cell signalling pathways on nuclear Nrf2 protein levels

The transcription factor Nrf2 and its negative regulator Keap1 play important roles in transcriptional induction of phase II detoxifying enzymes, in response to reactive electrophiles and antioxidants. To examine the involvement of Nrf2 in the EGCG-induced *HMOX-1* expression, B lymphoblasts were treated with EGCG (30μM) for 2, 4 or 8 hours, in the presence or absence of LY294002 (30μM for 30 minutes pre-treatment) or SB203580 (1μM for 30 minutes pre-treatment). Nuclear proteins were isolated and equal amounts separated by SDS-PAGE. Westerns were then immunoblotted with an antibody directed against Nrf2. A number of immunoreactive bands were detected (figure 5.7), however the major band at around 110 kDa, which was larger than expected, is reported to correspond to Nrf2 (Chan *et al.* 1993; Moi *et al.* 1994).

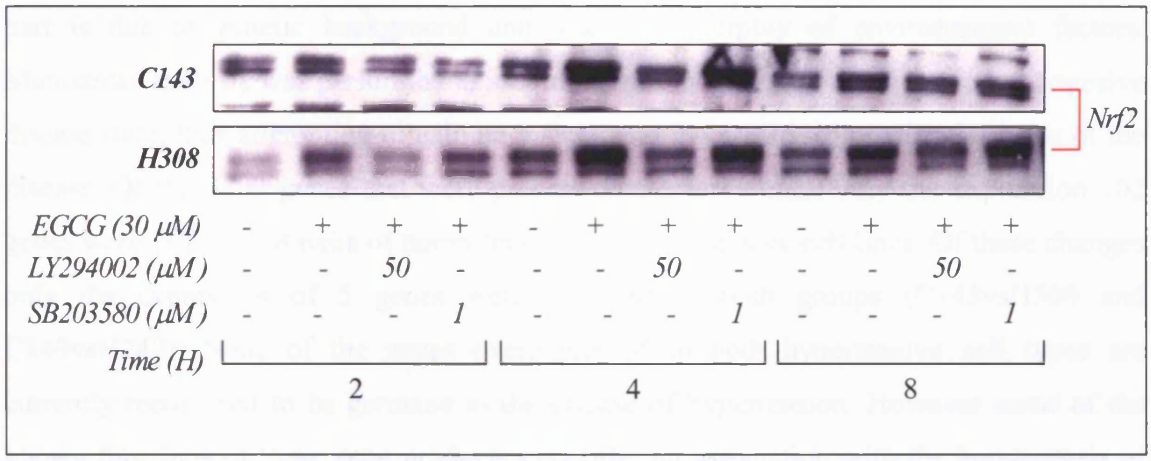

Figure 5.7 – Effect of EGCG, LY294002, and SB203580 on nuclear protein levels of Nrf2 in B lymphoblasts. *C143 and H308 B lymphoblasts were treated with EGCG (30μM) in the presence or absence of LY294002 (50μM/30 minutes pretreatment) or SB203580 (1μM/30 minutes pretreatment). Nuclear proteins were isolated and equal amounts separated by SDS-PAGE, before immunoblotting with an antibody directed against Nrf2. Data are a preliminary study and are the result of one independent experiment on each cell line.*

It was noted that this major band appeared as a doublet, and it may be that the Nrf2 antibody cross-reacts with Nrf1 protein, which has a similar migratory distance. It was evident that constitutive levels of Nrf2 were present in the untreated samples, which may explain the constitutive levels of HMOX-1 protein. In both C143 and H308 B lymphoblasts, EGCG treatment caused an increase in the nuclear levels of Nrf2 compared

to the untreated samples. This increase in protein levels was evident at 2 hours, and peaked at 4 hours EGCG treatment, a time point preceding the increase in HMOX-1 protein. Pre-treatment with LY294002 and SB203580 attenuated the EGCG-induced increase in nuclear Nrf2. The PI3K inhibitor LY294002 was more effective at reducing nuclear Nrf2, compared to the p38 inhibitor SB203580.

5.6 Discussion

5.6.1 Gene changes in the hypertensive cell lines

More than 70 million people display blood pressures that are outside normal physiological parameters, typically systolic 120 mmHg and diastolic 80mmHg for healthy adults. For many of these hypertensive subjects the precise molecular mechanisms behind the disease are undefined. This is due to the abnormal expression of many genes, which in part is due to genetic background and also the interplay of environmental factors. Microarray analysis was performed to identify genes that may give rise to the hypertensive disease state, thus attempting to gain an insight into the underlying molecular basis of the disease. Of the 5800 genes that were present on the array chip, only the expression 102 genes were different in pairs of normotensive and hypertensive cell lines. Of these changes only the expression of 5 genes were different in both groups (C143vsH308 and C149vsH242). None of the genes overexpressed in both hypertensive cell types are currently recognised to be germane to the disease of hypertension. However some of the known functions of these gene products may infer an association with the homeostasis of blood pressure.

The *IGHG3* gene was overexpressed by the greatest amount and codes for the protein immunoglobulin heavy chain G. IGHG3 is part of the immunoglobulin heavy chain that constitutes antibody molecules and also the B lymphocyte receptor. IGHG3 has recently been recognised to be involved in immune disorders such as ulcerative colitis (Lawrance *et al.* 2001) and also asthma (Oxelius *et al.* 1998), hence may play a role in immune type reactions involved in arterial disease.

There is a suggestion in the literature that tissue remodelling in the arterial vessels can have an effect on systemic blood pressure (Mulvany *et al.* 1987). The expression of *PLOD2*, which codes for the protein lysyl hydroxylase 2 was increased, and is reported to be involved in tissue remodelling (Uzawa *et al.* 1999).

The up-regulated gene *NR5A2* codes for nuclear receptor subfamily 5, group A and is part of the orphan nuclear receptor family, indicating that its physiological ligand has not been clearly identified. The protein regulates the expression of a number of genes involved in the bile acid biosynthesis pathway (Goodwin *et al.* 2000; Lu *et al.* 2000). In particular the regulation of cholesterol 11β-hydroxylase (CYP11B1), cholesterol 11α-hydroxylase (CYP11A1), 3β hydroxysteroid dehydrogenase type II (HSD3B2) and cholesterol 7α-hydroxylase (CYP7A1) has been demonstrated (Zhang *et al.* 2001; Wang *et al.* 2001; Sirianni *et al.* 2002). The conversion of cholesterol to bile acids is the most significant pathway for cholesterol disposal (Russell *et al.* 1992a, 1992b). Clearly high serum cholesterol contributes to atherosclerosis and other forms of cardiovascular disease (Brown *et al.* 1986) and thus gene up-regulation would be beneficial. Interestingly, other members of the cytochrome p450 (CYP) family of enzymes have been implicated in the regulation of blood pressure. In the kidney, CYPs generate two major classes of arachidonic acid (AA) metabolites, namely epoxyeicosatetraenoic acids (EETs) and hydroxyeicosatetraenoic acids (HETE) (Makita *et al.* 1996; Capdevila *et al.* 2000). Both EETs and HETEs contribute to the regulation of renal vascular tone and tubular sodium and water transport (Zeldin *et al.* 2001; McGiff *et al.* 1999; Roman *et al.* 2000). Impaired renal hemodynamics and increased salt retention was associated with altered EET- and HETE-generating P450 expression in a number of hypertensive rat and mouse models (Makita *et al.* 1994; Roman *et al.* 1997; Su *et al.* 1998; Honeck *et al.* 2000). Considering the above evidence, if validated by Western analysis, regulation of these enzymes possibly through EGCG-induced modulation of NR5A2 could provide a novel target for regulating blood pressure.

Other genes noteworthy of mention include immune/inflammatory genes such as *SCYA3*, *SCYA3L1*, and *CD9*, which code for small inducible cytokine A3, small inducible cytokine A3-like 1, and CD9 antigen respectively. SCYA3 and SCYA3L1 are monokines with inflammatory and chemokinetic properties. CD9 antigen is involved in platelet activation and aggregation and hence may well be involved in plaque formation. *MGST1* was overexpressed in H242 compared with the C149 cell line. *MGST1* codes for the protein microsomal glutathione S-transferase and functions to provide protection of intracellular membranes from oxidative modification as a result of oxidative stress (Mosialou *et al.* 1993), which is pro-atherogenic. Also the gene *HSD17B3*, which was underexpressed in H242 cell line, codes for the protein hydroxysteroid (17-β) dehydrogenase 3 and may be important with respect to hypertension. This notion is based

on evidence in the literature, where defects in other members of the hydroxysteroid family such as HSD11B2 have been implicated in various forms of hypertension (As reviewed in Garbers *et al.* 1999).

Several reports have suggested that both familial primary pulmonary hypertension and sporadic primary pulmonary hypertension may have a common aetiology that is associated with deficiencies in bone morphogenetic protein receptors (Thompson *et al.* 2000; Deng *et al.* 2000). These findings suggest that bone morphogenetic proteins play an important role in the maintenance of normal pulmonary blood pressure through the modification of vascular biology. The gene *BMP4,* which codes for bone morphogenetic protein 4 was underexpressed in H242 compared to the C149 cell line. Potentially this in part may explain the hypertensive disease status of the cell line.

Intriguingly, the gene *RGS2*, appeared to be repressed in both the hypertensive cell lines, compared to the normotensive cell lines. In the pairing H308 vs C143, *RGS2* was underexpressed 4 fold and by 3.5 fold in the H242 vs C149 pairing. However, this gene was omitted from the final selection of genes as only one of the three replicates in each experiment gave a reading. The other two replicates in each case, gave a value of zero, for which there could be several explanations. However, on closer inspection, it was clear that these values of zero were common to the same array slide prints, for both pairings of hypertensive vs normotensive. The array slide prints from which values of zero were given, were earlier print runs and in actual fact contained a lower number of spotted oligonucleotides or gene sequences, and did not contain the *RGS2* sequence. Later array slides or prints contained additional genes of which *RGS2* was included. Although only one observation was noted from each pairing, it was deemed to be significant ($p<0.1$) by the statistical test performed when analysing the data in the convertData (3.1.2) software.

RGS2 has been implicated to play a significant role in the maintenance of blood pressure, with an absence being linked to hypertension Oliveira-Dos-Santos *et al.* (2000), (see section 5.6.5). Although this response was noted in B lymphoblasts, it may in fact be indicative of a phenomenon in other more disease-relevant cells including vascular endothelial cells, and nephron cells of the kidney. It would be of interest in future studies in examine if this difference in fold expression in hypertensive cells was ubiquitous or specific to certain cells.

5.6.2 EGCG-induced gene changes in C143

Of the genes that were down-regulated at 4 and 24 hours following exposure to EGCG, one of the more relevant in terms of atherosclerosis is matrix metalloproteinase 1 (*MMP-1*). Elevated activity of MMP proteins has been implicated in a number of stages involved in plaque formation (Faia *et al.* 2002) (see 5.6.5), thus down-regulation may be beneficial in the prevention of atherosclerosis. *H4FG* encodes for a protein known as H4 histone family member G. The acetylation status of this protein is important to the regulation of expression of a plethora of genes, as acetylation makes the DNA less accessible to transcriptional apparatus. Histone deacetylation also plays a role in cell cycle progression (Attwood *et al.* 2002). GAPD, glyceraldehyde-3-phosphate dehydrogenase, has an important role in the glycolytic pathway (Liaud *et al.* 2000). It is a multifunctional protein with a number of other diverse activities such as protein kinase activity (Kawamoto *et al.* 1986), a role in RNA transport (Singh *et al.* 1993), ADP-ribosylation activity in humans (Kots *et al.* 1992; Dimmeler *et al.* 1992) and also a potential role in regulating cell growth of human cells (Mansur *et al.* 1993).

TCRA or T-cell receptor α polymorphisms have been associated with certain immunological disease states including rheumatoid arthritis (Cornelis *et al.* 1997), T cell leukaemias (Rabbitts *et al.* 1985), and ankylosing spondylitis (Brown *et al.* 1998).

Other interesting genes with altered expression at either 4 or 24 hours EGCG treatment, include those that code for cell growth regulatory proteins DEDD (-2.2), PDCD5 (2.0), CDKN1A (-4.4), CDC10 (-2.2), CDKL1 (-2.7), APEG1 (-3.0), PLK (-2.5), PLAB (-3.8), also cell signalling intermediates such as ILK (-2.4), junD (-2.7), EPAS1 (-2.5), VASP (-2.4), PPP2R1A (-2.5) and finally genes that may be associated with heart disease *GSTP1* (-3.0), *PRKAG1* (-3.2), and *LRP1* (-2.9). DEDD or death effector domain containing molecule and PDCD5 or programmed cell death 5 may function in the process of apoptosis (Stegh *et al.* 1998; Liu *et al.* 1999). CDKN1A cyclin-dependent kinase inhibitor 1A and CDKL1 cyclin-dependent kinase-like 1 (CDC2 related kinase) are important regulators of the cell cycle with CDKN1A interacting with p53, which itself acts as an inhibitor to cellular proliferation (Harper *et al.* 1993). *APEG1* codes for the protein aortic preferentially expressed protein 1 and may have a role in regulating the growth and differentiation of arterial smooth muscle cells (Hsieh *et al.* 1996), an important stage in plaque formation. Given that APEG1 has been implicated in regulating cell growth in smooth muscle cells it remains feasible that it may affect B lymphoblast cell growth in the

same way. PLK or polo-like kinase is a serine/threonine protein kinase and may function to promote progression of the cell cycle. It has been found to accumulate to a maximum during the G2 and M phases, declining to nearly undetectable levels following mitosis and throughout G1 phase (Holtrich *et al.* 1994). PLAB is part of the TGFβ superfamily and hence there is a possibility that it is involved in proliferation and differentiation or apoptosis (See 5.6.5). Decreasing the expression of these growth regulatory genes may help to prevent the proliferative phases of atherosclerosis.

The signalling proteins include ILK or integrin-linked kinase, which is part of the ILK-pinch complex and is considered to be one of the convergence points of integrin and growth factor signalling pathways. It phosphorylates β1 and β3 integrin subunit on serine/threonine residues, but also Akt1 and GSK3 (as reviewed in Dedhar S, 2000). The gene *junD* codes for transcriptional protein jun D, which can bind to c-fos to make up the AP-1 transcription factor. The AP-1 transcription factor can modulate the expression of genes that regulate cell proliferation. EPAS1 or endothelial PAS domain protein 1 functions as a transcription factor involved in the induction of oxygen-regulated genes. It is pertinent to atherosclerosis affecting vascular plasticity and endothelial cell growth through the regulation of VEGF expression. Thus repression of this gene may be beneficial to the prevention of plaque formation. The *VASP* gene codes for a protein known as vasodilator-stimulated phosphoprotein. This protein acts in concert with profilin to convey signal transduction to actin filament production (Reinhard *et al.* 1995), hence may be pertinent to atherosclerosis as it is involved in cellular proliferation and also tissue remodelling. The importance of phosphatases are often overlooked with respect to signal transduction. PPP2R1A or protein phosphatase 2 (formerly 2A), regulatory subunit A (PR 65), alpha isoform is a serine/threonine protein phosphatase that serves to coordinate assembly of the catalytic subunit of the phosphatase complex.

The genes *GSTP1*, *PRKAG1*, and *LRP*, which were down-regulated by EGCG, may be involved in atherosclerosis. GSTP1 or glutathione s-transferase is a phase II drug metabolising enzyme (Hayes *et al.* 1995) and can buffer cells from attack by reactive oxygen species, thus preventing the initial causes of cellular damage leading to the early stages of plaque formation. However, GSTP1 can also deplete a cell of its substrate glutathione (an antioxidant), and as a consequence could be detrimental. The gene *PRKAG1* codes for a protein known as protein kinase, AMP-activated, gamma 1 (non-catalytic subunit) and is responsible for the regulation of fatty acid synthesis by phosphorylation of acetyl-coA carboxylase. It also regulates cholesterol synthesis via

phosphorylation and inactivation of hydroxymethylglutaryl-coA reductase and hormone-sensitive lipase. Repression of this protein may be help to prevent plaque formation by reducing cholesterol-like proteins. The protein low density lipoprotein-related protein 1 is involved in the plasma clearance of chylomicron remnants and activated α-macroglobulin, as well as the local metabolism of complexes between plasminogen activators and their endogenous inhibitors. Repression of *LRP1* may prevent chylomicron clearance and thus be deleterious in the prevention and therapy of atherosclerosis.

5.6.3 EGCG-induced gene changes in H308

LGALS9 codes for the protein lectin, galactoside-binding soluble 9. This protein binds galactosides and may play a role in thymocyte-epithelial interactions relevant to the thymus. Also, one isoform acts as an eosinophil chemoattractant thus demonstrating its role in immune type reactions. *MGST2* codes for the microsomal glutathione s-transferase 2 protein which may act to protect the cell from the pro-atherosclerotic damage caused by reactive oxygen species (Townsend *et al.* 1998).

Genes that are germane to atherosclerosis included those that code for proteins which effect cell growth, enhance the inflammatory/immune response or aid in the tissue remodelling that occurs during the disease progression. The growth regulatory genes included *CKS1* (2.0), *CDC7L1* (2.1), *CD69* (2.1), *EXTL3* (2.0), and *KSR* (2.1). CKS1 or CDC28 protein kinase 1 and CDC7-like homologue were both up-regulated after 24 hours EGCG exposure. CD69 is involved in lymphocyte proliferation and also functions as a signal transmitting receptor in lymphocytes, natural killer cells and platelets (Ziegler *et al.* 1994). Both EXTL or exostoses and KSR or kinase suppressor of ras, are putative tumour suppressor genes and hence possibly cause a reduction of cell growth by direct or indirect signalling mechanisms (Xing *et al.* 2000). Thus up-regulation of these two genes may be beneficial in preventing the proliferative stages during plaque formation.

The genes that are important for immunological functions included *SCYA4* (-2.2), *SCYA18* (-2.4), *SCYD1* (2.3), *TNFSF8* (-2.0), and *IL13RA1* (2.2). SCYA4 or small inducible cytokine A4, is a monokine with inflammatory and chemokinetic properties. SCYA18 or small inducible cytokine subfamily A, member 18 functions as a chemotactic factor acting on lymphocytes but not monocytes. SCYD1 or small inducible cytokine subfamily D member 1 acts as a chemotactic factor for lymphocytes and also monocytes but not neutrophils. TNFSF8 or tumour necrosis factor superfamily member 8 (CD30L)

binds to the CD30 receptor and induces proliferation of immune cells. *IL13RA1* codes for the IL-13 receptor and hence is involved in the activation of immune cells but also endothelial cells (Murata *et al.* 1997). It is thought that suppression in the levels of some of the above gene products would be beneficial in the prevention and therapy of atherosclerosis, by suppressing the immunological and inflammatory responses associated with atherosclerosis.

Other interesting genes which might be relevant to atherosclerosis include those which code for proteins involved in tissue remodelling such as *MMP-14* (-2.4), *ADAM11* (2.0), *CTSB* (-2.0), and *ITGB3* (-2.0). MMP-14, CTSB (cathepsin b) and ADAM11 (disintegrin and metalloproteinase domain 11) are proteinases and hence may aid in the infiltration of circulating monocytes but also in the removal of old and laying down of new extracellular matrix proteins during plaque formation. ITGB3 or integrin β 3-glycoprotein acts as a receptor for a number of extracellular matrix proteins but also for MMP-2 and plasminogen, which may be involved in plaque formation (see 5.6.5). Also ITGB3 through its binding of fibrinogen brings about platelet/platelet interaction and thus aggregation which is thought to play a role in the early stages of atherosclerosis.

Cholesterol and LDL are thought to play significant roles in the development of atherosclerosis (Ross R *et al.* 1993). Interestingly two genes involved in cholesterol and lipid movement were modulated by EGCG in H308 cell line. *CETP* codes for a protein known as cholesteryl ester transfer protein, and is involved in the transfer of insoluble cholesteryl esters in the reverse transport of cholesterol (BolanosGarcia *et al.* 1997). LRP2 or low-density lipoprotein-related protein 2 functions to bind clusterin but also with less affinity, matrix proteins, plasminogen activator inhibitor type 1 complex and also apolipoprotein e-enriched β-vLDL.

Also worthy of mention is the gene *ATP1B1*, which codes for the protein ATPase (Na+/K+) transporting β1 peptide, and is important in the maintenance of cation homeostasis across cellular membranes (see 5.6.5).

5.6.4 EGCG-induced gene changes in HUVEC

The expression of the *HMOX-1* gene was significantly altered following EGCG treatment (see 5.6.5). This gene was up-regulated by 2.4 fold at 24 hours, and 1.3 fold at 4 hours EGCG. At 4 hours this gene was excluded from table 5.2 as it was below the 2.0 fold or above criterion. Other genes of interest that were modulated by EGCG in this cell type

included *PAI2* or plasminogen activator inhibitor 2. *PAI2* was found to be up-regulated (2.0) following treatment with EGCG at 4 hours. PAI2 inhibits the activity of plasminogens, which are enzymes pivotal in the tissue remodelling that occurs in arterial disease (see 5.6.5). Also of interest to cardiovascular disease are the genes *BHMT* coding for betaine-homocysteine methyltransferase, regulator of G protein signalling (RGS2) and *SCYA2* small inducible cytokine A2 (SCYA2). The modulation of these genes could be either, detrimental or beneficial to cardiovascular disease. BHMT functions to regulate the conversion of betaine and homocysteine to dimethylglycine and methionine, respectively. In theory, repression of the *BHMT* gene or defects in the function of this enzyme could lead to hyperhomocystinemia, an independent risk factor for the development of atherosclerotic vascular disease (Clarke *et al.* 1991, Duell *et al.* 1997). However, such a defect in this enzyme has not been reported in the literature.

The gene *RGS2* codes for the protein regulator of G protein signalling, and is a member of the family of proteins with GAP activity. RGS2 has been implicated to be involved in regulation of blood pressure though a number of mechanisms such as maintenance of vascular homeostasis (see section 5.6.5). Thus the two-fold induction in HUVEC following treatment with EGCG (4 hours) may be beneficial in the prevention and/or treatment of hypertension.

SCYA2 (down-regulated at 24 hours) is a chemotactic factor that attracts monocytes and basophils but not neutrophils or eosinophils to the site of injury. SCYA2 has been detected in human atherosclerotic samples (Takeya *et al.* 1993; Economou *et al.* 2001) and has been implicated in the recruitment of monocytes into the arterial wall during atherosclerotic plaque formation (Rollins *et al.* 1990; Nelken *et al.* 1991). Interestingly it has been suggested that NF-κB regulates the expression of SCYA2 (Collins *et al.* 1995; Marumo *et al.* 1999; Wang *et al.* 2000). Both NF-κB and the expression of SCYA2 were reported to be reduced by antioxidants in cultured endothelial cells (Satriano *et al.* 1993; Ishikawa *et al.* 1999). As discussed in chapter 3, EGCG was found in this study to inhibit basal NF-κB nuclear translocation in HUVEC. This may suggest that the observed decrease in *SCYA2* gene expression (in HUVEC) was due to the EGCG-induced inhibition of NF-κB nuclear translocation.

5.6.5 Gene changes common to B lymphoblasts and HUVEC

To date there are two reports in the literature that examine the effect of EGCG on the expression of genes using microarray analysis (Okabe *et al.* 2001; Wang SI *et al.* 2002). In the report by Okabe *et al.* (2001), the authors examined the effects of EGCG on the expression of 558 genes in the human lung cancer cell line PC-9. Following treatment, down-regulation of 12 genes (under 0.5 fold) and up-regulation of 4 genes (at least 2 fold) was noted. The down-regulated genes included NF-κB inducing kinase (*NIK*), which was also found to be down-regulated in the C143 cell line (1.4 fold at 4 hours; 1.1 fold at 24 hours) H308 (1.2 fold at 4 hours; 1.1 at 24 hours) and HUVEC (1.3 fold at 24 hours) following EGCG treatment. These data were excluded from the tables, as the fold change did not meet the two-fold or greater cut-off point. Given that Okabe *et al.* disclose that *NIK* was only down-regulated by what appeared to be 0.2 fold, it may mean that the observed fold decreases (1.1 minimum) were in fact physiologically relevant. Using RT-PCR the group confirmed the EGCG-induced inhibition of *NIK*. However, the authors did not reveal if they had attempted to examine the effect of EGCG on NIK protein levels, to examine if the changes in mRNA were reflected by changes in protein. Hence it remains problematic to conclude if 0.2 fold decrease was physiologically relevant.

The second report by Wang SI *et al.* (2002) investigated the effect of a 12-hour exposure of EGCG (12 μM) on the expression of 250 kinases and phosphatases in human prostate LNCaP cancer cells. A total of 25 genes showed a significant response to EGCG. Of these, 16 genes had increased expression and 9 genes had reduced expression. Out of the genes with increased expression, were the genes that encode for the ribosomal protein kinase B *RPSKB* and mevalonate kinase and *PMVK*. The gene *RPS6KB1* which encodes for the protein ribosomal protein S6 kinase polypeptide B1 was also up-regulated in the H308 cell line (2.0). However *PMVK* which encodes for phosphomevalonate kinase, was down-regulated in the C143 (-2.2), and thus differed from the LNCaP cells. These data indicate that some of the changes in gene expression in LNCaP were consistent with changes in B lymphoblast cells, but also shows a possible cell type specific response.

Another important gene with an altered expression following treatment with EGCG is *RGS2*. *RGS2* was up-regulated at 4 hours EGCG treatment in HUVEC (2 fold), and in the C143 and H308 (1.2 fold in each cell). The role of G protein coupled receptors in hypertension and cardiovascular disease is well established (Rockman *et al.* 2002). As mentioned previously, RGS2 protein is a member of the family of proteins with GAP

activity. GAP proteins accelerate the hydrolysis of Gα-bound GTP of G proteins, returning the Gα subunit to an inactive state. Recent studies have assigned physiological roles to some of the RGS family members. RGS2 has been shown to be involved in immune and neurological responses (Heximer *et al.* 1997; Ingi *et al.* 1998; Burchett *et al.* 1998). Using an RGS2-deficent murine model, Oliveira-Dos-Santos *et al.* (2000) showed a striking relationship between RGS2 deficiency and blood pressure, demonstrating that the absence of RGS2 caused hypertension. RGS2 impacts on a number of important processes involved in blood pressure maintenance including the angiotensin II system (via AngII receptor), regulating water and sodium homeostasis, and also vascular homeostasis. Thus the two-fold induction in HUVEC (and to a lesser extent in the B lymphoblasts) following treatment with EGCG (4 hours) may be beneficial in the prevention and/or treatment of hypertension. Interestingly, it appears that *RGS2* expression is repressed in hypertensive lymphoblasts (see section 5.6.1). Although the RGS2 protein in B cell lineages may not contribute to hypertension *per se*, it may be indicative of a ubiquitous cellular effect.

Many types of cardiovascular disease such as atherosclerosis, involve a significant modification of the extracellular matrix. Alteration of the extracellular matrix is most likely to occur through the action of two families of matrix degrading proteinases, the plasminogen activator (PA) and the matrix metalloproteinase (MMP) system. The plasminogen system is composed of an inactive pro-enzyme plasminogen (Plg) that can be converted to plasmin by either of two PAs, tissue-type PA (t-PA) or urokinase-type PA (u-PA). This system is controlled at the level of the PAs by plasminogen activator inhibitors (PAIs) (Wiman *et al.* 1995; Schneiderman *et al.* 1991). As t-PA has fibrin specificity it is primarily involved in clot dissolution, while u-PA has been implicated in cell migration and tissue remodelling. Plasmin is able to degrade fibrin and extracellular matrix proteins directly or indirectly, via activation of other proteinases including MMPs (Saksela *et al.* 1988; Carmeliet *et al.* 1997) and also activate or liberate growth factors from the extracellular matrix (Ferrara *et al.* 1997; Keyt *et al.* 1996). The MMPs consist of a family of proteinases able to degrade most extracellular matrix components in the vessel wall (as reviewed in Galis *et al.* 2002; Dollery *et al.* 1995). Control of MMP activity is mediated by tissue inhibitors of MMPs (TIMPs) in a tissue- and substrate specific manner. MMPs are secreted as zymogens and hence they require extracellular activation, which can occur through the u-PA generated plasmin.

Microarray analysis has shown that, in C143 and H308, a number of MMP genes were down-regulated following exposure to EGCG. MMP-1 mRNA was found to be

reduced following 4 and 24 hours of EGCG treatment in both the B lymphoblast cell lines. *MMP-14* mRNA was also shown to decrease after 24 hours of EGCG, but only in H308. Interestingly an inhibitor of PAs, *PAI-2* mRNA, was found to increase following EGCG treatment, but in HUVEC only. It must also be noted that *PAI-1* was down-regulated in C143 after 24 hour EGCG treatment. Given that *MMP-1* gene expression was down-regulated in both B lymphoblast cell lines (and at both time points) this would suggest that EGCG-induced inhibition was a valid result. However, in HUVEC *MMP-1* gene expression was not significantly changed using the 2 fold and above cut off. If a laxer criterion was applied (as noted above for HMOX1) then *MMP-1* gene expression increases by approximately 1.4 fold at both 4 and 24 hours respectively. This difference may simply be due to the fact that HUVEC are different cell types. Alternatively it may be due to the heterogeneity of subjects from which the endothelial cells were derived. With this in mind, it would be beneficial to perform more replicates and pool endothelial cells from a number of different subjects. This would give a more representative population of cells and hence a more typical response for HUVEC.

Hitherto, this is the only report that demonstrates EGCG causes a decrease in the mRNA levels of these tissue-remodelling proteins, using cDNA microarray. There have been several other reports that have investigated the effects of tea polyphenols on the expression and activity of MMPs (Liu *et al.* 2001; Maeda-Yamamoto *et al.* 1999; Annabi *et al.* 2002). In these reports the authors demonstrate that EGCG inhibited the activity of MMP-2 and MMP-9 using gelatin zymography assays. However only the report by Annabi *et al.* (2002) demonstrated that EGCG caused a reduction in the expression of an activator to proMMP-2 (inactive preform of MMP-2) referred to as MT1-MMP. Although MMP-2 was not present on the array used in these experiments, MMP-9 was present and data indicated that there was a reduction (-1.7) in gene expression. However these data were excluded as they were below the two-fold or greater cut-off point in gene expression.

Expression of various MMPs and/or their inhibitors is enhanced in injured or atherosclerotic arteries (as reviewed in Galis *et al.* 2002; George SJ, 2000; Pasterkamp *et al.* 2000). Regulation of PAIs and MMPs by EGCG may be beneficial in terms of cardiovascular chemoprevention. The growth of atherosclerotic plaque occurs through structural changes that lead to the accumulation of cells, extracellular matrix and lipids within the intimal layer of the disease artery. Increases in MMP expression and activity were associated with development of neonatal arterial lesions and smooth muscle cell (SMC) migration after arterial balloon injury in experimental models, whereas MMP

inhibition decreases SMC migration *in vitro* and *in vivo* (Zempo *et al.* 1994; Bendeck *et al.* 1994; Forough *et al.* 1996; Southgate *et al.* 1996). Cell migration, including endothelial and monocytic infiltration are also significant early stages in plaque formation during atherosclerosis. Recent experiments suggest the involvement of MMP action may facilitate this step. Cellular interaction between in vitro between T lymphocytes and EC monolayers was shown to trigger T cell secretion of MMP-2 (Romanic *et al.* 1994). Direct interaction of monocytic cells with paraformaldehyde-fixed monolayer of human endothelial cells (EC) was shown to increase monocyte MMP-9 production severalfold (Amorino *et al.* 1998). Furthermore macrophage foam cells derived from experimental atherosclerotic plaques have been found to produce matrix-degrading proteinases and migration of these cells is dependent on plasmin-induced proteolysis (Galis *et al.* 1995). Proteinases also participate in proliferation of certain cell types including SMCs (as reviewed in Galis *et al.* 2002; Dollery *et al.* 1995).

The above evidence would suggest that proteinases play a vital role in cardiovascular biology. Not only do they aid in cell migration i.e. leukocytes, endothelial and SMCs during the initial stages of disease, they also have a prevalent function in the intermediate stage as macrophages release proteinases to remove necrotic cellular debris. Proteinases also play important roles in the later stages of disease. The demise of atherosclerotic plaque occurs through structural disruption of the arterial wall, which triggers thrombosis, the cause of occlusion and the majority of acute vascular events (Falk *et al.* 1995). Plaque disruption takes one of two forms, frank rupture and superficial erosion (Falk *et al.* 1995; Burke *et al.* 1997). Rupture is associated with fracture of the fibrous cap with exposure of the prothrombotic core (Davies *et al.* 1984). MMP overexpression and *in situ* matrix degrading activity has been discovered in the vulnerable shoulder regions of human atheroma (Galis *et al.* 1994). The majority of MMPs released are from the resident macrophages and also to a certain extent T cells. B cells make up only a small proportion of cells present in the plaque, and thus their contribution to plaque stability is only minor. However, if the EGCG response is evident in both macrophages and T cells, then by reducing MMP expression or by enhancing levels of PAIs following EGCG treatment, in theory it is possible that this would slow down or regress atherosclerosis.

Interestingly, another polyphenolic antioxidant curcumin, examined by microarray, has also been shown bring about a decrease in gene expression of some of these MMPs (M Squires, PhD Thesis, University of Leicester, 2001). There is a possibility that EGCG and

curcumin may alter the expression of *MMP* genes through a common mechanism. In the B lymphoblast cells, EGCG and curcumin inhibited nuclear translocation of NF-κB, with EGCG also inhibiting NF-κB DNA binding (chapter 3). Inhibition of the transactivation potential of NF-κB by curcumin has also been confirmed by number of independent groups (Plummer *et al.* 1999; Singh *et al.* 1995; Pendurthi *et al.* 1997). NF-κB activation has been correlated with MMP expression in a number of independent studies. There is strong evidence to suggest MMP-1 expression is dependent on NF-κB activity (Sakai *et al.* 2001; Bond *et al.* 1999; Bond *et al.* 2001), and the study by Chase *et al.* (2002) demonstrated that NF-κB was necessary for the pathology-related upregulation of MMP-1 and MMP-3 in foam cells elicited during atherosclerosis formation *in vivo*. Thus considering this evidence, the possibility that NF-κB regulates the expression of MMP-1 in the B lymphoblast cell lines remains likely.

The exact mechanisms by which NF-κB regulates the expression of many of these MMPs has not been clearly defined. Although there is no evidence of an NF-κB binding element in the promoter region of MMP-1 in the human B lymphoblasts or HUVEC, in cells of some species such as rabbit fibroblasts, there is evidence of an atypical NF-κB binding *Drosophila* dorsal-like element in the distal MMP-1 promoter (Vincenti *et al.* 1998). However, in the human breast cancer cell line BC-8701, MMP-1 is regulated by NF-κB, with no evidence of a distal NF-κB binding element (Rutter *et al.* 1997). This suggests that NF-κB acts on another signalling intermediate, which in turn regulates the expression of MMP-1. In fact, both MMP-1 and MMP-3 have been found to be up-regulated by NF-κB-dependent induction of IL-1 in a number of cell types (Lee *et al.* 1995a, 1995b). Also Liacini *et al.* (2002) demonstrated that NF-κB can potentiate transcription of MMP-3 that lacks an NF-κB binding element, via interactions with an AP-1 transcription factor.

Another gene, whose expression was modulated by EGCG in C143 (reduced at 24 hours) and H308 (reduced at 4 hours) was *PLAB*. It should be noted that in one HUVEC sample, *PLAB* mRNA increased by 1.2 fold at 4 hours and 1.5 fold at 24 hours. Again as discussed above, this difference may be due to the heterogeneity of individual subjects, as two experiments out of the three suggested that *PLAB* mRNA was reduced at 4 and 24 hour EGCG. Therefore there is a need for experimental replicates to help determine the typical result for HUVEC.

PLAB is a member of the TGFβ superfamily and is also known as prostate differentiation factor. *PLAB* mRNA as determined by microarray, was also reduced in MDA 468 cells following treatment with curcumin (at 3 hours) (M Squires, personal communications). As PLAB is part of the TGFβ superfamily, it may play a role in cellular proliferation or apoptosis. In addition, PLAB may also be involved in macrophage activation (Bootcov *et al.* 1997). Therefore it is conceivable that inhibition of PLAB may be beneficial with respect to atherosclerotic chemoprevention through cell growth regulation and macrophage inhibition.

Precisely how both EGCG and curcumin act to induce a decrease in the levels of *PLAB* mRNA is open to suggestion. It has been shown that the *PLAB* gene is regulated by wild type p53 protein (Li *et al.* 2000; Tan MJ *et al.* 2000). Data from this chapter and also chapter 4 show that *p53* mRNA and protein respectively, are not obviously affected in the B lymphoblast cell types, following EGCG treatment. This suggests that p53 is not involved in *PLAB* gene regulation in these cell lines.

One gene from these series of experiments that was particularly interesting was a gene (*HMOX-1*) that encodes for a protein known as heme-oxygenase 1. This protein is the rate-limiting enzyme responsible for the breakdown of heme. Through its actions, HMOX-1 possesses potent anti-atherogenic qualities, by enhancing the antioxidant status of a cell, and also modulating the plasticity of the arterial lumen (Gonzalez *et al.* 2000). The expression of *HMOX-1* mRNA was enhanced more than 2 fold in HUVEC at 24 hours and in C143 at 4 and 24 hours. Referring to the original microarray data, *HMOX1* gene expression was also elevated at 4 hours for both HUVEC (1.3 fold increase) and H308 (1.3 fold increase). Although these data were not included in the results, they may be physiologically relevant, since a 0.2 fold change detected by microarray was confirmed by RT-PCR, as reported in Okabe *et al.* (2001).

It was decided to investigate whether altered levels of HMOX-1 protein, following exposure to EGCG, reflected changes in *HMOX-1* expression. This gene was selected as *HMOX-1* expression was enhanced in all cell types (to some degree) following treatment with EGCG. Moreover, there is accumulating evidence in the literature that suggests a cytoprotective function, which may be beneficial in the prevention and/or therapy of atherosclerosis.

The increase in *HMOX-1* mRNA following EGCG treatment, was translated to the level of HMOX-1 protein. This was demonstrated in both the B lymphoblast cell lines (C143 and H308) and HUVEC, where HMOX-1 protein was enhanced following treatment

with 30 μM EGCG, a slightly higher dose than used in the microarray. It was interesting that the HUVEC appeared to have higher levels of constitutive HMOX-1 compared to the B lymphoblasts, thus making the increase by EGCG less striking. It may be that the HUVECs, being a primary cell line are more stressed by the culture conditions than the B lymphoblast cell lines during treatment, which may result in an increase of the stress-response protein HMOX-1. In addition, HUVEC being a different cell type may in fact have higher constitutive expression and thus higher levels of HMOX-1 protein than B cell lineages. However it is inappropriate to make unequivocal comparisons, as there is a need for more experimental replicates, and also slightly different protein levels were loaded on to the SDS-gel, with different film exposure times between experiments were used to detect the proteins.

To date, the precise signalling mechanisms behind this induction have not been described. However there are reports in the literature, on the effects of different antioxidants on the regulation of *HMOX-1* gene expression. Using microarray analysis, one such study by Li *et al.* (2002) demonstrated the induction of *HMOX-1* mRNA following treatment with the synthetic antioxidant, tBHQ. The group implicate the involvement of the Nrf2/ARE in the regulatory mechanisms behind the tBHQ-induced *HMOX-1* gene regulation, through the PI3K signalling pathway. Curcumin has also been shown to up-regulate *HMOX-1* gene expression (Hill-kapturczak *et al.* 2001; Motterlini *et al.* 2000; M. Squires, PhD Thesis, 2001). Motterlini *et al.* failed to elucidate the exact mechanisms controlling *HMOX-1* gene expression. However, the report by Hill-kapturczak *et al.* suggested the involvement of the NF-κB transcription factor. This was deduced as an inhibitor to IκB phosphorylation abolished curcumin-mediated induction of *HMOX-1* mRNA. The group further disclosed that *HMOX-1* gene regulation was not dependent on tyrosine phosphorylation, as demonstrated with the use of tyrosine kinase inhibitors (genistein and herbamycin A). In addition to this study, it has been reported that the *HMOX-1* contains NF-κB binding domains in the promoter regions of the gene (Lavrovosky *et al.* 1994, 2000). With this in mind, it is possible that NF-κB may play a role in the regulation of *HMOX-1* gene transcription in our cell types. However, data from chapter 3 demonstrate that EGCG inhibited the nuclear translocation and DNA binding activities of NF-κB at times preceding the observed increase in *HMOX-1* mRNA. This was suggestive that the EGCG-mediated increase in *HMOX-1* mRNA did not require positive regulation by NF-κB. This supposition is consistent with other independent reports in the literature (Stuhlmeier *et al.* 2000), which demonstrate the activation of *HMOX-1* through a

NF-κB independent mechanism. However, although unlikely, it does not rule out the possibility that NF-κB may regulate *HMOX-1* gene expression in a negative manner. In fact, there is evidence that may substantiate this conjecture. Hartsfield *et al.* (1998) reported a dramatic induction of NO-mediated HMOX-1 with pyrrolidine dithiocarbamate (PDTC), an inhibitor of NF-κB. This finding has also been corroborated by other independent sources (Stuhlmeier *et al.* 2000; deMeester *et al.* 1998). Interestingly, the report by Stuhlmeier *et al.* (2000) found that this inhibitor did not completely block NF-κB translocation. However by over-expressing IκBα, the group demonstrated that *HMOX-1* induction was again independent of NF-κB activation. Therefore due to the contradictory evidence further studies appraising the involvement of NF-κB in the regulation of *HMOX-1* would be of interest.

Other possible regulatory pathways behind induction of HMOX-1 are fairly diverse. Given the complexity of the *HMOX-1* promoter (Muller *et al.* 1987; Lavrovsky *et al.* 1994), it is likely that several signalling mechanisms and/or transcription factors are involved in its regulation. Reports by several groups have suggested that ERK1/2 and/or p38 and/or PI3K are positive regulators of the ARE in several cell lines (Yu *et al.* 1999, 2000a; Wild *et al.* 1999). P38 can also negatively regulate the induction of ARE (Yu *et al.* 2000b). Dissection of the signalling pathways involved in the EGCG-induced response was approached using various known signal-transduction pathway inhibitors. From these series of experiments it was apparent that HMOX-1 protein induction in B lymphoblasts was dependent on the PI3K pathway and partially dependent on the p38 pathway. This was concluded as HMOX-1 protein levels decreased in cells which had been pre-treated with LY294002 (an inhibitor to PI3K) and SB203580 (an inhibitor to p38). There were no observed decreases in HMOX-1 protein levels following pre-treatment with SP600125 (a SAPK/JNK inhibitor) or U0126 (an inhibitor of MEK, an upstream kinase of ERK1/2) in C143, but maybe a small decrease in H308 following U0126 treatment, indicating the specificity of this response. In HUVEC SB203580 showed some inhibition of EGCG-induced HMOX-1 protein, while U0126, LY294002 and SP600125 were not effective. The ERK inhibitor U0126 did reduce basal levels of HMOX-1 protein.

Until now the induction of HMOX-1 by EGCG, involving the p38 pathway has not been reported. There are reports demonstrating regulation of Nrf2 (a positive regulator of *HMOX-1*) independently of p38 signalling (Huang *et al.* 2000). However other similar studies concur with the data presented here, and thus have shown that HMOX-1 regulation was dependent on the p38 pathway, following a stress insult (Luss *et al.* 2002; Kacimi *et*

al. 2000; Chen K *et al.* 2000). In one such report by Kacimi *et al.* hypoxia induced in cardiac myocytes resulted in an increase a HMOX-1 protein level, which was abrogated following treatment with SB203580. The group also show that hypoxic conditions brought about the activation of the p38 pathway as detected by *in vitro* kinase assays. An independent report by Chen K *et al.* (2000) also demonstrated that p38 activation was required for HMOX-1 induction, following exposure to sodium nitroprusside (SNP), a nitric oxide donor.

Although SB203580 partially inhibited HMOX-1 protein levels, there were no significant changes in phosphorylated p38 between untreated and EGCG-treated cells, as detected using a phospho-specific p38 antibody (data not shown). It may be that any potential phosphorylation of p38 by EGCG was transient and occurred at time points earlier than those examined (2 hours), and thus were not detected.

In the EGCG-induced response, U0126 or SP600125 did not significantly affect the levels of HMOX-1 protein in B lymphoblasts, but reduced levels in HUVEC. This suggests that the ERK and SAPK/JNK pathways are not involved in the regulation of HMOX-1 levels in B lymphoblasts. This is supported by Kacimi *et al.* (2000) and also Chen K *et al.* (2000), who exclude the involvement of the ERK pathway and SAPK/JNK pathway in stress-induced HMOX-1 induction. The hypoxic response described by Kacimi *et al.* (2000) was also partially blocked by the PKC inhibitor chelerythrine. Intriguingly, it has been reported that pre-treatment of HeLa cells with PD98059 (an inhibitor of MEK) suppressed induction of HMOX-1 following SNP insult (Chen K *et al.* 2000), which is consistent with the HUVEC data. Although conflicting, these findings are not surprising as different cell types were examined in each investigation. Further differences may be explained in some instances due to the different stimulants used to induce HMOX-1 protein. It is thus clear from these studies that the involvement of specific signalling pathways in the regulation of HMOX-1 is dependent on the nature of the inducing signal, and also on the cell type under investigation. At present there have been no reports that demonstrate the involvement of the JNK pathway (although AP1 binding sites have been found in the HMOX-1 promoter (Alam *et al.* 1995, Choi *et al.* 1996).

As previously mentioned, it is unlikely that the NF-κB transcription factor regulates the positive transcription of *HMOX-1*. However, with respect to other transcription factors, HMOX-1 promoter contains specific binding sites for AP-1 (Zwacka *et al.* 1998), Nrf2 (Alam *et al.* 2000; Li *et al.* 2002; Yu *et al.* 1999; Kang *et al.* 2000) and hypoxia-inducible factor-1α (HIF-1) (Hoetzel *et al.* 2001; Lee PJ *et al.* 1997; Gong *et al.* 2001).

Lee PJ *et al.* (1997) reported that hypoxia treatment of rat aortic vascular smooth muscle cells resulted in the expression of *HMOX-1* by a HIF-1-dependent mechanism. However, in contrast to these findings, Wood *et al.* (1998) using Chinese hamster ovary cells deficient in the HIF-1α subunit demonstrated that, although activation of certain hypoxic-responsive genes such as *glut1* was attenuated in these cells, induction of *HMOX-1* by hypoxia and cobalt was not impaired. Taken together, these studies imply that *HMOX-1* regulation can be HIF-dependent or HIF-independent following hypoxia and hypoxia mimetics. Interestingly a report by Gong *et al.* (2001) has indicated that induction of *HMOX-1* by hypoxia is HIF-1 dependent, while induction by cobalt is mediated by Nrf2.

In fact it is commonly accepted that the transcription factor Nrf2 and its negative regulator Keap1 play important roles in transcriptional induction of phase II detoxifying enzymes, in response to reactive electrophiles and antioxidants. The PI3K inhibitor LY294002 had a pronounced effect on HMOX-1 induction by EGCG in B lymphoblasts. To date with respect to ARE, there has only been one other report in the literature that investigates the effect of LY294002 on the induction of a plethora of genes including HMOX-1 (Li *et al.* 2002). In this report, the authors suggest the involvement of the PI3K pathway in the tBHQ-induced increase in HMOX-1 gene expression. In addition, tBHQ was found to mediate nuclear translocation of Nrf2 and activation of the ARE, which led to the induction of HMOX-1. Treatment with LY294002 abolished this response. This would suggest that tBHQ activates HMOX-1 via the PI3K pathway, which occurs through an Nrf2/ARE dependent regulatory mechanism.

The data from a preliminary study examining the nuclear levels of Nrf2, suggest the involvement of this transcription factor in the EGCG-induction of *HMOX-1*. Using an antibody directed against Nrf2, the presence of a band of 110kDa, was identified. A number of other less intense, non-specific bands were also visible. Nrf2 is reported to be 66kDa. The larger than expected protein may however correspond to Nrf2. Interestingly, there are reports in the literature which demonstrate a higher than expected molecular weight of both Nrf1 and Nrf2 proteins (Venugopal *et al.* 1998; Chan *et al.* 1993; Moi *et al.* 1994). In Venugopal *et al.* (1998), detection of a 110kDa and a 66kDa band were detected in both cases using an Nrf1 and an Nrf2 antibody. It is reported that the two different sized Nrf1 or Nrf2 proteins are coded for by the same gene and are thus a result of different post-translational processing (Chan *et al.* 1993; Moi *et al.* 1994).

The presence of the higher mobility band detected using Nrf2 was approximately 110kDa, and thus based on the above evidence is likely to be Nrf2. This immunoreactive band appears to increase following treatment with EGCG, reaching a maximum at 4 hours, that is at time points preceding the EGCG-induced increase in HMOX-1 protein levels. At time points after 4 hours, levels of nuclear Nrf2 remain elevated, but progressively decrease at longer treatment times up to 96 hours (data not shown). In B lymphoblasts both LY294002 and SB203580, appear to reduce nuclear levels of Nrf2, which match the observed decrease in HMOX-1 protein levels with these inhibitors.

Figure 5.7 represents the proposed mechanisms regulating HMOX-1 protein levels in B lymphoblasts. It is important to mention that the experimental evidence, demonstrating that p38 and PI3K regulate the translocation of Nrf2, and thus may in part regulate the expression of HMOX-1, is an initial result of one experiment performed in two B lymphoblast cell lines. However the notion that p38 and PI3K regulate Nrf2 is supported by several other reports in the literature (Alam *et al.* 2000; Li *et al.* 2002; Yu *et al.* 1999; Kang *et al.* 2000). One thing that is apparent from the literature, is that regulation of Nrf2 is complex and involves various signalling pathways that are likely to be inducer- and cell-specific. Thus, although these data implicates the involvement of the Nrf2 transcription factor in EGCG-induced induction of *HMOX-1* in B lymphoblasts, it does not rule out the involvement of either AP-1 or HIF-1, which would be of interest to examine in future studies.

In conclusion these studies have provided an insight to the underlying molecular mechanisms, which may contribute to the atherosclerotic predisposing condition of hypertension. These experiments identified *RGS2* which was down-regulated in the hypertensives, providing further support to (Oliveira-Dos-Santos *et al.* 2000) to the involvement of this gene in hypertension. In addition these studies provided information to the downstream consequences of EGCG treatment on the gene expression profiles of two types of cells. From these experiments, identification of a potential novel therapeutic target gene were identified, namely *HMOX-1*.

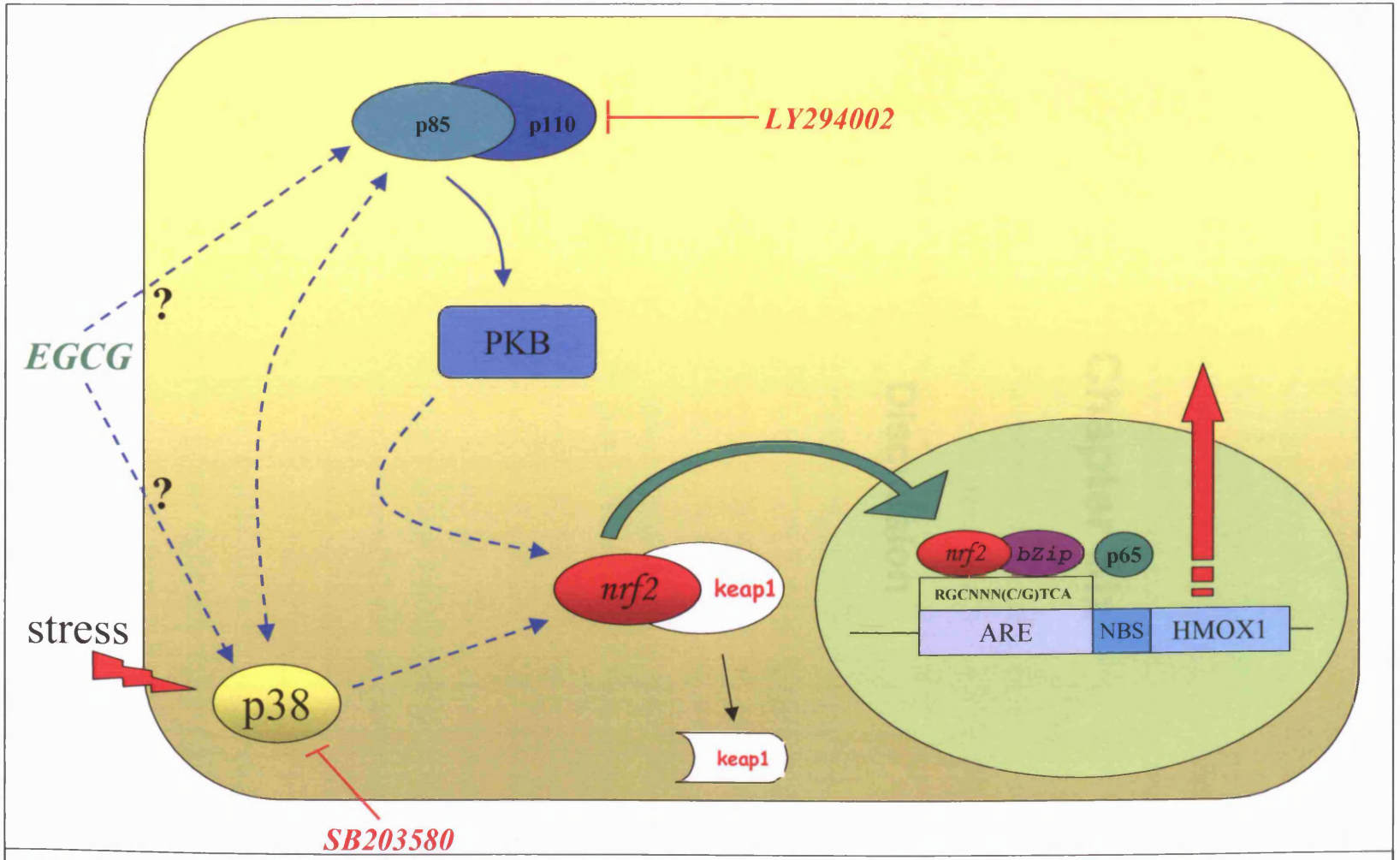

Figure 5.8 – The proposed mechanisms regulating the EGCG-induced increases in HMOX-1 protein in B lymphoblasts. *EGCG appears to induce an increase in HMOX-1 protein by increasing the nuclear levels of the transcription factor Nrf2. Both the PI3K and to a lesser extent the p38 MAPK pathways seem to regulate the EGCG-induced increase in nuclear Nrf2, thus controlling the protein levels of HMOX-1. ? - pathways linking EGCG to the PI3K and p38 pathways are yet to be fully elucidated, but may involve ROS such as H_2O_2.*

Chapter Six

Discussion

The chemopreventive benefit of dietary constituents has been recognised for a number of years, but only more recently have the mechanisms of action at a cellular level been investigated. There is a need for a better understanding of these mechanisms as emphasised by a number of intervention studies. The Alpha-Tocopherol, Beta Carotene Cancer Prevention (ATBC) study in Finland (Heinonen *et al.* 1994), and the Carotene and Retinal Efficacy Trial (CARET) study in the USA (Omenn *et al.* 1996), were conducted among high-risk subjects (smokers and asbestos-exposed workers, respectively) and found significantly higher cancer incidence rates in groups receiving relatively high levels of beta-carotene. In the ATBC study, subjects receiving high doses of vitamin E showed no significant decrease in cardiovascular disease morbidity but did experience significantly more hemorrhagic strokes than did controls. Those assigned to receive beta-carotene experienced an 11 % increase in ischemic heart disease deaths. In the CARET trial, a nonsignificant 26 % increase in cardiovascular disease mortality was reported in the treated group. Conversely, the Cambridge Heart Antioxidant Study (CHAOS) prevention controlled trial conducted on heart disease patients with angiographically proven coronary artherosclerosis (Stephens *et al.* 1996), found that vitamin E supplementation significantly reduced by 77 % the risk of recurrent nonfatal myocardial infarction. The apparent discrepancies in results among the different intervention trials may be explained by the types of populations recruited (general population or high risk subjects), the supplementation levels employed (nutritional or higher doses) and the form of administration (alone or in combination with other constituents).

Therefore, one of the aims of this thesis was to investigate the mechanisms behind the chemopreventive properties of four dietary constituents, namely curcumin, indole-3-carbinol, resveratrol and epigallocatechin 3 gallate. These constituents are seen as simple, accessible, and cost-effective preventive measures that can generally improve the public's health, as evidenced by epidemiological studies. This investigation was performed in a number of cell types, which included a panel of B lymphoblast cell lines derived from normotensive subjects and hypertensive subjects as a surrogate for vascular endothelial cells, and also HUVEC, as a model for the endothelial cells that line the arterial lumen. A comparison between the normotensive and hypertensive B lymphoblasts may identify potential differences that contribute to the disease state. This would give an idea of the chemopreventive efficacy of the agents in subjects with a high risk, compared to subjects with a low risk of developing heart disease.

A further aim was to evaluate if the more readily available B lymphoblasts, would make a suitable surrogate biomarker cell for vascular endothelial cells, using the endothelial cell model, HUVEC, as a comparison. Biomarkers are an extremely useful prognostic tool in identifying the existence of disease but also in predicting the likely efficacy of chemopreventive agents. It is acknowledged that *in vitro* cell systems have their limitations. However they provide a valuable basis for further studies *in vivo*.

The effect of the four chemopreventive agents on the NF-κB signalling pathway was examined. Curcumin, resveratrol, and EGCG inhibited activation of this pathway in B lymphoblasts and HUVEC at low micromolar concentrations. EGCG also blocked NF-κB DNA binding in B cells and HUVEC. The blockade of NF-κB translocation in HUVEC appeared to be mediated through inhibition of IKKα activity, and subsequent phosphorylation and degradation of IκBα. Although EGCG blocked activation of NF-κB in B lymphoblasts, it did not affect phosphorylation or degradation of IκBα, suggesting an alternate mechanism.

There was a noted difference between the normotensive and hypertensive cell line following treatment with I3C. In C143 cell line, I3C appeared to enhance PMA-induced translocation of NF-κB p65, whereas in the H308 cell line, I3C (50 μM) decreased PMA-stimulated but enhanced constitutive nuclear NF-κB p65. However I3C blocked TNFα-induced translocation of NF-κB p65 in HUVEC. This may indicate that the I3C response was cell type specific.

It was evident from these experiments that the B cells had a higher constitutive nuclear NF-κB p65 compared to the HUVEC, which was due to a number of putative mechanisms including transformations with EBV (as discussed in chapter 3).

From the initial studies in chapter 3, it was resolved to focus on one of the chemopreventive agents with the most potential. EGCG was selected due to a number of considerations. Firstly, out of the four agents, EGCG was one of the more efficacious at blocking activation of NF-κB. Also EGCG being the major polyphenolic component of green tea, next to water, is the most widely consumed beverage in the world, already demonstrating human acquiescence. The *in vivo* bioavailabilty of EGCG is good, with biological effects having been observed at physiologically relevant concentrations (refer to chapter 1).

EGCG was found to inhibit cell growth of normotensive and hypertensive B lymphoblasts, and also HUVEC, at low micromolar concentrations. These data are in agreement with more recent reports in the literature (Locher *et al.* 2002; Liang *et al.* 1999;

Yang GY *et al.* 2000). This growth inhibition appeared to be in part due to blockade of the cell cycle, but more notably due to induction of apoptosis. There were no significant differences in the growth, cell cycle and apoptotic responses between normotensive and hypertensive B lymphoblasts. HUVEC differed only in the effect of EGCG on the cell cycle, where there was a suggestion of a G_2/M phase arrest in addition to the G_0/G_1 arrest observed in the B lymphoblasts. This inhibition of proliferation and induction of apoptosis correlated with changes in some important cell cycle and apoptotic regulators. Cyclin D1 protein levels were unaffected by EGCG in B lymphoblast cells, and although not statistically significant, appeared to decrease in HUVEC at treatment times preceding cell cycle inhibition and induction of apoptosis. The p53 protein was found to increase in HUVECs, but was unchanged in B lymphoblasts, at EGCG treatment times preceding and during the observed cell cycle inhibition and induction of apoptosis. There was no significant change in Pin1 or XIAP in either B lymphoblasts or HUVEC following treatment with EGCG. The protein levels of CDK1 were reduced in HUVEC following EGCG treatment, which may have contributed to the G_2/M phase arrest of the cell cycle. Interestingly, HMOX-1 protein has recently been implicated in cell growth regulation by inhibiting cell cycle phase progression (Duckers *et al.* 2001), and was increased in both the B lymphoblasts and HUVEC following treatment with EGCG (chapter 5).

Thus it was concluded that in HUVEC, inhibition of cell cycle progression and induction of apoptosis appeared to involve an increase in p53 and HMOX-1, but a decrease in cyclin D1 and CDK1 protein and a loss of NF-κB activity. In B lymphoblasts inhibition of the cell cycle and induction of apoptosis appeared to be independent of changes in p53, cyclin D1, Pin1, CDK1, and XIAP proteins, but may involve blockade of the NF-κB pathway, which protects the cells from apoptosis. Feuillard *et al.* (2000) showed that a loss of NF-κB activity in EBV transformed B cells resulted in an increase in apoptosis.

It was next decided to examine the effect of EGCG on potential down-stream gene targets of NF-κB using the technique of microarray. Also of particular interest was the comparison between normotensive and hypertensive B lymphoblast cell lines, to identify potential differences in the gene expression profile that may explain the hypertensive disease status of the cells and thus attempt to gain an insight into the underlying molecular basis of the disease. It was apparent from the analysis that only a few genes were consistently overexpressed in the hypertensive lymphoblasts compared to the normotensive lymphoblasts. Interestingly one gene whose expression was repressed in both hypertensive comparisons was *RGS2*. A recent report by Oliveira-Dos-Santos *et al.* (2000) has

implicated RGS2 to play a significant role in the maintenance of blood pressure, with an absence being linked to hypertension.

Evaluating the gene changes in both the B lymphoblasts and HUVEC following EGCG treatment identified a number of interesting genes that potentially could be beneficial in the prevention or suppression of atherosclerosis. One of these was the suppression of MMP1 expression. The involvement of MMP family members in the initiation and later phases of the atherosclerotic disease process has been documented (Faia *et al.* 2002). Therefore suppression of MMP1 by EGCG may prevent either the onset of, or problems associated with the progressive disease. Another gene change following EGCG treatment, which was particularly interesting was the induction of *HMOX-1* expression. This was evident to some degree in both B lymphoblasts and also HUVEC. There is accumulating evidence in the literature that HMOX-1 provides a cytoprotective function, which may be beneficial in the prevention and/or therapy of atherosclerosis (Platt *et al.* 1998; Schwartz *et al.* 2001; Luss *et al.* 2002). The induction of *HMOX-1* by EGCG was further examined to see if this effect was observed at the levels of the protein. HMOX-1 protein was also upregulated in B lymphoblasts and HUVEC, albeit at slightly different EGCG treatment times. However, the pathways leading to induction of *HMOX-1* and thus increase in HMOX-1 protein appear to be different between the B lymphoblasts and HUVEC. In the B lymphoblasts the increase in HMOX-1 protein appeared to be dependent partly on the p38 MAPK pathway, but more so on the PI3K pathway, whereas in HUVEC, inhibitors to the PI3K and p38 pathway did not significantly change the protein levels of HMOX-1 following EGCG treatment. When evaluating the overall effectiveness of the microarray experiments it was encouraging to note some patterns, which were revealing as to biological processes and were also found in independent studies. It was also pleasing to demonstrate that changes in the gene expression of *HMOX-1*, corresponded to changes in HMOX-1 protein.

In conclusion, it was apparent that the B lymphoblasts reflected certain changes and thus responded in a similar manner to HUVEC in some of the experiments. However, this investigation highlighted important differences between the two cell types. One of the fundamental problems when comparisons were made was that the B lymphoblasts were a transformed cell line, and hence many of the noted differences may have been attributable to this phenomenon. As evidenced in the literature, EBV transformation can potentially impinge on a number of signalling pathways and thus orchestrate a number of important cellular responses. On the basis of work presented here and using the endothelial cell

model HUVEC, it was concluded that the B lymphoblasts would not make an appropriate surrogate cell for vascular endothelial cells.

In future it would be of interest to compare the HUVEC or B lymphoblasts, with a human aortic endothelial cell line, to see if the later responded in a similar manner to either cell type. Alternatively, perhaps examining the responses to EGCG in a primary B cell, would indicate if they behaved similarly to the B lymphoblast cell lines. This may then be a better comparison and allow for a final decision to be made regarding the suitability of the B lymphoblast cells as a surrogate for vascular endothelial cells.

Reviewing the data in this thesis indicated that the hypertensive cell lines usually responded in a similar manner to the two normotensive cell lines. This implies that there were no differences between them, at least with the endpoints examined here, such as the NF-κB pathway. However, it should be pointed out that only two normotensive and two hypertensive B lymphoblasts were used in these series of experiments. Therefore in the future it would be prudent to compare cellular responses in a greater number of normotensive and hypertensive cell lines, which may increase the validity and statistical significance of the data.

This investigation provided information on how these chemopreventive agents act and thus may be beneficial when attempting to design preventive dietary intervention studies in healthy volunteers or groups of at risk patients.

Bibliography

Acheson RM, Williams DRR. Does consumption of fruit and vegetables protect against stroke. Lancet 1983; 1: 1191-1193.

Agarwal R. Cell signaling and regulators of cell cycle as molecular targets for prostate cancer prevention by dietary agents. Biochemical Pharmacology 2000; 60: 1051-1059.

Ahmad N, Feyes DK, Nieminen AL, Agarwal R, Mukhtar H. Green tea constituent epigallocatechin-3-gallate and induction of apoptosis and cell cycle arrest in human carcinoma cells. Journal of the National Cancer Institute 1997; 89: 1881-1886.

Ahmad N, Gupta S, Mukhtar H. Green tea polyphenol epigallocatechin-3-gallate differentially modulates nuclear factor kappa B in cancer cells versus normal cells. Archives of Biochemistry and Biophysics 2000; 376: 338-346.

Ahn HY, Hadizadeh KR, Seul C, Yun YP, Vetter H, Sachinidis A. Epigallocathechin-3 gallate selectively inhibits the PDGF-BB- induced intracellular signaling transduction pathway in vascular smooth muscle cells and inhibits transformation of sis-transfected NIH 3T3 fibroblasts and human glioblastoma cells (A172). Molecular Biology of the Cell 1999; 10: 1093-1104.

Alam J, Camhi S, Choi AMK. Identification of a 2nd region upstream of the mouse heme oxygenase-1 gene that functions as a basal level and inducer-dependent transcription enhancer. Journal of Biological Chemistry 1995; 270: 11977-11984.

Alam J, Wicks C, Stewart D, Gong PF, Touchard C, Otterbein S, Choi AMK, Burow ME, Tou JS. Mechanism of heme oxygenase-1 gene activation by cadmium in MCF-7 mammary epithelial cells - Role of p38 kinase and Nrf2 transcription factor. Journal of Biological Chemistry 2000; 275: 27694-27702.

Alexandrova ML, Bochev PG, Markova VI, Bechev BG, Popova MA, Danovska MP, and Simeonova VK. Changes in phagocyte activity in patients with ischaemic stroke. Luminescence 16(6), 357-365. 2001.

Alvarez E, Leiro J, Orallo F. Effect of (-)-epigallocatechin-3-gallate on respiratory burst of rat macrophages. International Immunopharmacology 2002; 2: 849-855.

Amorino GP, Hoover RL. Interactions of monocytic cells with human endothelial cells stimulate monocytic metalloproteinase production. American Journal of Pathology 1998; 152: 199-207.

Amundson SA, Myers TG, Fornace AJ. Roles for p53 in growth arrest and apoptosis: putting on the brakes after genotoxic stress. Oncogene 1998; 17: 3287-3299.

Anderson RF, Fisher LJ, Hara Y, Harris T, Mak WB, Melton LD, Packer JE. Green tea catechins partially protect DNA from OH radical-induced strand breaks and base damage through fast chemical repair of DNA radicals. Carcinogenesis 2001; 22: 1189-1193.

Annabi B, Lachambre MP, Bousquet-Gagnon N, Page M, Gingras D, Beliveau R. Green tea polyphenol (-)-epigallocatechin 3-gallate inhibits MMP-2 secretion and MT1-MMP-driven migration in glioblastoma cells. Biochimica et Biophysica Acta-Molecular Cell Research 2002; 1542: 209-220.

Anrather J, Csizmadia V, Soares MP, Winkler H. Regulation of NF-kappa B RelA phosphorylation and transcriptional activity by p21(ras) and protein kinase C zeta in primary endothelial cells. Journal of Biological Chemistry 1999; 274: 13594-13603.

Anto RJ, Maliekal TT, Karunagaran D. L-929 cells harboring ectopically expressed RelA resist curcumin-induced apoptosis. Journal of Biological Chemistry 2000; 275: 15601-15604.

Arber N, Doki Y, Han EKH, Sgambato A, Zhou P, Kim NH, Delohery T, Klein MG, Holt PR, Weinstein IB. Antisense to cyclin D1 inhibits the growth and tumorigenicity of human colon cancer cells. Cancer Research 1997; 57: 1569-1574.

ArenzanaSeisdedos F, Turpin P, Rodriguez M, Thomas D, Hay RT, Virelizier JL, Dargemont C. Nuclear localization of I kappa B alpha promotes active transport of NF-kappa B from the nucleus to the cytoplasm. Journal of Cell Science 1997; 110: 369-378.

Attwood JT, Yung RL, and Richardson BC. DNA methylation and the regulation of gene transcription. Cellular and molecular life science 59(2), 241-257. 2002.

August DA, Landau J, Caputo D, Hong JG, Lee MJ, Yang CS. Ingestion of green tea rapidly decreases prostaglandin E-2 levels in rectal mucosa in humans. Cancer Epidemiology Biomarkers & Prevention 1999; 8: 709-713.

Azzam EI, deToledo SM, Pykett MJ, Nagasawa H, Little JB. CDC2 is down-regulated by ionizing radiation in a p53-dependent manner. Cell Growth and Differentiation 1997; 8: 1161-1169.

Babu PS, Srinivasan K. Hypolipidemic action of curcumin, the active principle of turmeric (Curcuma longa) in streptozotocin induced diabetic rats. Molecular and Cellular Biochemistry 1997; 166: 169-175.

Baeuerle PA, Henkel T. Function amd activation of NF-kappa-B in the immune-system. Annual Review of Immunology 1994; 12: 141-179.

Bailey GS, Hendricks JD, Shelton DW, Nixon JE, Pawlowski NE. Enhancement of carcinogenesis by the natural anticarcinogen indole-3-carbinol. Journal of the National Cancer Institute 1987; 78: 931-934.

Bajpai UD, Zhang KM, Teutsch M, SEN R, Wortis HH. Bruton's tyrosine kinase links the B cell receptor to nuclear factor kappa B activation. Journal of Experimental Medicine 2000; 191: 1735-1744.

Baldi L, Brown K, Franzoso G, Siebenlist U. Critical role for lysines 21 and 22 in signal-induced, ubiquitin-mediated proteolysis of I kappa B-alpha. Journal of Biological Chemistry 1996; 271: 376-379.

Baldwin AS. The NF-kappa B and I kappa B proteins: New discoveries and insights. Annual Review of Immunology 1996; 14: 649-683.

Baldwin AS, Azizkhan JC, Jensen DE, Beg AA, Coodly LR. Induction of NF-kappa-B DNA-binding activity during the G0-to- G1 transition in mouse fibroblasts. Molecular and Cellular Biology 1991; 11: 4943-4951.

Balentine DA, Wiseman SA, Bouwens LCM. The chemistry of tea flavonoids. Critical Reviews in Food Science and Nutrition 1997; 37: 693-704.

Balla G, Jacob HS, Balla J, Rosenberg M, Nath K, Apple F, Eaton JW, Vercellotti GM. Ferritin - a cytoprotective antioxidant strategem of endothelium. Journal of Biological Chemistry 1992; 267: 18148-18153.

Bargou RC, Emmerich F, Krappmann D, Bommert K, Mapara MY, Arnold W, Royer HD, Grinstein E, Greiner A, Scheidereit C, Dorken B. Constitutive nuclear factor-kappa B-RelA activation is required for proliferation and survival of Hodgkin's disease tumor cells. Journal of Clinical Investigation 1997; 100: 2961-2969.

Barthelman M, Bair WB, Stickland KK, Chen WX, Timmermann N, Valcic S, Dong ZG, Bowden GT. (-)-epigallocatechin-3-gallate inhibition of ultraviolet B induced AP-1 activity. Carcinogenesis 1998; 19: 2201-2204.

Bash J, Zong WX, Gelinas C. c-Rel arrests the proliferation of HeLa cells and affects critical regulators of the G(1)/S-phase transition. Molecular and Cellular Biology 1997; 17: 6526-6536.

Bauer I, Vollmar B, Jaeschke H, Rensing H, Kraemer T, Larsen R, Bauer M. Transcriptional activation of heme oxygenase-1 and its functional significance in acetaminophen-induced hepatitis and hepatocellular injury in the rat. Journal of Hepatology 2000; 33: 395-406.

Beg AA, Finco TS, Nantermet PV, Baldwin AS. Tumor-necrosis-factor and interleukin-1 lead to phosphorylation and loss of I-kappa-B-alpha - a mechanism for NF-kappa-B activation. Molecular and Cellular Biology 1993; 13: 3301-3310.

Beg AA, Sha WC, Bronson RT, Ghosh S, Baltimore D. Embryonic lethality and liver degeneration in mice lacking the Rela component of NF-kappa-B. Nature 1995; 376: 167-170.

Belguendouz L, Fremont L, Linard A. Resveratrol inhibits metal ion-dependent and independent peroxidation of porcine low-density lipoproteins. Biochemical Pharmacology 1997; 53: 1347-1355.

Bendeck MP, Zempo N, Clowes AW, Galardy RE, Reidy MA. Smooth-muscle cell-migration and matrix metalloproteinase expression after arterial injury in the rat. Circulation Research 1994; 75: 539-545.

Bentires-Alj M, Dejardin E, Viatour P, Van Lint C, Froesch B, Reed JC, Merville MP, Bours V. Inhibition of the NF-kappa B transcription factor increases Bax expression in cancer cell lines. Oncogene 2001; 20: 2805-2813.

Bhalla RC, Toth KF, Bhatty RA, Thompson LP, Sharma RV. Estrogen reduces proliferation and agonist-induced calcium increase in coronary artery smooth muscle cells. American Journal of Physiology-Heart and Circulatory Physiology 1997; 41: H1996-H2003.

Bhatia N, Agarwal R. Detrimental effect of cancer preventive phytochemicals silymarin, genistein and epigallocatechin 3-gallate on epigenetic events in human prostate carcinoma DU145 cells. Prostate 2001; 46: 98-107.

Bhullar IS, Li YS, Miao H, Zandi E, Kim M, Shyy JYJ, Chien S. Fluid shear stress activation of I kappa B kinase is integrin-dependent. Journal of Biological Chemistry 1998; 273: 30544-30549.

Bierhaus A, Zhang YM, Quehenberger P, Luther T, Haase M, Muller M, Mackman N, Ziegler R, Nawroth PP. The dietary pigment curcumin reduces endothelial tissue factor gene expression by inhibiting binding of AP-1 to the DNA and activation of NF-kappa B. Thrombosis and Haemostasis 1997; 77: 772-782.

Binko J, Majewski H. 17 beta-estradiol reduces vasoconstriction in endothelium-denuded rat aortas through inducible NOS. American Journal of Physiology-Heart and Circulatory Physiology 1998; 43: H853-H859.

Bird AG, McLachlan SM, Britton S. Cyclosporin-a promotes spontaneous outgrowth in vitro of Epstein-Barr virus-induced B-cell lines. Nature 1981; 289: 300-301.

Bird TA, Schooley K, Dower SK, Hagen H, Virca GD. Activation of nuclear transcription factor NF-kappa B by interleukin-1 is accompanied by casein kinase II-mediated phosphorylation of the p65 subunit. Journal of Biological Chemistry 1997; 272: 32606-32612.

Birt DF, Walker B, Tibbels MG, Bresnick E. Anti-mutagenesis and anti-promotion by apigenin, robinetin and indole-3-carbinol. Carcinogenesis 1986; 7: 959-963.

Biswas DK, Cruz AP, Gansberger E, Pardee AB. Epidermal growth factor-induced nuclear factor kappa B activation: A major pathway of cell-cycle progression in estrogen-receptor negative breast cancer cells. Proceedings of the National Academy of Sciences of the United States of America 2000; 97: 8542-8547.

Blagosklonny MV, Wu GS, Omura S, Eldeiry WS. Proteasome-dependent regulation of p21(WAF1/CIP1) expression. Biochemical and Biophysical Research Communications 1996; 227: 564-569.

Blond JP, Denis MP, Bezard J. Antioxidant action of resveratrol in lipid-peroxidation. Sciences des Aliments 1995; 15: 347-357.

BolanosGarcia VM, SorianoGarcia M, and MasOliva J. CETP and exchangeable apoproteins:common features in lipid binding activity. Molecular and cellular biochemistry 175(1-2), 1-10. 1997.

Boldin MP, Goncharov TM, Goltsev YV, Wallach D. Involvement of MACH, a novel MORT1/FADD-interacting protease, in Fas/APO-1- and TNF receptor-induced cell death. Cell 1996; 85: 803-815.

Bond M, Baker AH, Newby AC. Nuclear factor kappa B activity is essential for matrix metalloproteinase-1 and -3 upregulation in rabbit dermal fibroblasts. Biochemical and Biophysical Research Communications 1999; 264: 561-567.

Bond M, Chase AJ, Baker AH, Newby AC. Inhibition of transcription factor NF-kappa B reduces matrix metalloproteinase-1,-3 and -9 production by vascular smooth muscle cells. Cardiovascular Research 2001; 50: 556-565.

Bootcov MR, Bauskin AR, Valenzuela SM, Moore AG, Bansal M, He XY, Zhang HP, Donnellan M, Mahler S, Pryor K, Walsh BJ, Nicholson RC, Fairlie WD, Por SB, Robbins JM, Breit SN. MIC-1, a novel macrophage inhibitory cytokine, is a divergent member of the TGF-beta superfamily. Proceedings of the National Academy of Sciences of the United States of America 1997; 94: 11514-11519.

Borden P, Heller RA. Transcriptional control of matrix metalloproteinases and the tissue inhibitors of matrix metalloproteinases. Critical Reviews in Eukaryotic Gene Expression 1997; 7: 159-178.

Bosca AR, Gutierrez MAC, Soler A Puerta C, Diez A, Quintanilla E, Bernd A, Miquel J. Effects of the antioxidant turmeric on lipoprotein peroxides: Implications for the prevention of atherosclerosis. Age 1997; 20: 165-168.

Bowie AG, O'Neill LAJ. Vitamin C inhibits NF-kappa B activation by TNF via the activation of p38 mitogen-activated protein kinase. Journal of Immunology 2000; 165: 7180-7188.

Brand K, Page S, Rogler G, Bartsch A, Brandl R, Knuechel R, Page M, Kaltschmidt C, Baeuerle PA, Neumeier D. Activated transcription factor nuclear factor-kappa B is present in the atherosclerotic lesion. Journal of Clinical Investigation 1996; 97: 1715-1722.

Brand K, Page S, Walli AK, Neumeier D, Baeuerle PA. Role of nuclear factor-kappa B in atherogenesis. Experimental Physiology 1997; 82: 297-304.

British Heart Foundation. www.bhf.org.uk

Brown G, Albers JJ, Fisher LD Schaefer SM, Lin JT, Kaplan C, Zhao XQ, Bisson BD, Fitzpatrick VF, Dodge HT. Regression of coronary-artery disease as a result of intensive lipid-lowering therapy in men with high-levels of apolipoprotein-B. New England Journal of Medicine 1990; 323: 1289-1298.

Brown MA, Rudwaleit M, Pile KD, Kennedy LG, Shatford J, Amos CI, Siminovitch K, Rubin L, Calin A, Wordsworth BP. The role of germline polymorphisms in the T-cell receptor in susceptibility to ankylosing spondylitis. British Journal of Rheumatology 1998; 37: 454-458.

Brown MS, Goldstein JL. A receptor-mediated pathway for cholesterol homeostasis. Science 1986; 232: 34-47.

Bunz F, Dutriaux A, Lengauer C, Waldman T, Zhou S, Brown JP, Sedivy JM, Kinzler KW, Vogelstein B. Requirement for p53 and p21 to sustain G(2) arrest after DNA damage. Science 1998; 282: 1497-1501.

Burchett SA, Volk ML, Bannon MJ, Granneman JG. Regulators of G protein signaling: Rapid changes in mRNA abundance in response to amphetamine. Journal of Neurochemistry 1998; 70: 2216-2219.

Burke AP, Farb A, Malcom GT, Liang YH, Smialek J, Virmani R. Coronary risk factors and plaque morphology in men with coronary disease who died suddenly. New England Journal of Medicine 1997; 336: 1276-1282.

Burns TF, El Deiry WS. The p53 pathway and apoptosis. Journal of Cellular Physiology 1999; 181: 231-239.

Cahir-McFarland ED, Davidson DM, Schauer SL, Duong J, Kieff E. NF-kappa B inhibition causes spontaneous apoptosis in Epstein- Barr virus-transformed lymphoblastoid cells. Proceedings of the National Academy of Sciences of the United States of America 2000; 97: 6055-6060.

Capdevila JH, Falck JR, Harris RC. Cytochrome P450 and arachidonic acid bioactivation: molecular and functional properties of the arachidonate monooxygenase. Journal of Lipid Research 2000; 41: 163-181.

Carmeliet P, Moons L, Lijnen R, Baes M, Lemaitre V, Tipping P, Drew A, Eeckhout Y, Shapiro S, Lupu F, Collen D. Urokinase-generated plasmin activates matrix metalloproteinases during aneurysm formation. Nature Genetics 1997; 17: 439-444.

CaulinGlaser T, GarciaCardena G, Sarrel P, Sessa WC, Bender JR. 17 beta-Estradiol regulation of human endothelial cell basal nitric oxide release, independent of cytosolic Ca2+ mobilization. Circulation Research 1997; 81: 885-892.

CaulinGlaser T, Watson CA, Pardi R, Bender JR. Effects of 17 beta-estradiol on cytokine-induced endothelial cell adhesion molecule expression. Journal of Clinical Investigation 1996; 98: 36-42.

Chan H, Bartos DP, Owen-Schaub LB. Activation-dependent transcriptional regulation of the human fas promoter requires NF-kappa B p50-p65 recruitment. Molecular and Cellular Biology 1999; 19: 2098-2108.

Chan JY, Han XL, Kan YW. Cloning of NRF1, an NF-E2-related transcription factor, by genetic selection in yeast. Proceedings of the National Academy of Sciences of the United States of America 1993; 90: 11371-11375.

Chan MY. Inhibition of tumor-necrosis-factor by curcumin, a phytochemical. Biochemical Pharmacology 1995; 49: 1551-1556.

Chang BD, Broude EV, Fang J, Kalinichenko TV, Abdryashitov R, Poole JC, Roninson IB. p21(Waf1/Cip1/Sdi1)-induced growth arrest is associated with depletion of mitosis-

control proteins and leads to abnormal mitosis and endoreduplication in recovering cells. Oncogene 2000; 19: 2165-2170.

Chase AJ, Bond M, Crook MF, Newby AC. Role of nuclear factor-kappa B activation in metalloproteinase- 1,-3, and -9 secretion by human macrophages in vitro and rabbit foam cells produced in vivo. Arteriosclerosis Thrombosis and Vascular Biology 2002; 22: 765-771.

Chen CK, PaceAsciak CR. Vasorelaxing activity of resveratrol and quercetin in isolated rat aorta. General Pharmacology 1996; 27: 363-366.

Chen CL, Edelstein LC, Gelinas C. The Rel/NF-kappa B family directly activates expression of the apoptosis inhibitor Bcl-x(L). Molecular and Cellular Biology 2000; 20: 2687-2695.

Chen F, Castranova V, Shi XL. New insights into the role of nuclear factor-kappa B in cell growth regulation. American Journal of Pathology 2001; 159: 387-397.

Chen FE, Ghosh G. Regulation of DNA binding by Rel/NF-kappa B transcription factors: structural views. Oncogene 1999; 18: 6845-6852.

Chen FE, Huang DB, Chen YQ, Ghosh G. Crystal structure of p50/p65 heterodimer of transcription factor NF-kappa B bound to DNA. Nature 1998; 391: 410-413.

Chen HW, Huang HC. Effect of curcumin on cell cycle progression and apoptosis in vascular smooth muscle cells. British Journal of Pharmacology 1998; 124: 1029-1040.

Chen K, Maines MD. Nitric oxide induces heme oxygenase-1 via mitogen-activated protein kinases ERK and p38. Cellular and Molecular Biology 2000; 46: 609-617.

Chen LS, Lee MJ, Li H, Yang CS. Absorption, distribution, and elimination of tea polyphenols in rats. Drug Metabolism and Disposition 1997; 25: 1045-1050.

Chen PC, Wheeler DS, Malhotra V, Odoms K, Denenberg AG, Wong HR. A green tea-derived polyphenol, epigallocatechin-3-gallate, inhibits I kappa B kinase activation and IL-8 gene expression in respiratory epithelium. Inflammation 2002; 26: 233-241.

Chen WX, Dong ZG, Valcic S, Timmermann BN, Bowden GT. Inhibition of ultraviolet B-induced c-fos gene expression and p38 mitogen-activated protein kinase activation by (-)-

epigallocatechin gallate in a human keratinocyte cell line. Molecular Carcinogenesis 1999; 24: 79-84.

Chen XB, Bargonetti J, Prives C. P53, through P21 (WAF1/CIP1), induces cyclin D1 synthesis. Cancer Research 1995; 55: 4257-4263.

Chen YQ, Ghosh S, Ghosh G. A novel DNA recognition mode by the NF-kappa B p65 homodimer. Nature Structural Biology 1998; 5: 67-73.

Chen YR, Tan TH. Inhibition of the c-Jun N-terminal kinase (JNK) signaling pathway by curcumin. Oncogene 1998; 17: 173-178.

Chen ZJ, Hagler J, Palombella VJ, Melandri F, Scherer D, Ballard D, Maniatis T. Signal-induced site-specific phosphorylation targets I-kappa-B- alpha to the ubiquitin-proteasome pathway. Genes & Development 1995; 9: 1586-1597.

Cho S, Cho SG, Shim YS, Lee WK. Sequence analysis of the latent membrane protein 1 genes of Epstein-Barr virus isolates in Korea. Journal of Microbiology 1998; 36: 130-138.

Choi AMK, Alam J. Heme oxygenase-1: Function, regulation, and implication of a novel stress-inducible protein in oxidant-induced lung injury. American Journal of Respiratory Cell and Molecular Biology 1996; 15: 9-19.

Christou H, Morita T, Hsieh CM, Koike H, Arkonac B, Perrella MA, Kourembanas S. Prevention of hypoxia-induced pulmonary hypertension by enhancement of endogenous heme oxygenase-1 in the rat. Circulation Research 2000; 86: 1224-1229.

Chu ZL, McKinsey TA, Liu L, Gentry JJ, Malim MH, Ballard DW. Suppression of tumor necrosis factor-induced cell death by inhibitor of apoptosis c-IAP2 is under NF-kappa B control. Proceedings of the National Academy of Sciences of the United States of America 1997; 94: 10057-10062.

Chung JY, Huang CS, Meng XF, Dong ZG, Yang CS. Inhibition of activator protein 1 activity and cell growth by purified green tea and black tea polyphenols in H-ras-transformed cells: Structure-activity relationship and mechanisms involved. Cancer Research 1999; 59: 4610-4617.

Ciechanover A. The ubiquitin-proteasome proteolytic pathway. Cell 1994; 79: 13-21.

Cines DB, Pollak ES, Buck CA, Loscalzo J, Zimmerman GA, McEver RP, Pober JS, Wick TM, Konkle BA, Schwartz BS, Barnathan ES, McCrae KR, Hug BA, Schmidt AM, Stern DM. Endothelial cells in physiology and in the pathophysiology of vascular disorders. Blood 1998; 91: 3527-3561.

Clark JE, Foresti R, Sarathchandra P, Kaur H, Green CJ, Motterlini R. Heme oxygenase-1-derived bilirubin ameliorates postischemic myocardial dysfunction. American Journal of Physiology-Heart and Circulatory Physiology 2000; 278: H643-H651.

Clarke R, Daly L, Robinson K, Naughten E, Cahalane S, Fowler B, Graham I. Hyperhomocysteinemia - an independent risk factor for vascular- disease. New England Journal of Medicine 1991; 324: 1149-1155.

Clemens JA, Stephenson DT, Dixon EP, Smalstig EB, Mincy RE, Rash KS, Little S. Global cerebral ischemia activates nuclear factor-kappa B prior to evidence of DNA fragmentation. Molecular Brain Research 1997; 48: 187-196.

Cohen GM, Sun XM, Fearnhead H, MacFarlane M, Brown DG, Snowden RT, Dinsdale D. Formation of large molecular-weight fragments of DNA is a key committed step of apoptosis in thymocytes. Journal of Immunology 1994; 153: 507-516.

Collins T. Biology of disease - endothelial nuclear factor-kappa-B and the initiation of the atherosclerotic lesion. Laboratory Investigation 1993; 68: 499-508.

Collins T, Read MA, Neish AS, Whitley MZ, Thanos D, Maniatis T. Transcriptional regulation of endothelial-cell adhesion molecules - NF-kappa-B and cytokine-inducible enhancers. FASEB Journal 1995; 9: 899-909.

Corda S, Laplace C, Vicaut E, Duranteau J. Rapid reactive oxygen species production by mitochondria in endothelial cells exposed to tumor necrosis factor-alpha is mediated by ceramide. American Journal of Respiratory Cell and Molecular Biology 2001; 24: 762-768.

Cordle SR, Donald R, Read MA, Hawiger J. Lipopolysaccharide induces phosphorylation of mad3 and activation of c-rel and related NF-kappa-B proteins in human monocytic thp-1 cells. Journal of Biological Chemistry 1993; 268: 11803-11810.

Cornelis F, Hardwick L, Flipo RM, Martinez M, Lasbleiz S, Prudhomme JF, Tran TH, Walsh S, Delaye A, Nicod A, Loste MN, Lepage V, Gibson K, Pile K, Djoulah S, Danze PM, Liote F, Charron D, Weissenbach J, Kuntz D, Bardin T, Wordsworth BP. Association of rheumatoid arthritis with an amino acid allelic variation of the T cell receptor. Arthritis and Rheumatism 1997; 40: 1387-1390.

Cover CM, Hsieh SJ, Cram EJ, Hong CB, Riby JE, Bjeldanes LF, Firestone GL. Indole-3-carbinol and tamoxifen cooperate to arrest the cell cycle of MCF-7 human breast cancer cells. Cancer Research 1999; 59: 1244-1251.

Cover CM, Hsieh SJ, Tran SH, Hallden G, Kim GS, Bjeldanes LF, Firestone GL. Indole-3-carbinol inhibits the expression of cyclin-dependent kinase-6 and induces a G(1) cell cycle arrest of human breast cancer cells independent of estrogen receptor signaling. Journal of Biological Chemistry 1998; 273: 3838-3847.

Cramer P, Larson CJ, Verdine GL, Muller CW. Structure of the human NF-kappa B p52 homodimer-DNA complex at 2.1 angstrom resolution. EMBO Journal 1997; 16: 7078-7090.

Crenshaw DG, Yang J, Means AR, Kornbluth S. The mitotic peptidyl-prolyl isomerase, Pin1, interacts with Cdc25 and Plx1. EMBO Journal 1998; 17: 1315-1327.

Cressman DE, Greenbaum LE, Haber BA, Taub R. Rapid activation of post-hepatectomy factor nuclear factor kappa-B in hepatocytes, a primary response in the regenerating liver. Journal of Biological Chemistry 1994; 269: 30429-30435.

Crouvezier S, Powell B, Keir D, Yaqoob P. The effects of phenolic components of tea on the production of pro- and anti-inflammatory cytokines by human leukocytes in vitro. Cytokine 2001; 13: 280-286.

Cybulsky MI, Gimbrone MA. Endothelial expression of a mononuclear leukocyte adhesion molecule during atherogenesis. Science 1991; 251: 788-791.

Das NP. Studies on flavonoid metabolism. Absorption and metabolism of (+)-catechin in man. Biochemical Pharmacology 20(12), 3435-3445. 1971.

Dashwood WM, Orner GA, Dashwood RH. Inhibition of beta-catenin/Tcf activity by white tea, green tea, and epigallocatechin-3-gallate (EGCG): minor contribution of H_2O_2 at

physiologically relevant EGCG concentrations. Biochemical and Biophysical Research Communications 2002; 296: 584-588.

Davies MJ. Apoptosis in cardiovascular disease. Heart 1997; 77: 498-501

Davies MJ, Thomas A. Thrombosis and acute coronary-artery lesions in sudden cardiac ischemic death. New England Journal of Medicine 1984; 310: 1137-1140.

De Laurenzi V, Melino G. Apoptosis - The little devil of death. Nature 2000; 406: 135-136.

Dedhar S. Cell-substrate interactions and signalling through ILK. Current Opinion in Cell Biology 12, 250-256. 2000.

Dejardin E, Bonizzi G, Bellahcene A, Castronovo V, Merville MP, Bours V. Highly-expressed p100/p52 (NFKB2) sequesters other NF-kappa-B-related proteins in the cytoplasm of human breast-cancer cells. Oncogene 1995; 11: 1835-1841.

Dejardin E, Deregowski V, Chapelier M, Jacobs N, Gielen J, Merville MP, Bours V. Regulation of NF-kappa B activity by I kappa B-related proteins in adenocarcinoma cells. Oncogene 1999; 18: 2567-2577.

DeMeester SL, Buchman TG, Qiu YY, Dunnigan K, Hotchkiss RS, Karl IE, Cobb JP. Pyrrolidine dithiocarbamate activates the heat shock response and thereby induces apoptosis in primed endothelial cells. Shock 1998; 10: 1-6.

Demeule M, Brossard M, Page M, Gingras D, Beliveau R. Matrix metalloproteinase inhibition by green tea catechins. Biochimica et Biophysica Acta-Protein Structure and Molecular Enzymology 2000; 1478: 51-60.

Demrow HS, Slane PR, Folts JD. Administration of wine and grape juice inhibits in-vivo platelet activity and thrombosis in stenosed canine coronary- arteries. Circulation 1995; 91: 1182-1188.

Deng ZM, Morse JH, Slager SL, Cuervo N, Moore KJ, Venetos G, Kalachikov S, Cayanis E, Fischer SG, Barst RJ, Hodge SE, Knowles JA. Familial primary pulmonary hypertension (gene PPH1) is caused by mutations in the bone morphogenetic protein receptor-II gene. American Journal of Human Genetics 2000; 67: 737-744.

DiDonato J, Mercurio F, Rosette C, WuLi J, Suyang H, Ghosh S, Karin M. Mapping of the inducible I kappa B phosphorylation sites that signal its ubiquitination and degradation. Molecular and Cellular Biology 1996; 16: 1295-1304.

DiDonato JA, Hayakawa M, Rothwarf DM, Zandi E, Karin M. A cytokine-responsive I kappa B kinase that activates the transcription factor NF-kappa B. Nature 1997; 388: 548-554.

DiDonato JA, Mercurio F, Karin M. phosphorylation of I-kappa-b-alpha precedes but is not sufficient for its dissociation from NF-kappa-B. Molecular and Cellular Biology 1995; 15: 1302-1311.

Dietrich C, Bartsch T, Schanz F, Oesch F, Wieser RJ. p53-dependent cell cycle arrest induced by N-acetyl-L-leucinyl-L-leucinyl-L-norleucinal in platelet-derived growth factor-stimulated human fibroblasts. Proceedings of the National Academy of Sciences of the United States of America 1996; 93: 10815-10819.

Dimmeler S, Lottspeich F, Brune B. Nitric-oxide causes ADP-ribosylation and inhibition of glyceraldehyde-3-phosphate dehydrogenase. Journal of Biological Chemistry 1992; 267: 16771-16774.

Doi TS, Marino MW, Takahashi T, Yoshida T, Sakakura T, Old LJ, Obata Y. Absence of tumor necrosis factor rescues RelA-deficient mice from embryonic lethality. Proceedings of the National Academy of Sciences of the United States of America 1999; 96: 2994-2999.

Dollery CM, McEwan JR, Henney AM. Matrix metalloproteinases and cardiovascular-disease. Circulation Research 1995; 77: 863-868.

Dominguez I, Sanz L, ArenzanaSeisdedos F, Diazmeco MT, Virelizier JL, Moscat J. Inhibition of protein-kinase-c zeta-subspecies blocks the activation of an NF-kappa-B-like activity in xenopus-laevis oocytes. Molecular and Cellular Biology 1993; 13: 1290-1295.

Dong ZG, Ma WY, Huang CS, Yang CS. Inhibition of tumor promoter-induced activator protein 1 activation and cell transformation by tea polyphenols, (-)- epigallocatechin gallate, and theaflavins. Cancer Research 1997; 57: 4414-4419.

Dore S, Takahashi M, Ferris CD, Hester LD, Guastella D, Snyder SH. Bilirubin, formed by activation of heme oxygenase-2, protects neurons against oxidative stress injury. Proceedings of the National Academy of Sciences of the United States of America 1999; 96: 2445-2450.

Du CY, Fang M, Li YC, Li L, Wang XD. Smac, a mitochondrial protein that promotes cytochrome c- dependent caspase activation by eliminating IAP inhibition. Cell 2000; 102: 33-42.

Du XL, Stockklauser-Farber K, Rosen P. Generation of reactive oxygen intermediates, activation of NF-Kappa B, and induction of apoptosis in human endothelial cells by glucose: Role of nitric oxide synthase? Free Radical Biology and Medicine 1999; 27: 752-763.

Duckers HJ, Boehm M, True AL, Yet S-F, San H, Park JL, Webb RC, Lee M-E, Nabel GJ, and Nabel EG. Heme oxygenase-1 protects against vascular constriction and proliferation. Nature Medicine 7,693-698. 2001.

Duckett CS, Perkins ND, Leung K, AgranofF AB, Nabel GJ. Cytokine induction of nuclear factor kappa-b in cycling and growth-arrested cells - evidence for cell cycle-independent activation. Journal of Biological Chemistry 1995; 270: 18836-18840.

Duell PB, Malinow MR. Homocyst(e)ine: An important risk factor for atherosclerotic vascular disease. Current Opinion in Lipidology 1997; 8: 28-34.

Duggan DJ, Bittner M, Chen YD, Meltzer P, Trent JM. Expression profiling using cDNA microarrays. Nature Genetics 1999; 21: 10-14.

Dzau VJ, Braun-Dullaeus RC, Sedding DG. Vascular proliferation and atherosclerosis: New perspectives and therapeutic strategies. Nature Medicine 2002; 8: 1249-1256

Economou E, Tousoulis D, Katinioti A, Stefanadis C, Trikas A, Pitsavos C, Tentolouris C, Toutouza MG, Toutouzas P. Chemokines in patients with ischaemic heart disease and the effect of coronary angioplasty. International Journal of Cardiology 2001; 80: 55-60.

Eisen MB, Spellman PT, Brown PO, Botstein D. Cluster analysis and display of genome-wide expression patterns. Proceedings of the National Academy of Sciences of the United States of America 1998; 95: 14863-14868.

El Deiry WS. p21/p53, cellular growth control and genomic integrity. Cyclin Dependent Kinase (Cdk) Inhibitors 1998a; 227: 121-137.

El Deiry WS. The p53 pathway and cancer therapy. Cancer Journal 1998b; 11: 229-236.

El-Deiry WS, Harper JW, O'connor PM, Velculescu VE, Canman CE, Jackman J, Pietenpol JA, Burrell M, Hill DE, Wang YS, Wiman KG, Mercer WE, Kastan MB, Kohn KW, Elledge SJ, Kinzler KW, Vogelstein B. WAF1/CIP1 is induced in p53-mediated G(1) arrest and apoptosis. Cancer Research 1994; 54: 1169-1174.

El Mowafy AM, White RE. Resveratrol inhibits MAPK activity and nuclear translocation in coronary artery smooth muscle: reversal of endothelin-1 stimulatory effects. Febs Letters 1999; 451: 63-67.

Eliopoulos AG, Blake SMS, Floettmann JE, Rowe M, Young LS. Epstein-Barr virus-encoded latent membrane protein 1 activates the JNK pathway through its extreme C terminus via a mechanism involving TRADD and TRAF2. Journal of Virology 1999; 73: 1023-1035.

Ellmeier W, Jung S, Sunshine MJ, Hatam F, Xu Y, Baltimore D, Mano H, Littman DR. Severe B cell deficiency in mice lacking the Tec kinase family members Tec and Btk. Journal of Experimental Medicine 2000; 192: 1611-1623.

Faia KL, Davis WP, Marone AJ, Foxall TL. Matrix metalloproteinases and tissue inhibitors of metalloproteinases in hamster aortic atherosclerosis: correlation with in-situ zymography. Atherosclerosis 2002; 160: 325-337.

Falk E, Shah PK, Fuster V. Coronary plaque disruption. Circulation 1995; 92: 657-671.

Favreau LV, Pickett CB. Transcriptional regulation of the rat NAD(P)H-quinone reductase gene - identification of regulatory elements controlling basal level expression and inducible expression by planar aromatic compounds and phenolic antioxidants. Journal of Biological Chemistry 1991; 266: 4556-4561.

Feng Q, Kumagai T, Torii Y, Nakamura Y, Osawa T, Uchida K. Anticarcinogenic antioxidants as inhibitors against intracellular oxidative stress. Free Radical Research 2001; 35: 779-788.

Fernandes-Alnemri T, Armstrong RC, Krebs J, Srinivasula SM, Wang L, Bullrich F, Fritz LC, Trapani JA, Tomaselli KJ, Litwack G, Alnemri ES. In vitro activation of CPP32 and Mch3 by Mch4, a novel human apoptotic cysteine protease containing two FADD-like domains. Proceedings of the National Academy of Sciences of the United States of America 1996; 93: 7464-7469.

Ferrara N, DavisSmyth T. The biology of vascular endothelial growth factor. Endocrine Reviews 1997; 18: 4-25.

Ferrero ME, Bertelli AAE, Fulgenzi A, Pellegatta F, Corsi MM, Bonfrate M, Ferrara F, De Caterina R, Giovannini L, Bertelli A. Activity in vitro of resveratrol on granulocyte and monocyte adhesion to endothelium. American Journal of Clinical Nutrition 1998; 68: 1208-1214.

Feuillard J, Schuhmacher M, Kohanna S, Asso-Bonnet M, Ledeur F, Joubert-Caron R, Bissieres P, Polack A, Bornkamm GW, Raphael M. Inducible loss of NF-kappa B activity is associated with apoptosis and Bcl-2 down-regulation in Epstein-Barr virus- transformed B lymphocytes. Blood 2000; 95: 2068-2075.

Fields ER, Seufzer BJ, Oltz EM, Miyamoto S. A switch in distinct I kappa B alpha degradation mechanisms mediates constitutive NF-kappa B activation in mature B cells. Journal of Immunology 2000; 164: 4762-4767.

Fleming I. Cytochrome P450 enzymes in vascular homeostasis. Circulation Research 2001; 89: 753-762.

Folgueira L, McElhinny JA, Bren GD, MacMorran WS, DiazMeco MT, Moscat J, Paya CV. Protein kinase C-zeta mediates NF-kappa B activation in human immunodeficiency virus-infected monocytes. Journal of Virology 1996; 70: 223-231.

Forough R, Koyama N, Hasenstab D, Lea H, Clowes M, Nikkari ST, Clowes AW. Overexpression of tissue inhibitor of matrix metalloproteinase- 1 inhibits vascular smooth muscle cell functions in vitro and in vivo. Circulation Research 1996; 79: 812-820.

Forsburg SL, Nurse P. Cell-cycle regulation in the yeasts Saccharomyces cerevisiae and Schizosaccharomyces pombe. Annual Review of Cell Biology 1991; 7: 227-256.

Frankel EN, Kanner J, German JB, Parks E, Kinsella JE. Inhibition of oxidation of human low-density-lipoprotein by phenolic substances in red wine. Lancet 1993; 341: 454-457.

Francis DA, Sen R, Rice N, Rothstein TL. Receptor-specific induction of NF-κB components in primary B cells. International Immunology 1998; 10: 285-293.

Freay AD, Curtis SW, Korach KS, Rubanyi GM. Mechanism of vascular smooth muscle relaxation by estrogen in depolarized rat and mouse aorta - Role of nuclear estrogen receptor and Ca2+ uptake. Circulation Research 1997; 81: 242-248.

Freeman RS, Estus S, Johnson EM. Analysis of cell cycle-related gene-expression in postmitotic neurons - selective induction of cyclin D1 during programmed cell-death. Neuron 1994; 12: 343-355.

Fremont L, Belguendouz L, Delpal S. Antioxidant activity of resveratrol and alcohol-free wine polyphenols related to LDL oxidation and polyunsaturated fatty acids. Life Sciences 1999; 64: 2511-2521.

Frick MH, Elo O, Haapa K, Heinonen OP, Heinsalmi P, Helo P, Huttunen JK, Kaitaniemi P, Koskinen P, Manninen V, Maenpaa H, Malkonen M, Manttari M, Norola S, Pasternack A, Pikkarainen J, Romo M, Sjoblom T, Nikkila EA. Helsinki heart-study - primary-prevention trial with gemfibrozil in middle-aged men with dyslipidemia - safety of treatment, changes in risk-factors, and incidence of coronary heart-disease. New England Journal of Medicine 1987; 317: 1237-1245.

Frost V, Delikat S, Al Mehairi S, Sinclair AJ. Regulation of p27(KIP1) in Epstein-Barr virus-immortalized lymphoblastoid cell lines involves non-apoptotic caspase cleavage. Journal of General Virology 2001; 82: 3057-3066.

Furukawa Y, Piwnicaworms H, Ernst TJ, Kanakura Y, Griffin JD. CDC2 gene-expression at the G1-transition to S-transition in human T-lymphocytes. Science 1990; 250: 805-808.

Galis ZS, Khatri JJ. Matrix metalloproteinases in vascular remodeling and atherogenesis - The good, the bad, and the ugly. Circulation Research 2002; 90: 251-262.

Galis ZS, Sukhova GK, Lark MW, Libby P. Increased expression of matrix metalloproteinases and matrix-degrading activity in vulnerable regions of human atherosclerotic plaques. Journal of Clinical Investigation 1994; 94: 2493-2503

Galis ZS, Sukhova GK, Kranzhofer R, Clark S, Libby P. Macrophage foam cells from experimental atheroma constitutively produce matrix-degrading proteinases. Proceedings of the National Academy of Sciences of the United States of America 1995; 92: 402-406.

Gao XH, Xu YX, Janakiraman N, Chapman RA, Gautam SC. Immunomodulatory activity of resveratrol: suppression of lymphocyte proliferation, development of cell-mediated cytotoxicity, and cytokine production. Biochemical Pharmacology 2001; 62: 1299-1308.

Garbers DL, Dubois SK. The molecular basis of hypertension. Annual Review of Biochemistry 1999; 68: 127-155.

Gaziano JM, Buring JE, Breslow JL, Goldhaber SZ, Rosner B, Vandenburgh M, Willett W, Hennekens CH. Moderate alcohol intake, increased levels of high-density-lipoprotein and its subfractions, and decreased risk of myocardial-infarction. New England Journal of Medicine 1993; 329: 1829-1834.

Geng YJ. Molecular signal transduction in vascular cell apoptosis. Cell Research 2001; 11: 253-264.

George SJ. Therapeutic potential of matrix metalloproteinase inhibitors in atherosclerosis. Expert Opinion on Investigational Drugs 2000; 9: 993-1007.

Gey KF, Brubacher GB, Stahelin HB. Plasma-levels of antioxidant vitamins in relation to ischemic-heart disease and cancer. American Journal of Clinical Nutrition 1987a; 45: 1368-1377.

Gey KF, Puska P. Plasma vitamin-E and vitamin-A inversely correlated to mortality from ischemic-heart-disease in cross-cultural epidemiology. Annals of the New York Academy of Sciences 1989; 570: 268-282.

Gey KF, Stahelin HB, Puska P, Evans A. Relationship of plasma-level of vitamin-C to mortality from ischemic-heart-disease. Annals of the New York Academy of Sciences 1987b; 498: 110-123.

Ghosh G, Vanduyne G, Ghosh S, Sigler PB. Structure of NF-kappa-B p50 homodimer bound to a kappa-B site. Nature 1995; 373: 303-310.

Ghosh S, May MJ, Kopp EB. NF-kappa B and rel proteins: Evolutionarily conserved mediators of immune responses. Annual Review of Immunology 1998; 16: 225-260.

Gierse JK, Hauser SD, Creely DP, Koboldt C, Rangwala SH, Isakson PC, Seibert K. Expression and selective-inhibition of the constitutive and inducible forms of human cyclooxygenase. Biochemical Journal 1995; 305: 479-484.

Gilchrist R, Lomax ME, Camplejohn RS. The need for dynamic methods for measuring cell cycle perturbations: a study in radiation-treated lymphoblastoid cell lines of varying p53 status. Cell Proliferation 1999; 32: 15-24.

Gonen H, Shkedy D, Barnoy S, Kosower NS, Ciechanover A. On the involvement of calpains in the degradation of the tumor suppressor protein p53. Febs Letters 1997; 406: 17-22.

Gong PF, Hu B, Stewart D, Ellerbe M, Figueroa YG, Blank V, Beckman BS, Alam J. Cobalt induces heme oxygenase-1 expression by a hypoxia-inducible factor-independent mechanism in Chinese hamster ovary cells - Regulation by Nrf2 and MafG transcription factors. Journal of Biological Chemistry 2001; 276: 27018-27025.

Gonzalez W, Fontaine V, Pueyo ME, Laquay N, Messika-Zeitoun D, Philippe M, Arnal JF, Jacob MP, Michel JB. Molecular plasticity of vascular wall during N-G-nitro-L-arginine methyl ester-induced hypertension - Modulation of proinflammatory signals. Hypertension 2000; 36: 103-109.

Goodwin B, Jones SA, Price RR, Watson MA, McKee DD, Moore LB, Galardi C, Wilson JG, Lewis MC, Roth ME, Maloney PR, Willson TM, Kliewer SA. A regulatory cascade of the nuclear receptors FXR, SHP-1, and LRH-1 represses bile acid biosynthesis. Molecular Cell 2000; 6: 517-526.

Gopinathan V, Miller NJ, Milner AD, Riceevans CA. Bilirubin and ascorbate antioxidant activity in neonatal plasma. Febs Letters 1994; 349: 197-200.

Graham HN. Green tea composition, consumption, and polyphenol chemistry. Preventive Medicine 1992; 21: 334-350.

Graves PR, Yu LJ, Schwarz JK, Gales J, Sausville EA, O'Connor PM, Piwnica-Worms H. The Chk1 protein kinase and the Cdc25C regulatory pathways are targets of the anticancer agent UCN-01. Journal of Biological Chemistry 2000; 275: 5600-5605.

Green DR, Reed JC. Mitochondria and apoptosis. Science 1998; 281: 1309-1312.

Grilli M, Pizzi M, Memo M, Spano P. Neuroprotection by aspirin and sodium salicylate through blockade of NF-kappa B activation. Science 1996; 274: 1383-1385.

Grimm S, Bauer MKA, Baeuerle PA, SchulzeOsthoff K. Bcl-2 down-regulates the activity of transcription factor NF- kappa B induced upon apoptosis. Journal of Cell Biology 1996; 134: 13-23.

Grossman SR, Johannsen E, Tong X, Yalamanchili R, Kieff E. The Epstein-Barr-virus nuclear antigen-2 transactivator is directed to response elements by the j-kappa recombination signal binding-protein. Proceedings of the National Academy of Sciences of the United States of America 1994; 91: 7568-7572.

Grumont RJ, Gerondakis S. The subunit composition of NF-kappa-B complexes changes during B-cell development. Cell Growth & Differentiation 1994; 5: 1321-1331.

Grumont RJ, Rourke IJ, Gerondakis S. Rel-dependent induction of A1 transcription is required to protect B cells from antigen receptor ligation-induced apoptosis. Genes & Development 1999; 13: 400-411.

Grumont RJ, Rouke IJ, O'Reilly LA, Strasser A, Miyake K, Sha W, Gerondakis S. B lymphocytes differentially use the Rel and nuclear kappa B1 (NF-kappa B1) transcription factors to regulate cell cycle progression and apoptosis in quiescent and mitogen-activated cells. 1998; 187: 663-674.

Grutter MG. Caspases: key players in programmed cell death. Current Opinion in Structural Biology 2000; 10: 649-655.

Gu ZM, Flemington C, Chittenden T, Zambetti GP. ei24, a p53 response gene involved in growth suppression and apoptosis. Molecular and Cellular Biology 2000; 20: 233-241.

Guevara NV, Kim HS, Antonova EI, Chan L. The absence of p53 accelerates atherosclerosis by increasing cell proliferation in vivo. Nature Medicine 1999; 5: 335-339.

Gupta S, Ahmad N, Nieminen AL, Mukhtar H. Growth inhibition, cell-cycle dysregulation, and induction of apoptosis by green tea constituent (-)-epigallocatechin-3-gallate in androgen-sensitive and androgen-insensitive human prostate carcinoma cells. Toxicology and Applied Pharmacology 2000; 164: 82-90.

Guttridge DC, Albanese C, Reuther JY, Pestell RG, Baldwin AS. NF-kappa B controls cell growth and differentiation through transcriptional regulation of cyclin D1. Molecular and Cellular Biology 1999; 19: 5785-5799.

Hackett AM, Griffiths LA, Broillet A, Wermeille M. The metabolism and excretion of (+)-[C-14] cyanidanol-3 in man following oral-administration. Xenobiotica 1983; 13: 279-286.

Hajra L, Evans AI, Chen M, Hyduk SJ, Collins T, Cybulsky MI. The NF-kappa B signal transduction pathway in aortic endothelial cells is primed for activation in regions predisposed to atherosclerotic lesion formation. Proceedings of the National Academy of Sciences of the United States of America 2000; 97: 9052-9057.

Hammarskjold ML, Simurda MC. Epstein-Barr-Virus latent membrane-protein transactivates the human-immunodeficiency-virus type-1 long terminal repeat through induction of NF-kappa-B activity. Journal of Virology 1992; 66: 6496-6501.

Han EKH, Ng SC, Arber N, Begemann M, Weinstein IB. Roles of cyclin D1 and related genes in growth inhibition, senescence and apoptosis. Apoptosis 1999; 4: 213-219.

Haqqi TM, Anthony DD, Gupta S, Ahmad N, Lee MS, Kumar GK, Mukhtar H. Prevention of collagen-induced arthritis in mice by a polyphenolic fraction from green tea. Proceedings of the National Academy of Sciences of the United States of America 1999; 96: 4524-4529.

Harper JW, Adami GR, Wei N, Keyomarsi K, Elledge SJ. The p21 CDK-interacting protein CIP1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell 1993; 75: 805-816.

Hartsfield CL, Alam J, Choi AMK. Transcriptional regulation of the heme oxygenase 1 gene by pyrrolidine dithiocarbamate. FASEB Journal 1998; 12: 1675-1682.

Hayes JD, Pulford DJ. The glutathione S-Transferase supergene family: Regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. Critical Reviews in Biochemistry and Molecular Biology. 1995; 30: 445-600.

Huang DY, Prystowsky MB. Identification of an essential cis-element near the transcription start site for transcriptional activation of the proliferating cell nuclear antigen gene. Journal of Biological Chemistry 1996; 271: 1218-1225.

Huang HC, Nguyen T, and Pickett CB. Regulation of the antioxidant response element by protein kinase C-mediated phosphorylation of NF-E2 related factor 2. Proceedings of the National Academy of Sciences of the United States of America 97(23), 12475-12480. 2000.

Heinonen OP, Huttunen JK, Albanes D, Haapakoski J, Palmgren J, Pietinen P, Pikkarainen J, Rautalahti M *et al.* Effect of vitamin-E and beta-carotene on the incidence of lung-cancer and other cancers in male smokers. New England Journal of Medicine 1994; 330: 1029-1035.

Heitzer T, Wenzel U, Hink U, Krollner D, Skatchkov M, Stahl RAK, Macharzina R, Brasen JH, Meinertz T, Munzel T. Increased NAD(P)H oxidase-mediated superoxide production in renovascular hypertension: Evidence for an involvement of protein kinase C. Kidney International 1999; 55: 252-260.

Heldin CH, Westermark B. Mechanism of action and in vivo role of platelet-derived growth factor. Physiological Reviews 1999; 79: 1283-1316.

Hengartner MO. The biochemistry of apoptosis. Nature 2000; 407: 770-776.

Henle W, Diehl V Kohn G Zur Hausen H Henle G. Herpes-type virus and chromosome marker in normal leukocytes after growth with irradiated Burkitt cells. Science 1967 157(792), 1064-1065.

Hennig B, Toborek M. Nutrition and endothelial cell function: implications in atherosclerosis. Nutrition Research 2001; 21: 279-293.

Heximer SP, Cristillo AD, Forsdyke DR. Comparison of mRNA expression of two regulators of G-protein signaling, RGS1/BL34/1R20 and RGS2/G0S8, in cultured human blood mononuclear cells. DNA and Cell Biology 1997; 16: 589-598.

Hill-Kapturczak N, Thamilselvan V, Liu FY, Nick HS, Agarwal A. Mechanism of heme oxygenase-1 gene induction by curcumin in human renal proximal tubule cells. American Journal of Physiology-Renal Physiology 2001; 281: F851-F859.

Hinz M, Krappmann D, Eichten A, Heder A, Scheidereit C, Strauss M. NF-kappa B function in growth control: Regulation of cyclin D1 expression and G(0)G(1)-to-S-Phase transition. Molecular and Cellular Biology 1999; 19: 2690-2698.

Hiscott J, Marois J, Garoufalis J, Daddario M, Roulston A, Kwan I, Pepin N, Lacoste J, Nguyen H, Bensi G, Fenton M. Characterization of a functional NF-kappa-B site in the human interleukin-1-beta promoter - evidence for a positive autoregulatory loop. Molecular and Cellular Biology 1993; 13: 6231-6240.

Hiyama H, Reeves SA. Role for cyclin D1 in UVC-induced and p53-mediated apoptosis. Cell Death and Differentiation 1999; 6: 565-569.

Hjermann I, Holme I, Byre KV, Leren P. Effect of diet and smoking intervention on the incidence of coronary heart-disease - report from the Oslo study-group of a randomized trial in healthy-men. Lancet 1981; 2: 1303-1310.

Hodges LC, Kearse EC, Green K. Intraocular pressure-lowering activity of phenolic antioxidants in normotensive rabbits. Current Eye Research 1999; 19: 234-240.

Hodgson JM, Puddey IB, Burke V, Beilin LJ, Jordan N. Effects on blood pressure of drinking green and black tea. Journal of Hypertension 1999; 17: 457-463.

Hoetzel A, Vagts DA, Loop T, Humar M, Bauer M, Pahl HL, Geiger KK, Pannen BHJ. Effect of nitric oxide on shock-induced hepatic heme oxygenase- 1 expression in the rat. Hepatology 2001; 33: 925-937.

Hofbauer R, Frass M, Gmeiner B, Handler S, Speiser W, Kapiotis S. The green tea extract epigallocatechin gallate is able to reduce neutrophil transmigration through monolayers of endothelial cells. Wiener Klinische Wochenschrift 1999; 111: 278-282.

Hofer-Warbinek R, Schmid JA, Stehlik C, Binder BR, Lipp J, de Martin R. Activation of NF-kappa B by XIAP, the X chromosome-linked inhibitor of apoptosis, in endothelial cells involves TAK1. Journal of Biological Chemistry 2000; 275: 22064-22068.

Hollman PCH, Tijburg LBM, Yang CS. Bioavailability of flavonoids from tea. Critical Reviews in Food Science and Nutrition 1997; 37: 719-738.

Holtrich U, Wolf G, Brauninger A, Karn T, Bohme B, Rubsamenwaigmann H, Strebhardt K. Induction and down-regulation of plk, a human serine/threonine kinase expressed in proliferating cells and tumors. Proceedings of the National Academy of Sciences of the United States of America 1994; 91: 1736-1740.

Honeck H, Gross V, Erdmann B, Kargel E, Neunaber R, Milia AF, Schneider W, Luft FC, Schunck WH. Cytochrome P450-dependent renal arachidonic acid metabolism in desoxycorticosterone acetate-salt hypertensive mice. Hypertension 2000; 36: 610-616.

Hornstra G, Barth CA, Galli C, Mensink RP, Mutanen M, Riemersma RA, Roberfroid M, Salminen K, Vansant G, Verschuren PM. Functional food science and the cardiovascular system. British Journal of Nutrition 1998; 80: S113-S146.

Howells LM, Gallacher-Horley B, Houghton CE, Manson MM, Hudson EA. Indole-3-carbinol inhibits protein kinase B/Akt and induces apoptosis in the human breast tumor cell line MDA MB468 but not in the nontumorigenic HBL100 line. Molecular Cancer Therapeutics 2002; 1: 1161-1172.

Hsieh CM, Yoshizumi M, Endege WO, Kho CJ, Jain MK, Kashiki S, delosSantos R, Lee WS, Perrella MA, Lee ME. APEG-1, a novel gene preferentially expressed in aortic smooth muscle cells, is down-regulated by vascular injury. Journal of Biological Chemistry 1996; 271: 17354-17359.

Hsieh TC, Juan G, Darzynkiewicz Z, Wu JM. Resveratrol increases nitric oxide synthase, induces accumulation of p53 and p21(WAF1/CIP1) and suppresses cultured bovine pulmonary artery endothelial cell proliferation by perturbing progression through S and G(2). Cancer Research 1999; 59: 2596-2601.

Hsu HL, Xiong J, Goeddel DV. The TNF receptor 1-associated protein TRADD signals cell-death and NF-kappa-B activation. Cell 1995; 81: 495-504.

Hsu TC, Zhao YJ, Wang RY, Wu YF, Spitz MR, Hong WK. Comparative efficacy as antioxidants between ascorbic acid and epigallocatechin gallate on cells of two human lymphoblastoid lines. Cancer Genetics and Cytogenetics 2001; 124: 169-171.

Huang CS, Ma WY, Goranson A, Dong ZG. Resveratrol suppresses cell transformation and induces apoptosis through a p53-dependent pathway. Carcinogenesis 1999; 20: 237-242.

Huen DS, Henderson SA, Croomcarter D, Rowe M. The Epstein-Barr-Virus latent membrane protein-1 (LMP1) mediates activation of NF-kappa-B and cell-surface phenotype via 2 effector regions in its carboxy-terminal cytoplasmic domain. Oncogene 1995; 10: 549-560.

Hutchins JRA, Hughes M, Clarke PR. Substrate specificity determinants of the checkpoint protein kinase Chk1. Febs Letters 2000; 466: 91-95.

Iademarco MF, McQuillan JJ, Rosen GD, Dean DC. Characterization of the promoter for vascular cell-adhesion molecule-1 (VCAM-1). Journal of Biological Chemistry 1992; 267: 16323-16329.

Ihling C, Menzel G, Wellens E, Monting JS, Schaefer HE, Zeiher AM. Topographical association between the cyclin-dependent kinases inhibitor p21, p53 accumulation, and cellular proliferation in human atherosclerotic tissue. Arteriosclerosis Thrombosis and Vascular Biology 1997; 17: 2218-2224.

Ikigai H, Nakae T, Hara Y, Shimamura T. Bactericical catechins damage the lipid bilayer. Biochimica et Biophysica Acta 1993; 1147: 132-136

Imai K, Nakachi K. Cross-sectional study of effects of drinking green tea on cardiovascular and liver-diseases. British Medical Journal 1995; 310: 693-696.

Imbert V, Rupec RA, Livolsi A, Pahl HL, Traenckner EBM, MuellerDieckmann C, Farahifar D, Rossi B, Auberger P, Baeuerle PA, Peyron JF. Tyrosine phosphorylation of I kappa B-alpha activates NF-kappa B without proteolytic degradation of I kappa B-alpha. Cell 1996; 86: 787-798.

Inadera H, Sekiya T, Yoshimura T, Matsushima K. Molecular analysis of the inhibition of monocyte chemoattractant protein-1 gene expression by estrogens and xenoestrogens in MCF-7 cells. Endocrinology 2000; 141: 50-59.

Ingi T, Krumins AM, Chidiac P, Brothers GM, Chung S, Snow BE, Barnes CA, Lanahan AA, Siderovski DP, Ross EM, Gilman AG, Worley PF. Dynamic regulation of RGS2 suggests a novel mechanism in G- protein signaling and neuronal plasticity. Journal of Neuroscience 1998; 18: 7178-7188.

Ishikawa Y, Sugiyama H, Stylianou E, Kitamura M. Bioflavonoid quercetin inhibits interleukin-1-induced transcriptional expression of monocyte chemoattractant protein- 1 in glomerular cells via suppression of nuclear factor-kappa B. Journal of the American Society of Nephrology 1999; 10: 2290-2296.

Izumi KM, Kaye KM, Kieff ED. The Epstein-Barr virus LMP1 amino acid sequence that engages tumor necrosis factor receptor associated factors is critical for primary B lymphocyte growth transformation. Proceedings of the National Academy of Sciences of the United States of America 1997; 94: 1447-1452.

Izumi KM, Kieff ED. The Epstein-Barr virus oncogene product latent membrane protein 1 engages the tumor necrosis factor receptor-associated death domain protein to mediate B lymphocyte growth transformation and activate NF-kappa B. Proceedings of the National Academy of Sciences of the United States of America 1997; 94: 12592-12597.

Izumi KM, McFarland EC, Ting AT, Riley EA, Seed B, Kieff ED. The Epstein-Barr virus oncoprotein latent membrane protein 1 engages the tumor necrosis factor receptor-associated proteins TRADD and receptor-interacting protein (RIP) but does not induce apoptosis or require RIP for NF-kappa B activation. Molecular and Cellular Biology 1999; 19: 5759-5767.

Jiang CW, Sarrel PM, Lindsay DC, Poolewilson PA, Collins P. Endothelium-independent relaxation of rabbit coronary-artery by 17-beta-estradiol invitro. British Journal of Pharmacology 1991; 104: 1033-1037.

Jiang MC, YangYen HF, Yen JJY, Lin JK. Curcumin induces apoptosis in immortalized NIH 3T3 and malignant cancer cell lines. Nutrition and Cancer-An International Journal 1996; 26: 111-120.

Jimenez GS, Khan SH, Stommel JM, Wahl GM. p53 regulation by post-translational modification and nuclear retention in response to diverse stresses. Oncogene 1999; 18: 7656-7665.

Jitoe A, Masuda T, Tengah IGP, Suprapta DN, Gara IW, Nakatani N. Antioxidant activity of tropical ginger extracts and analysis of the contained curcuminoids. Journal of Agricultural and Food Chemistry 1992; 40: 1337-1340.

Jobin C, Bradham CA, Russo MP, Juma B, Narula AS, Brenner DA, Sartor RB. Curcumin blocks cytokine-mediated NF-kappa B activation and proinflammatory gene expression by inhibiting inhibitory factor I-kappa B kinase activity. Journal of Immunology 1999; 163: 3474-3483.

Joyce D, Bouzahzah B, Fu MF, Albanese C, D'Amico M, Steer J, Klein JU, Lee RJ, Segall JE, Westwick JK, Der CJ, Pestell RG. Integration of Rac-dependent regulation of cyclin D1 transcription through a nuclear factor-kappa B-dependent pathway. Journal of Biological Chemistry 1999; 274: 25245-25249.

Kacimi R, Chentoufi J, Honbo N, Long CS, Karliner JS. Hypoxia differentially regulates stress proteins in cultured cardiomyocytes: Role of the p38 stress-activated kinase signaling cascade, and relation to cytoprotection. Cardiovascular Research 2000; 46: 139-150.

Kane LP, Shapiro VS, Stokoe D, Weiss A. Induction of NF-kappa B by the Akt PKB kinase. Current Biology 1999; 9: 601-604.

Kang KW, Ryu JH, Kim SG. The essential role of phosphatidylinositol 3-kinase and of p38 mitogen-activated protein kinase activation in the antioxidant response element-mediated rGSTA2 induction by decreased glutathione in H4IIE hepatoma cells. Molecular Pharmacology 2000; 58: 1017-1025.

Karin M. How NF-kappa B is activated: the role of the I kappa B kinase (IKK) complex. Oncogene 1999a; 18: 6867-6874.

Karin M. The beginning of the end: I kappa B kinase (IKK) and NF-kappa B activation. Journal of Biological Chemistry 1999b; 274: 27339-27342.

Kawamori T, Lubet R, Steele VE, Kelloff GJ, Kaskey RB, Rao CV, Reddy BS. Chemopreventive effect of curcumin, a naturally occurring anti-inflammatory agent, during the promotion/progression stages of colon cancer. Cancer Research 1999; 59: 597-601.

Kawamoto RM, Caswell AH. Autophosphorylation of glyceraldehydephosphate dehydrogenase and phosphorylation of protein from skeletal-muscle microsomes. Biochemistry 1986; 25: 656-661.

Kaye KM, Devergne O, Harada JN, Izumi KM, Yalamanchili R, Kieff E, Mosialos G. Tumor necrosis factor receptor associated factor 2 is a mediator of NF-kappa B activation by latent infection membrane protein 1, the Epstein-Barr virus transforming protein. Proceedings of the National Academy of Sciences of the United States of America 1996; 93: 11085-11090.

Kaye KM, Izumi KM, Kieff E. Epstein-Barr-Virus latent membrane protein-1 is essential for B-lymphocyte growth transformation. Proceedings of the National Academy of Sciences of the United States of America 1993; 90: 9150-9154.

Kerenyi T, Voss B, Rauterberg J, Fromme HG, Jellinek H, Hauss WH. Connective-tissue proteins on the injured endothelium of the rat aorta. Experimental and Molecular Pathology 1985; 43: 151-161.

Kerner JD, Appleby MW, Mohr RN, Chien S, Rawlings DJ, Maliszewski CR, Witte ON, Perlmutter RM. Impaired expansion of mouse B-cell progenitors lacking BTK. Immunity 1995; 3: 301-312.

Kerr JF, Wyllie AH, and Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. British Journal of Cancer 26(4), 239-257. 1972.

Kesteloot H. Epidemiological-studies on the relationship between sodium, potassium, calcium, and magnesium and arterial blood-pressure. Journal of Cardiovascular Pharmacology 1984; 6: S192-S196.

Keyt BA, Berleau LT, Nguyen HV, Chen H, Heinsohn H, Vandlen R, Ferrara N. The carboxyl-terminal domain (111-165) of vascular endothelial growth factor is critical for its mitogenic potency. Journal of Biological Chemistry 1996; 271: 7788-7795.

Khokhar S, Magnusdottir SGM. Total phenol, catechin, and caffeine contents of teas commonly consumed in the United Kingdom. Journal of Agricultural and Food Chemistry 2002; 50: 565-570.

Kim J, Hwang JS, Cho YK, Han YK, Jeon YJ, Yang KH. Protective effects of (-)-epigallocatechin-3-gallate on UVA- and UVB-induced skin damage. Skin Pharmacology and Applied Skin Physiology 2001; 14: 11-19.

Kimura Y, Okuda H, Arichi S. Effects of stilbenes on arachidonate metabolism in leukocytes. Biochimica et Biophysica Acta 1985; 834: 275-278.

Kirch HC, Flaswinkel S, Rumpf H, Brockmann D, Esche H. Expression of human p53 requires synergistic activation of transcription from the p53 promoter by AP-1, NF-kappa B and Myc/Max. Oncogene 1999; 18: 2728-2738.

Kistler B, Rolink A, Marienfeld R, Neumann M, Wirth T. Induction of nuclear factor-kappa B during primary B cell differentiation. Journal of Immunology 1998; 160: 2308-2317.

Kitiyakara C, Wilcox CS. Antioxidants for hypertension. Current Opinion in Nephrology and Hypertension 1998; 7: 531-538.

Kluck RM, BossyWetzel E, Green DR, Newmeyer DD. The release of cytochrome c from mitochondria: A primary site for Bcl-2 regulation of apoptosis. Science 1997; 275: 1132-1136.

Kockx MM. Apoptosis in the atherosclerotic plaque - Quantitative and qualitative aspects. Arteriosclerosis Thrombosis and Vascular Biology 1998; 18: 1519-1522.

Kolodgie FD, Jacob A, Wilson PS, Carlson GC, Farb A, Verma A, Virmani R. Estradiol attenuates directed migration of vascular smooth muscle cells in vitro. American Journal of Pathology 1996; 148: 969-976.

Kondo K, Kurihara M, Miyata N, Suzuki T, Toyoda M. Scavenging mechanisms of (-)-epigallocatechin gallate and (-)- epicatechin gallate on peroxyl radicals and formation of superoxide during the inhibitory action. Free Radical Biology and Medicine 1999; 27: 855-863.

Kondo T, Ohta T, Igura K, Hara Y, Kaji K. Tea catechins inhibit angiogenesis endothelial cell growth, migration in vitro, measured by human tube formation, through inhibition of VEGF receptor binding. Cancer Letters 2002; 180: 139-144.

Kono S, Shinchi K, Wakabayashi K, Honjo S, Todoroki I, Sakurai Y, Imanishi K, Nishikawa H, Ogawa S, and Katsurada M. Relation of green tea consumption to serum lipids and lipoproteins in Japanese men. Journal of Epidemiology 6(3), 128-133. 1996.

Kots AY, Skurat AV, Sergienko EA, Bulargina TV, Severin ES. Nitroprusside stimulates the cysteine-specific mono(ADP-ribosylation) of glyceraldehyde-3-phosphate dehydrogenase from human erythrocytes. Febs Letters 1992; 300: 9-12.

Kovalenko M, Ronnstrand L, Heldin CH, Loubtchenkov M, Gazit A, Levitzki A, Bohmer FD. Phosphorylation site-specific inhibition of platelet-derived growth factor beta-receptor

autophosphorylation by the receptor blocking tyrphostin AG1296. Biochemistry 1997; 36: 6260-6269.

Krauss G. Biochemistry of signal transduction and regulation, second edition. Wiley-VCH, 2001.

Krek W, Nigg EA. Differential phosphorylation of vertebrate p34CDC2 kinase at the G1/S and G2/M transitions of the cell-cycle - identification of major phosphorylation sites. EMBO Journal 1991; 10: 305-316.

Kubbutat MHG, Jones SN, Vousden KH. Regulation of p53 stability by Mdm2. Nature 1997; 387: 299-303.

Kumar A, Dhawan S, Hardegen NJ, Aggarwal BB. Curcumin (diferuloylmethane) inhibition of tumor necrosis factor (TNF)-mediated adhesion of monocytes to endothelial cells by suppression of cell surface expression of adhesion molecules and of nuclear factor-kappa B activation. Biochemical Pharmacology 1998; 55: 775-783.

Laherty CD, Hu HM, Opipari AW, Wang F, Dixit VM. The Epstein-Barr-Virus LMP1-gene product induces A20-zinc finger protein expression by activating nuclear factor-kappa-B. Journal of Biological Chemistry 1992; 267: 24157-24160.

Lahti JM, Li HM, Kidd VJ. Elimination of cyclin D1 in vertebrate cells leads to an altered cell cycle phenotype, which is rescued by overexpression of murine cyclins D1, D2, or D3 but not by a mutant cyclin D1. Journal of Biological Chemistry 1997; 272: 10859-10869.

Lakin ND, Jackson SP. Regulation of p53 in response to DNA damage. Oncogene 1999; 18: 7644-7655.

Lallena MJ, Diaz-Meco MT, Bren G, Paya CV, Moscat J. Activation of I kappa B kinase beta by protein kinase C isoforms. Molecular and Cellular Biology 1999; 19: 2180-2188.

Lavrovsky Y, Schwartzman ML, Levere RD, Kappas A, Abraham NG. Identification of binding-sites for transcription factors NF- kappa-B and AP-2 in the promoter region of the human heme oxygenase-1 gene. Proceedings of the National Academy of Sciences of the United States of America 1994; 91: 5987-5991.

Lavrovsky Y, Song CS, Chatterjee B, Roy AK. Age-dependent increase of heme oxygenase-1 gene expression in the liver mediated by NF kappa B. Mechanisms of Ageing and Development 2000; 114: 49-60.

Lawrance LC, Fiocchi C, Chakravarti S. Ulcerative colitis and Crohn's disease: distinctive gene expression profiles and novel susceptibility candidate genes. Human Molecular Genetics 2001; 10: 445-456.

Leblanc GA, Stuart JD, Dunn SE, Baldwin WS. Effect of the plant-compound indole-3-carbinol on hepatic cholesterol homeostasis. Food and Chemical Toxicology 1994; 32: 633-639.

Lee E, Grodzinsky AJ, Libby P, Clinton SK, Lark MW, Lee RT. Human vascular smooth-muscle cell-monocyte interactions and metalloproteinase secretion in culture. Arteriosclerosis Thrombosis and Vascular Biology 1995a; 15: 2284-2289.

Lee E, Grodzinsky AJ, Libby P, Clinton SK, Lark MW, Lee RT. Monocytes induce metalloproteinase secretion by cultured human vascular smooth-muscle cells through interleukin-1. Circulation 1995b; 92: 2025-2025

Lee FYJ, Li YB, Zhu H, Yang SQ, Lin HZ, Trush M, Diehl AM. Tumor necrosis factor increases mitochondrial oxidant production and induces expression of uncoupling protein-2 in the regenerating rat liver. Hepatology 1999; 29: 677-687.

Lee HH, Dadgostar H, Cheng QW, Shu JY, Cheng GH. NF-kappa B-mediated up-regulation of Bcl-x and Bfl-1/A1 is required for CD40 survival signaling in B lymphocytes. Proceedings of the National Academy of Sciences of the United States of America 1999; 96: 9136-9141.

Lee MJ, Wang ZY, Li H, Chen LS, Sun Y, Gobbo S, Balentine DA, Yang CS. Analysis of plasma and urinary tea polyphenols in human- subjects. Cancer Epidemiology Biomarkers & Prevention 1995; 4: 393-399.

Lee MLT, Kuo FC, Whitmore GA, Sklar J. Importance of replication in microarray gene expression studies: Statistical methods and evidence from repetitive cDNA hybridizations. Proceedings of the National Academy of Sciences of the United States of America 2000; 97: 9834-9839.

Lee PJ, Jiang BH, Chin BY, Iyer NV, Alam J, Semenza GL, Choi AMK. Hypoxia-inducible factor-1 mediates transcriptional activation of the heme oxygenase-1 gene in response to hypoxia. Journal of Biological Chemistry 1997; 272: 5375-5381.

Lee RT, Libby P. The unstable atheroma. Arteriosclerosis Thrombosis and Vascular Biology 1997; 17: 1859-1867.

Levkau B, Garton KJ, Ferri N, Kloke K, Nofer JR, Baba HA, Raines EW, and Breithardt G. XIAP induces cell cycle arrest and activates nuclear factor-kappa B-New survival pathways dissabled by caspase-mediated cleavage during apoptosis of human endothelial cells. Circulation Research 88, 282-290. 2001.

Li HC, Yashiki S, Sonoda J, Lou H, Ghosh SK, Byrnes JJ, Lema C, Fujiyoshi T, Karasuyama M, Sonoda S. Green tea polyphenols induce apoptosis in vitro in peripheral blood T lymphocytes of adult T-cell leukemia patients. Japanese Journal of Cancer Research 2000; 91: 34-40.

Li J, Lee JM, Johnson JA. Microarray analysis reveals an antioxidant responsive element-driven gene set involved in conferring protection from an oxidative stress-induced apoptosis in IMR-32 cells. Journal of Biological Chemistry 2002; 277: 388-394.

Li JF, Schmidt AM. Characterization and functional analysis of the promoter of RAGE, the receptor for advanced glycation end products. Journal of Biological Chemistry 1997; 272: 16498-16506.

Li NX, Karin M. Ionizing radiation and short wavelength UV activate NF-kappa B through two distinct mechanisms. Proceedings of the National Academy of Sciences of the United States of America 1998; 95: 13012-13017.

Li PX, Wong J, Ayed A, Ngo D, Brade AM, Arrowsmith C, Austin RC, Klamut HJ. Placental transforming growth factor-beta is a downstream mediator of the growth arrest and apoptotic response of tumor cells to DNA damage and p53 overexpression. Journal of Biological Chemistry 2000; 275: 20127-20135.

Li ZW, Chu WM, Hu YL, Delhase M, Deerinck T, Ellisman M, Johnson R, Karin M. The IKK beta subunit of I kappa B kinase (IKK) is essential for nuclear factor kappa B activation and prevention of apoptosis. Journal of Experimental Medicine 1999; 189: 1839-1845.

Liacini A, Sylvester J, Li WQ, Zafarullah M. Inhibition of interleukin-1-stimulated MAP kinases, activating protein-1 (AP-1) and nuclear factor kappa B (NF-kappa B) transcription factors down-regulates matrix metalloproteinase gene expression in articular chondrocytes. Matrix Biology 2002; 21: 251-262.

Liang YC, Lin-Shiau SY, Chen CF, Lin JK. Inhibition of cyclin-dependent kinases 2 and 4 activities as well as induction of Cdk inhibitors p21 and p27 during growth arrest of human breast carcinoma cells by (-)-epigallocatechin- 3-gallate. Journal of Cellular Biochemistry 1999; 75: 1-12.

Liaud MF, Lichtle C, Apt K, Martin W, and Cerff R. Compartment-specific isoforms of TPI and GAPDH are important into diatom mitochondria as a fusion protein: Evidence in favour of mitochondrial origin of the eukaryotic glycolytic. Molecular Biology and Evolution 17(2), 213-223. 2000.

Liberto M, Cobrinik D. Growth factor-dependent induction of p21(CIP1) by the green tea by polyphenol, epigallocatechin gallate. Cancer Letters 2000; 154: 151-161.

Liehr JG, Ricci MJ. 4-hydroxylation of estrogens as marker of human mammary tumors. Proceedings of the National Academy of Sciences of the United States of America 1996; 93: 3294-3296.

Lin JK, Chen PC, Ho CT, Lin-Shiau SY. Inhibition of xanthine oxidase and suppression of intracellular reactive oxygen species in HL-60 cells by theaflavin-3,3'-digallate, (-)-epigallocatechin-3-gallate, and propyl gallate. Journal of Agricultural and Food Chemistry 2000; 48: 2736-2743.

Lin YL, Cheng CY, Lin YP, Lau YW, Juan IM, Lin JK. Hypolipidemic effect of green tea leaves through induction of antioxidant and phase II enzymes including superoxide dismutase, catalase, and glutathione S-transferase in rats. Journal of Agricultural and Food Chemistry 1998; 46: 1893-1899.

Lin YL, Lin JK. (-)-epigallocatechin-3-gallate blocks the induction of nitric oxide synthase by down-regulating lipopolysaccharide-induced activity of transcription factor nuclear factor-kappa B. Molecular Pharmacology 1997; 52: 465-472.

Liou HC, Sha WC, Scott ML, Baltimore D. Sequential induction of NF-kappa-B/rel family proteins during B-cell terminal differentiation. Molecular and Cellular Biology 1994; 14: 5349-5359.

Liou YC, Ryo A, Huang HK, Lu PJ, Bronson R, Fujimori F, Uchida T, Hunter T, Lu KP. Loss of Pin1 function in the mouse causes phenotypes resembling cyclin D1-null phenotypes. Proceedings of the National Academy of Sciences of the United States of America 2002; 99: 1335-1340.

Liu HT, Wang YG, Zhang YM, Song QS, Di CH, Chen GH, Tang J, Ma DL. TFAR19, a novel apoptosis-related gene cloned from human leukemia cell line TF-1, could enhance apoptosis of some tumor cells induced by growth factor withdrawal. Biochemical and Biophysical Research Communications 1999; 254: 203-210.

Liu JD, Chen SH, Lin CL, Tsai SH, Liang YC. Inhibition of melanoma growth and metastasis by combination with (-)-epigallocatechin-3-gallate and dacarbazine in mice. Journal of Cellular Biochemistry 2001; 83: 631-642.

Liu JY, Lin SJ, Lin JK. Inhibitory effects of curcumin on protein-kinase-C activity induced by 12-o-tetradecanoyl-phorbol-13-acetate in NIH 3T3 cells. Carcinogenesis 1993; 14: 857-861.

Liu L, Dahlgren C, Elwing H, and Lundqvist H. A simple chemoluminescence assay for the determination of reactive oxygen species produced by human neutrophils. Journal of Immunological Methods 192(1-2), 173-178. 1996.

Locher R, Emmanuele L, Suter PM, Vetter W, Barton M. Green tea polyphenols inhibit human vascular smooth muscle cell proliferation stimulated by native low-density lipoprotein. European Journal of Pharmacology 2002; 434: 1-7.

Long LH, Clement MV, Halliwell B. Artifacts in cell culture: Rapid generation of hydrogen peroxide on addition of (-)-epigallocatechin, (-)- epigallocatechin gallate, (+)-catechin, and quercetin to commonly used cell culture media. Biochemical and Biophysical Research Communications 2000; 273: 50-53.

Long LH, Halliwell B. Oxidation and generation of hydrogen peroxide by thiol compounds in commonly used cell culture media. Biochemical and Biophysical Research Communications 2001; 286: 991-994.

Lorenzo HK, Susin SA, Penninger J, Kroemer G. Apoptosis inducing factor (AIF): a phylogenetically old, caspase-independent effector of cell death. Cell Death and Differentiation 1999; 6: 516-524.

Lu KP, Hanes SD, Hunter T. A human peptidyl-prolyl isomerase essential for regulation of mitosis. Nature 1996; 380: 544-547.

Lu PJ, Zhou XY, Liou YC, Noel JP, Lu KP. Critical role of WW domain phosphorylation in regulating phosphoserine binding activity and Pin1 function. Journal of Biological Chemistry 2002; 277: 2381-2384.

Lu LH, Lee SS, Huang HC. Epigallocatechin suppression of proliferation of vascular smooth muscle cells: correlation with c-jun and JNK. British Journal of Pharmacology 1998; 124: 1227-1237.

Lu TT, Makishima M, Repa JJ, Schoonjans K, Kerr TA, Auwerx J, Mangelsdorf DJ. Molecular basis for feedback regulation of bile acid synthesis by nuclear receptors. Molecular Cell 2000; 6: 507-515.

Luss H, Schmitz W, Neumann J. A proteasome inhibitor confers cardioprotection. Cardiovascular Research 2002; 54: 140-151.

Ma Q, Kinneer K. Chemoprotection by phenolic antioxidants - Inhibition of tumor necrosis factor alpha induction in macrophages. Journal of Biological Chemistry 2002; 277: 2477-2484.

Mackman N. Regulation of the tissue factor gene. Thrombosis and Haemostasis. 1997; 78: 747-754.

MacLachlan TK, Somasundaram K, Sgagias M, Shifman Y, Muschel RJ, Cowan KH, El-Deiry WS. BRCA1 effects on the cell cycle and the DNA damage response are linked to altered gene expression. Journal of Biological Chemistry 2000; 275: 2777-2785.

Maeda-Yamamoto M, Kawahara H, Tahara N, Tsuji K, Hara Y, Isemura M. Effects of tea polyphenols on the invasion and matrix metalloproteinases activities of human fibrosarcoma HT1080 cells. Journal of Agricultural and Food Chemistry 1999; 47: 2350-2354.

Maki CG, Huibregtse JM, Howley PM. In vivo ubiquitination and proteasome-mediated degradation of p53. Cancer Research 1996; 56: 2649-2654.

Makita K, Falck JR, Capdevila JH. Cytochrome P450, the arachidonic acid cascade, and hypertension: New vistas for an old enzyme system. FASEB Journal 1996; 10: 1456-1463.

Makita K, Takahashi K, Karara A, Jacobson HR, Falck JR, Capdevila JH. Experimental and/or genetically controlled alterations of the renal microsomal cytochrome-p450 epoxygenase induce hypertension in rats fed a high-salt diet. Journal of Clinical Investigation 1994; 94: 2414-2420.

Makris C, Godfrey VL, Krahn-Senftleben G, Takahashi T, Roberts JL, Schwarz T, Feng LL, Johnson RS, Karin M. Female mice heterozygous for IKK gamma/NEMO deficiencies develop a dermatopathy similar to the human X-linked disorder incontinentia pigmenti. Molecular Cell 2000; 5: 969-979.

Malinin NL, Boldin MP, Kovalenko AV, Wallach D. MAP3K-related kinase involved in NF-kappa B induction by TNF, CD95 and IL-1. Nature 1997; 385: 540-544.

Maniatis T. A ubiquitin ligase complex essential for the NF-kappa B, Wnt/Wingless, and Hedgehog signaling pathways. Genes & Development 1999; 13: 505-510.

Manna SK, Mukhopadhyay A, Aggarwal BB. Resveratrol suppresses TNF-induced activation of nuclear transcription factors NF-kappa B, activator protein-1, and apoptosis: Potential role of reactive oxygen intermediates and lipid peroxidation. Journal of Immunology 2000; 164: 6509-6519.

Manna SK, Mukhopadhyay A, Van NT, Aggarwal BB. Silymarin suppresses TNF-induced activation of NF-kappa B, c- Jun N-terminal kinase, and apoptosis. Journal of Immunology 1999; 163: 6800-6809.

Manson MM, Ball HWL, Barrett MC, Clark HL, Judah DJ, Williamson G, Neal GE. Mechanism of action of dietary chemopreventive agents in rat liver: induction of phase I and II drug metabolizing enzymes and aflatoxin B-1 metabolism. Carcinogenesis 1997; 18: 1729-1738.

Manson MM, Hudson EA, Ball HWL, Barrett MC, Clark HL, Judah DJ, Verschoyle RD, Neal GE. Chemoprevention of aflatoxin B-1-induced carcinogenesis by indole-3-carbinol

in rat liver - predicting the outcome using early biomarkers. Carcinogenesis 1998; 19: 1829-1836.

Mansur NR, Meyersiegler K, Wurzer JC, Sirover MA. Cell-cycle regulation of the glyceraldehyde-3-phosphate dehydrogenase/uracil DNA glycosylase gene in normal human-cells. Nucleic Acids Research 1993; 21: 993-998.

Marianneau P, Cardona A, Edelman L, Deubel V, Despres P. Dengue virus replication in human hepatoma cells activates NF- kappa B which in turn induces apoptotic cell death. Journal of Virology 1997; 71: 3244-3249.

Marinovich M, Viviani B, Corsini E, Ghilardi F, Galli CL. NF-kappa B activation by triphenyltin triggers apoptosis in HL- 60 cells. Experimental Cell Research 1996; 226: 98-104.

Martin SJ, Reutelingsperger CPM, McGahon AJ, Rader JA, Vanschie RCAA, Laface DM, Green DR. Early redistribution of plasma-membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus - inhibition by overexpression of bcl-2 and abl. Journal of Experimental Medicine 1995a; 182: 1545-1556.

Martin SJ, Green DR. Protease activation during apoptosis - death by 1000 cuts. Cell 1995b; 82: 349-352.

Marumo T, Schini-Kerth VB, Busse R. Vascular endothelial growth factor activates nuclear factor- kappa B and induces monocyte chemoattractant protein-1 in bovine retinal endothelial cells. Diabetes 1999; 48: 1131-1137.

Marumo T, SchiniKerth VB, Fisslthaler B, Busse R. Platelet-derived growth factor-stimulated superoxide anion production modulates activation of transcription factor NF-kappa B and expression of monocyte chemoattractant protein 1 in human aortic smooth muscle cells. Circulation 1997; 96: 2361-2367.

Matsui K, Fine A, Zhu BM, Marshak-Rothstein A, Ju ST. Identification of two NF-kappa B sites in mouse CD95 ligand (Fas ligand) promoter: Functional analysis in T cell hybridoma. Journal of Immunology 1998; 161: 3469-3473.

Mattson MP, Camandola S. NF-kappa B in neuronal plasticity and neurodegenerative disorders. Journal of Clinical Investigation 2001; 107: 247-254.

McAnlis GT, McEneny J, Pearce J, Young IS. Black tea consumption does not protect low density lipoprotein from oxidative modification. European Journal of Clinical Nutrition 1998; 52: 202-206.

McCaffrey TA, Fu CZ, Du BH, Eksinar S, Kent KC, Bush H, Kreiger K, Rosengart T, Cybulsky MI, Silverman ES, Collins T. High-level expression of Egr-1 and Egr-1-inducible genes in mouse and human atherosclerosis. Journal of Clinical Investigation 2000; 105: 653-662.

McDanell R, Mclean AEM, Hanley AB, Heaney RK, Fenwick GR. Differential induction of mixed-function oxidase (MFO) activity in rat-liver and intestine by diets containing processed cabbage - correlation with cabbage levels of glucosinolates and glucosinolate hydrolysis products. Food and Chemical Toxicology 1987; 25: 363-368.

McDanell R, McLean AEM, Hanley AB, Heaney RK, Fenwick GR. Chemical and biological properties of indole glucosinolates (glucobrassicins) - a review. Food and Chemical Toxicology 1988; 26: 59-70.

McFarland EDC, Izumi KM, Mosialos G. Epstein-Barr virus transformation: involvement of latent membrane protein 1-mediated activation of NF-kappa B. Oncogene 1999; 18: 6959-6964.

McGiff JC, Quilley J. 20-HETE and the kidney: resolution of old problems and new beginnings. American Journal of Physiology-Regulatory Integrative and Comparative Physiology 1999; 277: R607-R623.

Mendelsohn ME, Karas RH. The protective effects of estrogen on the cardiovascular system. New England Journal of Medicine 1999; 340: 1801-1811.

Mercurio F, Zhu HY, Murray BW, Shevchenko A, Bennett BL, Li JW, Young DB, Barbosa M, Mann M. IKK-1 and IKK-2: Cytokine-activated I kappa B kinases essential for NF-kappa B activation. Science 1997; 278: 860-866.

Michiels C, Arnould T, Houbion A, Remacle J. Human umbilical vein endothelial-cells submitted to hypoxia-reoxygenation *in vitro* - implication of free-radicals, xanthine-oxidase, and energy deficiency. Journal of Cellular Physiology 1992; 153: 53-61.

Mikhailidis DP, Jeremy JY, Barradas MA, Green N, Dandona P. Effect of ethanol on vascular prostacyclin (prostaglandin-I-2) synthesis, platelet-aggregation, and platelet thromboxane release. British Medical Journal 1983; 287: 1495-1498.

Miura S, Watanabe J, Tomita T, Sano M, Tomita I. The inhibitory effects of tea polyphenols (flavan-3-ol derivatives) on Cu_2+ mediated oxidative modification of low-density-lipoprotein. Biological and Pharmaceutical Bulletin 1994; 17: 1567-1572.

Miyamoto S, Chiao PJ, Verma IM. Enhanced I-kappa-B-alpha degradation is responsible for constitutive NF-kappa-B activity in mature murine B-cell lines. Molecular and Cellular Biology 1994; 14: 3276-3282.

Miyamoto S, Seufzer BJ, Shumway SD. Novel I kappa B alpha proteolytic pathway in WEHI231 immature B cells. Molecular and Cellular Biology 1998; 18: 19-29.

Miyamoto S, Verma IM. REL/NF-kappa-B/I-kappa-B story. Advances in Cancer Research, Vol 66 1995; 66: 255-292.

Miyazawa T. Absorption, metabolism and antioxidative effects of tea catechin in humans. Biofactors 2000; 13: 55-59.

Moi P, Chan K, Asunis I, Cao A, Kan YW. Isolation of NF-E-related factor-2 (NRF2), a NF-E2-like basic leucine-zipper transcriptional activator that binds to the tandem NF-E2/AP-1 repeat of the beta-globin locus-control region. Proceedings of the National Academy of Sciences of the United States of America 1994; 91: 9926-9930.

Moncada S, Higgs A. Mechanisms of disease - the l-arginine nitric-oxide pathway. New England Journal of Medicine 1993; 329: 2002-2012.

Morita T, Kourembanas S. Endothelial cell expression of vasoconstrictors and growth factors is regulated by smooth muscle cell-derived carbon monoxide. Journal of Clinical Investigation 1995; 96: 2676-2682.

Moroianu J. Distinct nuclear import and export pathways mediated by members of the karyopherin beta family. Journal of Cellular Biochemistry 1998; 70: 231-239.

Mosialos G, Birkenbach M, Yalamanchili R, Vanarsdale T, Ware C, Kieff E. The Epstein-Barr-Virus transforming protein lmp1 engages signaling proteins for the tumor-necrosis-factor receptor family. Cell 1995; 80: 389-399.

Mosialou E, Ekstrom G, Adang AEP, Morgenstern R. Evidence that rat-liver microsomal glutathione transferase is responsible for glutathione-dependent protection against lipid-peroxidation. Biochemical Pharmacology 1993; 45: 1645-1651.

Motterlini R. Heme oxygenase-1-derived carbon monoxide contributes to the suppression of acute hypertensive responses in vivo (vol 83, pg 568, 1998). Circulation Research 1998; 83: 1289.

Motterlini R, Foresti R, Bassi R, Green CJ. Curcumin, an antioxidant and anti-inflammatory agent, induces heme oxygenase-1 and protects endothelial cells against oxidative stress. Free Radical Biology and Medicine 2000; 28: 1303-1312.

Mukhtar H, Gupta S, Ahmad N. Inhibition of nuclear transcription factor NF kappa B by green tea constituent epigallocatechin-3-gallate in human epidermoid carcinoma cells A431. Journal of Investigative Dermatology 1998; 110: 521.

Muller JM, Zieglerheitbrock HWL, Baeuerle PA. Nuclear factor kappa-B, a mediator of lipopolysaccharide effects. Immunobiology 1993; 187: 233-256.

Muller RM, Taguchi H, Shibahara S. Nucleotide-sequence and organization of the rat heme oxygenase gene. Journal of Biological Chemistry 1987; 262: 6795-6802.

Mulvany MJ. The structure of the resistance vasculature in essential-hypertension. Journal of Hypertension 1987; 5: 129-136.

Murata T, Obiri NI, Debinski W, Puri RK. Structure of IL-13 receptor: Analysis of subunit compostion in cancer and immune cells. Biochemical and Biophysical Research Communications 1997; 238: 90-94.

Murphy WJ, Muroi M, Zhang CX, Suzuki T, Russell SW. Both basal and enhancer kappa B elements are required for full induction of the mouse inducible nitric oxide synthase gene. Journal of Endotoxin Research 1996; 3: 381-393.

Murry CE, Gipaya CT, Bartosek T, Benditt EP, Schwartz SM. Monoclonality of smooth muscle cells in human atherosclerosis. American Journal of Pathology 1997; 151: 697-705.

Muzio M, Stockwell BR, Stennicke HR, Salvesen GS, Dixit VM. An induced proximity model for caspase-8 activation. Journal of Biological Chemistry 1998; 273: 2926-2930.

Nadkarni KM. Curcuma longa. Popular Prskashan publishing Co. Bombay 1976.

Nakagawa K, Miyazawa T. Absorption and distribution of tea catechin, (-)-epigallocatechin-3-gallate, in the rat. Journal of Nutritional Science and Vitaminology 1997a; 43: 679-684.

Nakagawa K, Okuda S, Miyazawa T. Dose-dependent incorporation of tea catechins, (-)-epigallocatechin-3-gallate and (-)-epigallocatechin, into human plasma. Bioscience Biotechnology and Biochemistry 1997b; 61: 1981-1985.

Nakayama T, Hashimoto T, Kajiya K, Kumazawa S. Affinity of polyphenols for lipid bilayers. Biofactors 2000; 13: 147-151.

Nakayama T, Ono K, Hashimoto K. Affinity of antioxidative polyphenols for lipid bilayers evaluated with a liposome system. Bioscience Biotechnology and Biochemistry 1998; 62: 1005-1007.

Nam S, Smith DM, Dou QP. Ester bond-containing tea polyphenols potently inhibit proteasome activity in vitro and in vivo. Journal of Biological Chemistry 2001; 276: 13322-13330.

Napoli C, de Nigris F, Palinski W. Multiple role of reactive oxygen species in the arterial wall. Journal of Cellular Biochemistry 2001; 82: 674-682.

Naumann M, Scheidereit C. Activation of NF-kappa-B in-vivo is regulated by multiple phosphorylations. EMBO Journal 1994; 13: 4597-4607.

Nelken NA, Coughlin SR, Gordon D, Wilcox JN. Monocyte chemoattractant protein-1 in human atheromatous plaques. Journal of Clinical Investigation 1991; 88: 1121-1127.

Neumann FJ, Marx N, Gawaz M, Brand K, Ott I, Rokitta C, Sticherling C, Meinl C, May A, Schomig A. Induction of cytokine expression in leukocytes by binding of thrombin-stimulated platelets. Circulation 1997; 95: 2387-2394.

Nevins JR. E2F - A link between the RB tumor suppressor protein and viral oncoproteins. Science 1992; 258: 424-429.

Ng LL, Davies JE, Siczkowski M, Sweeney FP, Quinn PA Krolewski B, Krolewski AS. Abnormal Na+/H+ antiporter phenotype and turnover of immortalized lymphoblasts from type-1 diabetic-patients with nephropathy. Journal of Clinical Investigation 1994; 93: 2750-2757.

Nguyen T, Huang HC, Pickett CB. Transcriptional regulation of the antioxidant response element - Activation by Nrf2 and repression by MafK. Journal of Biological Chemistry 2000; 275: 15466-15473.

Nishihira J, Oflaherty JT. Phorbol-myristate acetate receptors in human polymorphonuclear neutrophils. Journal of Immunology 1985; 135: 3439-3447.

Nomura M, Ma WY, Chen NY, Bode AM, Dong ZG. Inhibition of 12-O-tetradecanoylphorbol-13-acetate-induced NF- kappa B activation by tea polyphenols, (-)-epigallocatechin gallate and theaflavins. Carcinogenesis 2000; 21: 1885-1890.

Nurse P. Ordering S-phase and M-phase in the cell-cycle. Cell 1994; 79: 547-550.

Oda E, Ohki R, Murasawa H , Nemoto J, Shibue T, Yamashita T, Tokino T, Taniguchi T, Tanaka N. Nora, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. Science 2000; 288: 1053-1058.

Ohshima H, Yoshie Y, Auriol S, Gilibert I. Antioxidant and pro-oxidant actions of flavonoids: Effects on DNA damage induced by nitric oxide, peroxynitrite and nitroxyl anion. Free Radical Biology and Medicine 1998; 25: 1057-1065.

Okabe S, Fujimoto N, Sueoka N, Suganuma M, Fujiki H. Modulation of gene expression by (-)-epigallocatechin gallate in PC-9 cells using a cDNA expression array. Biological and Pharmaceutical Bulletin 2001; 24: 883-886.

Oliveira-dos-Santos AJ, Matsumoto G, Snow BE, Bai DL, Houston FP, Whishaw IQ, Mariathasan S, Sasaki T, Wakeham A, Ohashi PS, Roder JC, Barnes CA, Siderovski DP, Penninger JM. Regulation of T cell activation, anxiety, and male aggression by RGS2. Proceedings of the National Academy of Sciences of the United States of America 2000; 97: 12272-12277.

Omenn GS, Goodman GE, Thornquist MD, Balmes J, Cullen MR, Glass A, Keogh JP, Meyskens FL, Valanis B, Williams JH, Barnhart S, Hammar S. Effects of a combination of

beta carotene and vitamin A on lung cancer and cardiovascular disease. New England Journal of Medicine 1996; 334: 1150-1155.

Oppenheim JJ, Zachariae COC, Mukaida N, Matsushima K. Properties of the novel proinflammatory supergene intercrine cytokine family. Annual Review of Immunology 1991; 9: 617-648.

Otsuka T, Ogo T, Eto T, Asano Y, Suganuma M, Niho Y. Growth inhibition of leukemic-cells by (-)-epigallocatechin gallate, the main constituent of green tea. Life Sciences 1998; 63: 1397-1403.

Otsuki M, Saito H, Xu X, Sumitani S, Kouhara H, Kurabayashi M, Kasayama S. Cilostazol represses vascular cell adhesion molecule-1 gene transcription via inhibiting NF-κB binding to its recognition sequence. Atherosclerosis 2001; 158: 121-128.

Ott I, Fischer EG, Miyagi Y, Mueller BM, Ruf W. A role for tissue factor in cell adhesion and migration mediated by interaction with actin-binding protein 280. Journal of Cell Biology 1998; 140: 1241-1253.

Oxelius VA, Carlsson AM, Aurivillius M. Alternative G1m, G2m and G3m allotypes of IGHG genes correlate with atopic and nonatopic pathways of immune regulation in children with bronchial asthma. International Archives of Allergy and Immunology 1998; 115: 215-219.

PaceAsciak CR, Hahn S, Diamandis EP, Solea SG, Goldberg DM. The red wine phenolics trans-resveratrol and quercetin block human platelet-aggregation and eicosanoid synthesis - implications for protection against coronary heart-disease. Clinica Chimica Acta 1995; 235: 207-219.

Pagano M, Theodoras AM, Tam SW, Draetta GF. Cyclin D1-mediated inhibition of repair and replicative DNA-synthesis in human fibroblasts. Genes and Development 1994; 8: 1627-1639.

Pahl HL. Activators and target genes of Rel/NF-kappa B transcription factors. Oncogene 1999; 18: 6853-6866.

Pan MH, Liang YC, Lin-Shiau SY, Zhu NQ, Ho CT, Lin JK. Induction of apoptosis by the oolong tea polyphenol theasinensin A through cytochrome c release and activation of

caspase-9 and caspase-3 in human U937 cells. Journal of Agricultural and Food Chemistry 2000a; 48: 6337-6346.

Pan MH, Lin-Shiau SY, Ho CT, Lin JH, Lin JK. Suppression of lipopolysaccharide-induced nuclear factor-kappa B activity by theaflavin-3,3 '-digallate from black tea and other polyphenols through down-regulation of I kappa B kinase activity in macrophages. Biochemical Pharmacology 2000b; 59: 357-367.

Pan ZK, Christiansen SC, Ptasznik A, Zuraw BL. Requirement of phosphatidylinositol 3-kinase activity for bradykinin stimulation of NF-kappa B activation in cultured human epithelial cells. Journal of Biological Chemistry 1999; 274: 9918-9922.

Parhami F, Fang ZT, Fogelman AM, Andalibi A, Territo MC, Berliner JA. Minimally modified low-density-lipoprotein induced inflammatory responses in endothelial-cells are mediated by cyclic adenosine-monophosphate. Journal of Clinical Investigation 1993; 92: 471-478.

Parry GCN, Mackman N. A set of inducible genes expressed by activated human monocytic and endothelial-cells contain kappa-b-like sites that specifically bind C-Rel-p65 heterodimers. Journal of Biological Chemistry 1994; 269: 20823-20825.

Paschka AG, Butler R, Young CYF. Induction of apoptosis in prostate cancer cell lines by the green tea component, (-)-epigallocatechin-3-gallate. Cancer Letters 1998; 130: 1-7.

Passalaris TM, Benanti JA, Gewin L, Kiyono T, Galloway DA. The G(2) checkpoint is maintained by redundant pathways. Molecular and Cellular Biology 1999; 19: 5872-5881.

Pasterkamp G, Schoneveld AH, Hijnen DJ de Kleijn DPV, Teepen H, van der Wal AC, Borst C. Atherosclerotic arterial remodeling and the localization of macrophages and matrix metalloproteases 1, 2 and 9 in the human coronary artery. Atherosclerosis 2000; 150: 245-253.

Patra D, Wang SX, Kumagai A, Dunphy WG. The Xenopus Suc1/Cks protein promotes the phosphorylation of G(2)/M regulators. Journal of Biological Chemistry 1999; 274: 36839-36842.

Pearson DA, Frankel EN, Aeschbach R, German JB. Inhibition of endothelial cell mediated low-density lipoprotein oxidation by green tea extracts. Journal of Agricultural and Food Chemistry 1998; 46: 1445-1449.

Pedersen TR, Kjekshus J, Berg K, Haghfelt T, Faergeman O, Thorgeirsson G, Pyorala K, Miettinen T *et al.* Randomized Trial Of Cholesterol-Lowering In 4444 Patients With Coronary-Heart-Disease - The Scandinavian Simvastatin Survival Study (4s). Lancet 1994; 344: 1383-1389.

Pemberton LF, Blobel G, Rosenblum JS. Transport Routes Through The Nuclear Pore complex. Current Opinion in Cell Biology 1998; 10: 392-399.

Pendurthi UR, Nao LVM. Suppression of transcription factor Egr-1 by curcumin. Thrombosis Research 2000; 97: 179-189.

Pendurthi UR, Rao LVM. Effect of wine phenolics and stilbene analogues on tissue factor expression in endothelial cells. Thrombosis Research 2002a; 106: 205-211.

Pendurthi UR, Rao LVM. Resveratrol suppresses agonist-induced monocyte adhesion to cultured human endothelial cells. Thrombosis Research 2002b; 106: 243-248.

Pendurthi UR, Meng F, Mackman N, Rao LVM. Mechanism of resveratrol-mediated suppression of tissue factor gene expression. Thrombosis and Haemostasis 2002c; 87: 155-162.

Pendurthi UR, Williams JT, Rao LVM. Inhibition of tissue factor gene activation in cultured endothelial cells by curcumin - Suppression of activation of transcription factors Egr-1, AP-1, and NF-kappa B. Arteriosclerosis Thrombosis and Vascular Biology 1997; 17: 3406-3413.

Peng Q, Wei Z, Lau BHS. Pycnogenol inhibits tumor necrosis factor-alpha-induced nuclear factor kappa B activation and adhesion molecule expression in human vascular endothelial cells. Cellular and Molecular Life Sciences 2000; 57: 834-841.

Pettit AI, Wong RKM, Lee V, Jennings S, Quinn PA, Ng LL. Increased free radical production in hypertension due to increased expression of the NADPH oxidase subunit p22(phox) in lymphoblast cell lines. Journal of Hypertension 2002; 20: 677-683.

Phillips RJ, Ghosh S. Regulation of I kappa B beta in WEHI 231 mature B cells. Molecular and Cellular Biology 1997; 17: 4390-4396.

Platt JL, Nath KA. Heme oxygenase: Protective gene or Trojan horse. Nature Medicine 1998; 4: 1364-1365.

Plummer SM, Holloway KA, Manson MM, Munks RJL, Kaptein A, Farrow S, Howells L. Inhibition of cyclo-oxygenase 2 expression in colon cells by the chemopreventive agent curcumin involves inhibition of NF-kappa B activation via the NIK/IKK signalling complex. Oncogene 1999; 18: 6013-6020.

Polyak K, Xia Y, Zweier JL, Kinzler KW, Vogelstein B. A model for p53-induced apoptosis. Nature 1997; 389: 300-305.

Pontremoli S, Salamino F, Sparatore B, Detullio R, Patrone M, Tizianello A, Melloni E Enhanced activation of the respiratory burst oxidase in neutrophils from hypertensive patients. Biochemical and Biophysical Research Communications 1989; 158: 966-972.

Poston RN, Haskard DO, Coucher JR, Gall NP, Johnsontidey RR. Expression of intercellular-adhesion molecule-1 in atherosclerotic plaques. American Journal of Pathology 1992; 140: 665-673.

Qin ZH, Chen RW, Wang YM, Nakai M, Chuang DM, Chase TN. Nuclear factor kappa B nuclear translocation upregulates c-Myc and p53 expression during NMDA receptor-mediated apoptosis in rat striatum. Journal of Neuroscience 1999; 19: 4023-4033.

Rabbitts TH, Lefranc MP, Stinson MA, Sims JE, Schroder J, Steinmetz M, Spurr NL, Solomon E, Goodfellow PN The chromosomal location of T-cell receptor genes and a T-cell rearranging gene - possible correlation with specific translocations in human T-cell leukemia. EMBO Journal 1985; 4: 1461-1465.

RamirezBosca A, Soler A, Gutierrez MAC, Alvarez JL, Almagro EQ. Antioxidant Curcuma extracts decrease the blood lipid peroxide levels of human subjects. Age 1995; 18: 167-169.

Ramirez-Tortosa MC, Mesa MD, Aguilera MC, Quiles JL, Baro L, Ramirez-Tortosa CL, Martinez-Victoria E, Gil AOral administration of a turmeric extract inhibits LDL oxidation

and has hypocholesterolemic effects in rabbits with experimental atherosclerosis. Atherosclerosis 1999; 147: 371-378.

Ramirez-Tortosa. Functional food. The consumer, the products and the evidence. Sadler MJ, Saltmarsh M editors. London. 1998.

Rapaport SI, Rao LVM. The tissue factor pathway - how it has become a prima-ballerina. Thrombosis and Haemostasis 1995; 74: 7-17.

Reed SI. Control of the G(1)/S transition. Cancer Surveys 1997; 29: 7-23.

Reeve VE, Tyrrell RM. Heme oxygenase induction mediates the photoimmunoprotective activity of UVA radiation in the mouse. Proceedings of the National Academy of Sciences of the United States of America 1999; 96: 9317-9321.

Reinhard M, Giehl K, Abel K, Haffner C, Jarchau T, Hoppe V, Jockusch BM, and Walter U. The proline-rich focal adhesion and microfilament protein VASP is a ligand for profilins. EMBO Journal 14(8), 1583-1589. 1995.

Renaud S, Delorgeril M. Wine, alcohol, platelets, and the french paradox for coronary heart-disease. Lancet 1992; 339: 1523-1526.

RiceEvans CA, Miller NJ, Paganga G. Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free Radical Biology and Medicine 1996; 20: 933-956.

Riemersma RA, Wood DA, Macintyre CCA, Elton RA, Gey KF, Oliver MF. Risk of angina-pectoris and plasma-concentrations of vitamin-A, vitamin-C, and vitamin-E and carotene. Lancet 1991; 337: 1-5.

Rijnkels JM, Delsing DBJM, van der Reijden AC, Alink GM. Effects of vegetables fruit extracts and indole-3-carbinol on stearic acid-modulated intercellular communication and cytochrome P450-IA activity. Environmental Toxicology and Pharmacology 1998; 6: 103-109.

Rimm EB, Giovannucci EL, Willett WC Colditz GA, Ascherio A, Rosner B, Stampfer MJ. Prospective-study of alcohol-consumption and risk of coronary- disease in men. Lancet 1991; 338: 464-468.

Rimm EB, Klatsky A, Grobbee D, Stampfer MJ. Review of moderate alcohol consumption and reduced risk of coronary heart disease: Is the effect due to beer, wine, or spirits? British Medical Journal 1996; 312: 731-736.

Rimm EB, Stampfer MJ, Ascherio A, Giovannucci E, Colditz GA, Willett WC. Vitamin-E consumption and the risk of coronary heart-disease in men. New England Journal of Medicine 1993; 328: 1450-1456.

Rippmann JF, Hobbie S, Daiber C Guilliard B, Bauer M, Birk J, Nar H, Garin-Chesa P, Rettig WJ, Schnapp A. Phosphorylation-dependent proline isomerization catalyzed by Pin1 is essential for tumor cell survival and entry into mitosis. Cell Growth & Differentiation 2000; 11: 409-416.

Rizzardini M, Terao M, FalcianI F, Cantoni L. Cytokine induction of heme oxygenase messenger-RNA in mouse-liver - interleukin-1 transcriptionally activates the heme oxygenase gene. Biochemical Journal 1993; 290: 343-347.

Rockman HA, Koch WJ, Lefkowitz RJ. Seven-transmembrane-spanning receptors and heart function. Nature 2002; 415: 206-212.

Rollins BJ. Monocyte chemoattractant protein 1, a potential regulator of monocyte recruitment in inflammatory disease. Molecular Medicine Today 1996; 2: 198-204.

Rollins BJ, Yoshimura T, Leonard EJ, Pober JS. Cytokine-activated human endothelial-cells synthesize and secrete a monocyte chemoattractant, MCP-1/JE. American Journal of Pathology 1990; 136: 1229-1233.

Roman RJ, AlonsoGarcia M, Wilson TW. Renal P450 metabolites of arachidonic acid and the development of hypertension in Dahl salt-sensitive rats. American Journal of Hypertension 1997; 10: S63-S67.

Roman RJ, Maier KG, Sun CW, Harder DR, Alonso-Galicia M. Renal and cardiovascular actions of 20-hydroxyeicosatetraenoic acid and epoxyeicosatrienoic acids. Clinical and Experimental Pharmacology and Physiology 2000; 27: 855-865.

Romanic AM, Madri JA. The induction of 72-kd gelatinase in T-cells upon adhesion to endothelial-cells is VCAM-1 dependent. Journal of Cell Biology 1994; 125: 1165-1178.

Rosenfeld ME, Prichard L, Shiojiri N, Fausto N. Prevention of hepatic apoptosis and embryonic lethality in RelA/TNFR-1 double knockout mice. American Journal of Pathology 2000; 156: 997-1007.

Rosenfeld ME, Ylaherttuala S, Lipton BA, Ord VA, Witztum JL, Steinberg D. Macrophage colony-stimulating factor messenger-RNA and protein in atherosclerotic lesions of rabbits and humans. American Journal of Pathology 1992; 140: 291-300.

Ross R. The pathogenesis of atherosclerosis - a perspective for the 1990s. Nature 1993; 362: 801-809.

Ross R. Cell biology of atherosclerosis. Annual Review of Physiology 1995; 57: 791-804

Rothe M, Sarma V, Dixit VW, Goeddel DV. TRAF2-mediated activation of NF-kappa-B by TNF receptor-2 and CD40. Science 1995a; 269: 1424-1427.

Rothe M, Pan MG, Henzel WJ, Ayres TM, Goeddel DV. The TNFR2-TRAF signaling complex contains two novel proteins related to baculoviral-inhibitor of apoptosis proteins. Cell 1995b; 83: 1243-1252.

Rushmore TH, King RG, Paulson KE, Pickett CB. Regulation of glutathione s-transferase ya subunit gene-expression - identification of a unique xenobiotic-responsive element controlling inducible expression by planar aromatic-compounds. Proceedings of the National Academy of Sciences of the United States of America 1990; 87: 3826-3830.

Russell DW, Setchell KDR. Bile-acid biosynthesis. Biochemistry 1992a; 31: 4737-4749.

Russell DW. Cholesterol-biosynthesis and metabolism. Cardiovascular Drugs and Therapy 1992b; 6: 103-110.

Rutter JL, Benbow U, Coon CI, Brinckerhoff CE. Cell-type specific regulation of human interstitial collagenase-1 gene expression by interleukin-1 beta (IL-1 beta) in human fibroblasts and BC-8701 breast cancer cells. Journal of Cellular Biochemistry 1997; 66: 322-336.

Sacks FM, Pfeffer MA, Moye LA, Rouleau JL, Rutherford JD, Cole TG, Brown L, Warnica JW, Arnold JMO, Wun CC, Davis BR, Braunwald E. The effect of pravastatin on coronary events after myocardial infarction in patients with average cholesterol levels. New England Journal of Medicine 1996; 335: 1001-1009.

Sacks FM, Svetkey LP, Vollmer WM, Appel LJ, Bray GA, Harsha D, Obarzanek E, Conlin PR, Miller ER, Simons-Morton DG, Karanja N, Lin PH. Effects on blood pressure of reduced dietary sodium and the dietary approaches to stop hypertension (DASH) diet. New England Journal of Medicine 2001; 344: 3-10.

Sacks FM, Appel LJ, Moore TJ, Obarzanek E , Vollmer WM, Svetkey LP, Bray GA, Vogt TM, Cutler JA, Windhauser MM, Lin PH, Karanja N. A dietary approach to prevent hypertension: A review of the Dietary Approaches to Stop Hypertension (DASH) study. Clinical Cardiology 1999; 22 (7): 6-10.

Sakai T, Kambe F, Mitsuyama H, Ishiguro N, Kurokouchi K, Takigawa M, Iwata H, Seo H. Tumor necrosis factor alpha induces expression of genes for matrix degradation in human chondrocyte-like HCS-2/8 cells through activation of NF-kappa B: Abrogation of the tumor necrosis factor alpha effect by proteasome inhibitors. Journal of Bone and Mineral Research 2001; 16: 1272-1280.

Saksela O, Rifkin DB. Cell-associated plasminogen activation - regulation and physiological functions. Annual Review of Cell Biology 1988; 4: 93-126.

Salah N, Miller NJ, Paganga G, Tijburg L, Bolwell GP, Riceevans C. Polyphenolic flavanols as scavengers of aqueous-phase radicals and as chain-breaking antioxidants. Archives of Biochemistry and Biophysics 1995; 322: 339-346.

Sammut IA, Foresti R, Clark JE, Exon DJ, Vesely MJJ, Sarathchandra P, Green CJ, Motterlini R. Carbon monoxide is a major contributor to the regulation of vascular tone in aortas expressing high levels of haeme oxygenase-1. British Journal of Pharmacology 1998; 125: 1437-1444.

Sanders TH, McMichael RW, Hendrix KW. Occurrence of resveratrol in edible peanuts. Journal of Agricultural and Food Chemistry 2000; 48: 1243-1246.

Sandor V, Senderowicz A, Mertins S, Sackett D, Sausville E, Blagosklonny MV, Bates SE. P21-dependent G(1) arrest with downregulation of cyclin D1 and upregulation of cyclin E by the histone deacetylase inhibitor FR901228. British Journal of Cancer 2000; 83: 817-825.

Santee SM, OwenSchaub LB. Human tumor necrosis factor receptor p75/80 (CD120b) gene structure and promoter characterization. Journal of Biological Chemistry 1996; 271: 21151-21159.

Satriano JA, Shuldiner M, Hora K, Xing Y, Shan Z, Schlondorff D. Oxygen radicals as 2nd messengers for expression of the monocyte chemoattractant protein, JE/MCP-1, and the monocyte colony-stimulating factor, CSF-1, in response to tumor-necrosis-factor-alpha and immunoglobulin-g - evidence for involvement of reduced nicotinamide adenine-dinucleotide phosphate (NADPH)-dependent oxidase. Journal of Clinical Investigation 1993; 92: 1564-1571.

Sazuka M, Imazawa H, Shoji Y, Mita T, Hara Y, Isemura M. Inhibition of collagenases from mouse lung carcinoma cells by green tea catechins and black tea theaflavins. Bioscience Biotechnology and Biochemistry 1997; 61: 1504-1506.

Schmidt AM, Hori O, Chen JX, Li JF, Crandall J, Zhang JH, Cao R, Yan SD, Brett J, Stern D. Advanced glycation endproducts interacting with their endothelial receptor induce expression of vascular cell-adhesion molecule-1 (VCAM-1) in cultured human endothelial-cells and in mice - a potential mechanism for the accelerated vasculopathy of diabetes. Journal of Clinical Investigation 1995; 96: 1395-1403.

Schmidt KN, Traenckner EB-M, Meier B, Baeuerle P. Indiction of oxidative stress by okadaic acid is required for activation of transcription factor NF-κB. Journal of Biological Chemistry 1995; 270: 27136-27142.

Schneider A, Martin-Villalba A, Weih F, Vogel J, Wirth T, Schwaninger M. NF-kappa B is activated and promotes cell death in focal cerebral ischemia. Nature Medicine 1999; 5: 554-559.

Schneider E, Montenarh M, Wagner P. Regulation of CAK kinase activity by p53. Oncogene 1998; 17: 2733-2741.

Schneiderman J, Loskutoff DJ. Plasminogen-activator inhibitors. Trends in Cardiovascular Medicine 1991; 1: 99-102.

Schreck R, Rieber P, Baeuerle PA. Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-kappa-B transcription factor and HIV-1. EMBO Journal 1991; 10: 2247-2258.

Schwartz SM. A protective player in the vascular response to injury. Nature Medicine 2001; 7: 656-657.

Seifert R, Hilgenstock G, Fassbender M, Distler A. Regulation of the superoxide-forming NADPH oxidase of human neutrophils is not altered in essential-hypertension. Journal of Hypertension 1991; 9: 147-153.

Sen R, Baltimore D. Multiple nuclear factors interact with the immunoglobulin enhancer sequences. Cell 1986; 46: 705-716.

Shakhov AN, Collart MA, Vassalli P, Nedospasov SA, and Jongeeneel CV. Kappa-B-type enhancers are involved in lipopolysaccharide-mediated transcription activation of tumour necrosis factor-alpha gene in primary macrophages. Journal of Experimental Medicine 171(1), 35-47. 1990.

Shen MH, Stukenberg PT, Kirschner MW, Lu KP. The essential mitotic peptidyl-prolyl isomerase Pin1 binds and regulates mitosis-specific phosphoproteins. Genes and Development 1998; 12: 706-720.

Shertzer HG, Berger ML, Tabor MW. Intervention in free-radical mediated hepatotoxicity and lipid-peroxidation by indole-3-carbinol. Biochemical Pharmacology 1988; 37: 333-338.

Shiffman D, Mikita T, Tai JTN, Wade DP, Porter JG, Seilhamer JJ, Somogyi R, Liang SD, Lawn RM. Large scale gene expression analysis of cholesterol-loaded macrophages. Journal of Biological Chemistry 2000; 275: 37324-37332.

Siebenlist U, Franzoso G, Brown K. Structure, regulation and function of NF-kappa-B. Annual Review of Cell Biology 1994; 10: 405-455.

Siemann EH, Creasy LL. Concentration of the phytoalexin resveratrol in wine. American Journal of Enology and Viticulture 1992; 43: 49-52.

Singh R, Barden A, Mori T, Beilin L. Advanced glycation end-products: a review. Diabetologia 2001; 44: 129-146.

Singh R, Green MR. Sequence-specific binding of transfer-RNA by glyceraldehyde-3-phosphate dehydrogenase. Science 1993; 259: 365-368.

Singh S, Aggarwal BB. Activation of transcription factor NF-kappa-B is suppressed by curcumin (diferulolylmethane). Journal of Biological Chemistry 1995; 270: 24995-25000.

Sirianni R, Seely JB, Attia G Stocco DM, Carr BR, Pezzi V, Rainey WE. Liver receptor homologue-1 is expressed in human steroidogenic tissues and activates transcription of genes encoding steroidogenic enzymes. Journal of Endocrinology 2002; 174: R13-R17.

Sizemore N, Leung S, Stark GR. Activation of phosphatidylinositol 3-kinase in response to interleukin-1 leads to phosphorylation and activation of the NF-kappa B p65/RelA subunit. Molecular and Cellular Biology 1999; 19: 4798-4805.

Smith EB. Haemostatic factors and atherogenesis. Atherosclerosis 1996; 124: 137-143.

Snapper CM, Zelazowski P, Rosas FR, Kehry MR, Tian M, Baltimore D, Sha WC. B cells from p50/NF-kappa B knockout mice have selective defects in proliferation, differentiation, germ-line C-H transcription, and Ig class switching. Journal of Immunology 1996; 156: 183-191.

Sofer-Levi Y, Resnitzky D. Apoptosis induced by ectopic expression of cyclin D1 but not cyclin E. Oncogene 1996; 13: 2431-2437.

Soleas GJ, Diamandis EP, Goldberg DM. Wine as a biological fluid: History, production, and role in disease prevention. Journal of Clinical Laboratory Analysis 1997; 11: 287-313.

Sompayrac L, Jane S, Burn TC, Tenen DG, Danna KJ. Overcoming limitations of the messenger-RNA differential display technique. Nucleic Acids Research 1995; 23: 4738-4739.

Song HY, Rothe M, Goeddel DV. The tumor necrosis factor-inducible zinc finger protein A20 interacts with TRAF1/TRAF2 and inhibits NF-kappa B activation. Proceedings of the National Academy of Sciences of the United States of America 1996; 93: 6721-6725.

Southgate KM, Fisher M, Banning AP, Thurston VJ, Baker AH, Fabunmi RP, Groves PH, Davies M, Newby AC. Upregulation of basement membrane-degrading metalloproteinase secretion after balloon injury of pig carotid arteries. Circulation Research 1996; 79: 1177-1187.

Sovak MA, Bellas RE, Kim DW, Zanieski GJ, Rogers AE, Traish AM, Sonenshein GE. Aberrant nuclear factor-kappa B/Rel expression and the pathogenesis of breast cancer. Journal of Clinical Investigation 1997; 100: 2952-2960.

Speir E, Modali R, Huang ES Leon MB, Shawl F, Finkel T, Epstein SE. Potential role of human cytomegalovirus and p53 interaction in coronary restenosis. Science 1994; 265: 391-394.

Squires MS, Hudson EA, Howells L, Sale S, Houghton CE, Jones JL, Fox LH, Dickens M, Prigent SA, Manson MM. Relevance of mitogen activated protein kinase (MAPK) and phosphotidylinositol-3-kinase/protein kinase B (P13K/PKB) pathways to induction of apoptosis by curcumin in breast cells. Biochemical Pharmacology 2003; 65: 361-376.

Squires MS. PhD Thesis. University of Leicester 2001.

St leger AS, Cochrane AL, and Moore F. Factors associated with cardiac mortality in developed countries with particular reference to consumption of wine. Lancet 1979; 1: 1017-1020.

Stampfer MJ, Hennekens CH, Manson JE, Colditz GA, Rosner B, Willett WC. Vitamin-E consumption and the risk of coronary-disease in women. New England Journal of Medicine 1993; 328: 1444-1449.

Stegh AH, Schickling O, Ehret A, Scaffidi C, Peterhansel C, Hofmann TG, Grummt I, Krammer PH, Peter ME. DEDD, a novel death effector domain-containing protein, targeted to the nucleolus. EMBO Journal 1998; 17: 5974-5986.

Stehlik C, de Martin R, Kumabashiri I, Schmid JA, Binder BR, Lipp J. Nuclear factor (NF)-kappa B-regulated X-chromosome-linked IAP gene expression protects endothelial cells from tumor necrosis factor alpha-induced apoptosis. Journal of Experimental Medicine 1998; 188: 211-216.

Stennicke HR, Salvesen GS. Catalytic properties of the caspases. Cell Death and Differentiation 1999; 6: 1054-1059.

Stephens NG, Parsons A, Schofield PM, Kelly F, Cheeseman K, Mitchinson MJ, Brown MJ. Randomised controlled trial of vitamin E in patients with coronary disease: Cambridge Heart Antioxidant Study (CHAOS). Lancet 1996; 347: 781-786.

Stocker R, Yamamoto Y, McDonagh AF, Glazer AN, Ames BN. Bilirubin is an antioxidant of possible physiological importance. Science 1987; 235: 1043-1046.

Stuhlmeier KM. Activation and regulation of Hsp32 and Hsp70. European Journal of Biochemistry 2000; 267: 1161-1167.

Su P, Kaushal KM, Kroetz DL. Inhibition of renal arachidonic acid omega-hydroxylase activity with ABT reduces blood pressure in the SHR. American Journal of Physiology-Regulatory Integrative and Comparative Physiology 1998; 44: R426-R438.

Subbaramaiah K, Chung WJ, Michaluart P Telang N, Tanabe T, Inoue H, Jang MS, Pezzuto JM, Dannenberg AJ. Resveratrol inhibits cyclooxygenase-2 transcription and activity in phorbol ester-treated human mammary epithelial cells. Journal of Biological Chemistry 1998; 273: 21875-21882.

Sun AY, Chen YM, JamesKracke M , Wixom P, Cheng Y. Ethanol-induced cell death by lipid peroxidation in PC12 cells. Neurochemical Research 1997; 22: 1187-1192.

Susin SA, Lorenzo HK, Zamzami N, Marzo I, Snow BE, Brothers GM, Mangion J, Jacotot E, Costantini P, Loeffler M, Larochette N, Goodlett DR, Aebersold R, Siderovski DP, Penninger JM, Kroemer G. Molecular characterization of mitochondrial apoptosis-inducing factor. Nature 1999; 397: 441-446.

Suto A, Bradlow HL, Wong GY, Osborne MP, Telang NT. Persistent Estrogen responsiveness of ras oncogene-transformed mouse mammary epithelial-cells. Steroids 1992; 57: 262-268.

Suzuki A, Tsutomi Y, Akahane K, Araki T, Miura M. Resistance to Fas-mediated apoptosis: activation of caspase 3 is regulated by cell cycle regulator p21(WAF1) and IAP gene family ILP. Oncogene 1998; 17: 931-939.

Swei A, Lacy F, DeLano FA, Schmid-Schonbein GW. Oxidative stress in the Dahl hypertensive rat. Hypertension 1997; 30: 1628-1633.

Taketani S, Kohno H, Yoshinaga T, Tokunaga R. Induction of heme oxygenase in rat hepatoma-cells by exposure to heavy-metals and hyperthermia. Biochemistry International 1988; 17: 665-672.

Takeya M, Yoshimura T, Leonard EJ, Takahashi K. Detection of monocyte chemoattractant protein-1 in human atherosclerotic lesions by an anti-monocyte chemoattractant protein-1 monoclonal-antibody. Human Pathology 1993; 24: 534-539.

Tan MJ, Wang YX, Guan KL, Sun Y. PTCF-beta, a type beta transforming growth factor (TGF-beta) superfamily member, is a p53 target gene that inhibits tumor cell growth via TGF-beta signaling pathway. Proceedings of the National Academy of Sciences of the United States of America 2000; 97: 109-114.

Tan XH, Hu DR, Li SR, Han Y, Zhang YL, Zhou DY. Differences of four catechins in cell cycle arrest and induction of apoptosis in LoVo cells. Cancer Letters 2000; 158: 1-6.

Tanaka M, Fuentes ME, Yamaguchi K, Durnin MH, Dalrymple SA, Hardy KL, Goeddel DV. Embryonic lethality, liver degeneration, and impaired NF-kappa B activation in IKK-beta-deficient mice. Immunity 1999; 10: 421-429.

Taniguchi M, Miura K, Iwao H, Yamanaka S. Quantitative assessment of DNA microarrays - Comparison with Northern blot analyses. Genomics 2001; 71: 34-39.

Tanner FC, Yang ZY, Duckers E, Gordon D, Nabel GJ, Nabel EG. Expression of cyclin-dependent kinase inhibitors in vascular disease. Circulation Research 1998; 82: 396-403.

Taubman MB, Fallon JT, Schecter AD, Giesen P, Mendlowitz M, Fyfe BS, Marmur JD, Nemerson Y. Tissue factor in the pathogenesis of atherosclerosis. Thrombosis and Haemostasis 1997; 78: 200-204.

Taylor WR, DePrimo SE, Agarwal A, Agarwal ML, Schonthal AH, Katula KS, Stark GR. Mechanisms of G2 arrest in response to overexpression of p53. Molecular Biology of the Cell 1999; 10: 3607-3622.

Taylor WR, Schonthal AH, Galante J, Stark GR. p130/E2F4 binds to and represses the cdc2 promoter in response to p53. Journal of Biological Chemistry 2001; 276: 1998-2006.

Telang NT, Bradlow HL, Osborne MP. Molecular and endocrine biomarkers in noninvolved breast - relevance to cancer chemoprevention. Journal of Cellular Biochemistry 1992; 161-169.

Telang NT, Katdare M, Bradlow HL, Osborne MP, Fishman J. Inhibition of proliferation and modulation of estradiol metabolism: Novel mechanisms for breast cancer prevention

by the phytochemical indole-3-carbinol. Proceedings of the Society for Experimental Biology and Medicine 1997; 216: 246-252.

Telang NT, Narayanan R, Bradlow HL, Osborne MP. Coordinated expression of intermediate biomarkers for tumorigenic transformation in ras-transfected mouse mammary epithelial-cells. Breast Cancer Research and Treatment 1991; 18: 155-163.

Terry CM, Clikeman JA, Hoidal JR, Callahan KS. Effect of tumor necrosis factor-alpha and interleukin-1 alpha on heme oxygenase-1 expression in human endothelial cells. American Journal of Physiology-Heart and Circulatory Physiology 1998; 43: H883-H891.

Thaloor D, Singh AK, Sidhu GS, Prasad PV, Kleinman HK, Maheshwari RK. Inhibition of angiogenic differentiation of human umbilical vein endothelial cells by curcumin. Cell Growth and Differentiation 1998; 9: 305-312.

Thompson DA, Belinsky G, Chang THT, Jones DL, Schlegel R, Munger K. The human papillomavirus-16 E6 oncoprotein decreases the vigilance of mitotic checkpoints. Oncogene 1997; 15: 3025-3035.

Thompson JE, Phillips RJ, Erdjumentbromage H, Tempst P, Ghosh S. I-kappa-B-beta regulates the persistent response in a biphasic activation of NF-kappa-B. Cell 1995; 80: 573-582.

Thomson JR, Machado RD, Pauciulo MW,, Morgan NV, Humbert M, Elliott GC, Ward K, Yacoub M, Mikhail G, Rogers P, Newman J, Wheeler L, Higenbottam T, Gibbs JSR, Egan J, Crozier A, Peacock A, Allcock R, Corris P, Loyd JE, Trembath RC, Nichols WC. Sporadic primary pulmonary hypertension is associated with germline mutations of the gene encoding BMPR-II, a receptor member of the TGF-beta family. Journal of Medical Genetics 2000; 37: 741-745.

Tijburg LBM, Mattern T, Folts JD, Weisgerber UM, Katan MB. Tea flavonoids and cardiovascular diseases: A review. Critical Reviews in Food Science and Nutrition 1997; 37: 771-785.

Tobi SE, Gilbert M, Paul N, McMillan TJ. The green tea polyphenol, epigallocatechin-3-gallate, protects against the oxidative cellular and genotoxic damage of UVA radiation. International Journal of Cancer 2002; 102: 439-444.

Toda S, Miyase T, Arichi H, Tanizawa H, Takino Y. Natural antioxidants - antioxidative components isolated from rhizome of curcuma-longa. Chemical and Pharmaceutical Bulletin 1985; 33: 1725-1728.

Tojima Y, Fujimoto A, Delhase M, Chen Y, Hatakeyama S, Nakayama K, Kaneko Y, Nimura Y, Motoyama N, Ikeda K, Karin M, Nakanishi M. NAK is an I kappa B kinase-activating kinase. Nature 2000; 404: 778-782.

Tokunaga S, White IR, Frost C, Tanaka K, Kono S, Tokudome S, Akamatsu T, Moriyama T, Zakouji H. Green tea consumption and serum lipids and lipoproteins in a population of healthy workers in Japan. Annals of Epidemiology 2002; 12: 157-165.

Townsend AJ, Fields WR, Haynes RL, Doss AJ, Li Y, Doehmer J, Morrow CS. Chemoprotective functions of glutathione S-transferases in cell lines introduced to express specific isozymes by stable transfection. Chemico-Biological Interactions 1998; 112: 389-407.

Tsai SH, Lin-Shiau SY, Lin JK. Suppression of nitric oxide synthase and the down-regulation of the activation of NF kappa B in macrophages by resveratrol. British Journal of Pharmacology 1999; 126: 673-680.

Tsuchiya H. Effects of green tea catechins on membrane fluidity. Pharmacology 1999; 59: 34-44.

Turrens JF, Lariccia J, Nair MG. Resveratrol has no effect on lipoprotein profile and does not prevent peroxidation of serum lipids in normal rats. Free Radical Research 1997; 27: 557-562.

Turton NJ, Judah DJ, Riley J, Davies R, Lipson D, Styles JA, Smith AG, Gant TW. Gene expression and amplification in breast carcinoma cells with intrinsic and acquired doxorubicin resistance. Oncogene 2001; 20: 1300-1306.

Uzawa K, Grzesik WJ, Nishiura T, Kuznetsov SA, Robey PG, Brenner DA, Yamauchi M. Differential expression of human lysyl hydroxylase genes, lysine hydroxylation, and cross-linking of type I collagen during osteoblastic differentiation in vitro. Journal of Bone and Mineral Research 1999; 14: 1272-1280.

Vachharajani TJ, Work J, Issekutz AC, Granger DN. Heme oxygenase modulates selectin expression in different regional vascular beds. American Journal of Physiology-Heart and Circulatory Physiology 2000; 278: H1613-H1617.

Van Amelsvoort JMM, Hof KHV, Mathot JNJJ, Mulder TPJ, Wiersma A, Tijburg LBM. Plasma concentrations of individual tea catechins after a single oral dose in humans. Xenobiotica 2001; 31: 891-901.

Van Heerebeek L, Meischl C, Stooker W, Meijer CJLM, Niessen HWM, Roos D. NADPH oxidase(s): new source(s) of reactive oxygen species in the vascular system? Journal of Clinical Pathology 2002; 55: 561-568.

Van Vlijmen BJM, Gerritsen G, Franken AL, Boesten SM, Kockx MM, Gijbels MJ, Vierboom MP, van Eck M, van de Water B, van Berkel TJC, Havekes LM. Macrophage p53 deficiency leads to enhanced atherosclerosis in APOE*3-Leiden transgenic mice. Circulation Research 2001; 88: 780-786.

vanHetHof KH, deBoer HSM, Wiseman SA, Lien N, Weststrate JA, Tijburg LBM. Consumption of green or black tea does not increase resistance of low-density lipoprotein to oxidation in humans. American Journal of Clinical Nutrition 1997; 66: 1125-1132.

vanHetHof KH, Kivits GAA, Weststrate JA, Tijburg LBM. Bioavailability of catechins from tea: the effect of milk. European Journal of Clinical Nutrition 1998; 52: 356-359.

Vasquez-Vivar J, Kalyanaraman B, Martasek P, Hogg N, Masters BSS, Karoui H, Tordo P, Pritchard KA. Superoxide generation by endothelial nitric oxide synthase: The influence of cofactors. Proceedings of the National Academy of Sciences of the United States of America 1998; 95: 9220-9225.

Venugopal R, Jaiswal AK. Nrf2 and Nrf1 in association with Jun proteins regulate antioxidant response element-mediated expression and coordinated induction of genes encoding detoxifying enzymes. Oncogene 1998; 17: 3145-3156.

Verhagen AM, Ekert PG, Pakusch M, Silke J, Connolly LM, Reid GE, Moritz RL, Simpson RJ, Vaux DL. Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. Cell 2000; 102: 43-53.

Verlangieri AJ, Kapeghian JC, Eldean S, Bush M. Fruit and vegetable consumption and cardiovascular mortality. Medical Hypotheses 1985; 16: 7-15.

Vile GF, Tyrrell RM. Oxidative stress resulting from ultraviolet-A irradiation of human skin fibroblasts leads to a heme oxygenase-dependent increase in ferritin. Journal of Biological Chemistry 1993; 268: 14678-14681.

Vincenti MP, Coon CI, Brinckerhoff CE. Nuclear factor kappa B p50 activates an element in the distal matrix metalloproteinase 1 promoter in interleukin-1 beta- stimulated synovial fibroblasts. Arthritis and Rheumatism 1998; 41: 1987-1994.

Vinson JA, Jang JH, Dabbagh YA, Serry MM, Cal SH. Plant polyphenols exhibit lipoprotein-bound antioxidant activity using an in-vitro oxidation model for heart-disease. Journal of Agricultural and Food Chemistry 1995; 43: 2798-2799.

Wadsworth TL, Koop DR. Effects of the wine polyphenolics quercetin and resveratrol on pro-inflammatory cytokine expression in RAW 264.7 macrophages. Biochemical Pharmacology 1999; 57: 941-949.

Wang GP, Siow YL, O K. Homocysteine stimulates nuclear factor kappa B activity and monocyte chemoattractant protein-1 expression in vascular smooth-muscle cells: a possible role for protein kinase C. Biochemical Journal 2000; 352: 817-825.

Wang SI, Mukhtar H. Gene expression profile in human prostate LNCaP cancer cells by

(-)epigallocatechin-3-gallate. Cancer Letters 2002; 182: 43-51.

Wang ZN, Bassett M, Rainey WE. Liver receptor homologue-1 is expressed in the adrenal and can regulate transcription of 11 beta-hydroxylase. Journal of Molecular Endocrinology 2001; 27: 255-258.

Wang ZR, Zou JG, Huang YZ, Cao KJ, Xu YN, Wu JM. Effect of resveratrol on platelet aggregation in vivo and in vitro. Chinese Medical Journal 2002; 115: 378-380.

Webster GA, Perkins ND. Transcriptional cross talk between NF-kappa B and p53. Molecular and Cellular Biology 1999; 19: 3485-3495.

Wei GL, Krasinski K, Kearney M, Isner JM, Walsh K, Andres V. Temporally and spatially coordinated expression of cell cycle regulatory factors after angioplasty. Circulation Research 1997; 80: 418-426.

Weinberg RA. The retinoblastoma protein and cell-cycle control. Cell 1995; 81: 323-330.

Weiner CP, Lizasoain I, Baylis SA, Knowles RG, Charles IG, Moncada S. Induction of calcium-dependent nitric-oxide synthases by sex hormones. Proceedings of the National Academy of Sciences of the United States of America 1994; 91: 5212-5216.

Wellman GC, Bonev AD, Nelson MT, Brayden JE. Gender differences in coronary artery diameter involve estrogen, nitric oxide, and Ca2+-dependent K+ channels. Circulation Research 1996; 79: 1024-1030.

Westerheide SD, Mayo MW, Anest V, Hanson JL, Baldwin AS. The putative oncoprotein Bcl-3 induces cyclin D1 to stimulate G(1) transition. Molecular and Cellular Biology 2001; 21: 8428-8436.

White RE, Darkow DJ, Lang JLF. Estrogen relaxes coronary-arteries by opening K+/Ca+ channels through a CGMP-dependent mechanism. Circulation Research 1995; 77: 936-942.

Whitehead TP, Robinson D, Allaway S, Syms J, Hale A. Effect of red wine ingestion on the antioxidant capacity of serum. Clinical Chemistry 1995; 41: 32-35.

Whiteside ST, Epinat JC, Rice NR, Israel A. I kappa B epsilon, a novel member of the I kappa B family, controls RelA and cRel NF-kappa B activity. EMBO Journal 1997a; 16: 1413-1426.

Whiteside ST, Israel A. I kappa B proteins: structure, function and regulation. Seminars in Cancer Biology 1997b; 8: 75-82.

Wilcox JN, Smith KM, Schwartz SM, Gordon D. Localization of tissue factor in the normal vessel wall and in the atherosclerotic plaque. Proceedings of the National Academy of Sciences of the United States of America 1989; 86: 2839-2843.

Wild AC, Moinova HR, Mulcahy RT. Regulation of gamma-glutamylcysteine synthetase subunit gene expression by the transcription factor Nrf2. Journal of Biological Chemistry 1999; 274: 33627-33636.

Williams HT, Fenna D, and Macbeth RA. Alpha tocopherol in the treatment of intermittent claudication. Surgery, Gynecology & Obstetrics 132(4), 662-666. 1971.

Willis D, Moore AR, Frederick R, Willoughby DA. Heme oxygenase: A novel target for the modulation of the inflammatory response. Nature Medicine 1996; 2: 87-90.

Wilson SH, Best PJM, Edwards WD, Holmes DR, Carlson PJ, Celermajer DS, Lerman A. Nuclear factor-kappa B immunoreactivity is present in human coronary plaque and enhanced in patients with unstable angina pectoris. Atherosclerosis 2002; 160: 147-153.

Wiman B. Plasminogen-activator inhibitor-1 (PAI-1) in plasma - its role in thrombotic disease. Thrombosis and Haemostasis 1995; 74: 71-76.

Winkler KE, Swenson KI, Kornbluth S, Means AR. Requirement of the prolyl isomerase Pin1 for the replication checkpoint. Science 2000; 287: 1644-1647.

Wiseman S, Mulder T, Rietveld A. Tea flavonoids: Bioavailability in vivo and effects on cell signaling pathways in vitro. Antioxidants and Redox Signaling 2001; 3: 1009-1021.

Wiseman SA, Balentine DA, Frei B. Antioxidants in tea. Critical Reviews in Food Science and Nutrition 1997; 37: 705-718.

Wolff B, Naumann M. INK4 cell cycle inhibitors direct transcriptional inactivation of NF-kappa B. Oncogene 1999; 18: 2663-2666.

Wood SM, Wiesener MS, Yeates KM, Okada N, Pugh CW, Maxwell PH, Ratcliffe PJ. Selection and analysis of a mutant cell line defective in the hypoxia-inducible factor-1 alpha-subunit (HIF-1 alpha) - Characterization of HIF-1 alpha-dependent and -independent hypoxia-inducible Gene expression. Journal of Biological Chemistry 1998; 273: 8360-8368.

Woods DB, Vousden KH. Regulation of p53 function. Experimental Cell Research 2001; 264: 56-66.

Wu HY, Lozano G. NF-kappa-B activation of p53 - a potential mechanism for suppressing cell-growth in response to stress. Journal of Biological Chemistry 1994; 269: 20067-20074.

Wulf GM, Ryo A, Wulf GG Lee SW, Niu TH, Petkova V, Lu KP. Pin1 is overexpressed in breast cancer and cooperates with Ras signaling in increasing the transcriptional activity of c-Jun towards cyclin D1. EMBO Journal 2001; 20: 3459-3472.

Xing HR, Lozano J, Kolesnick R. Epidermal growth factor treatment enhances the kinase activity of kinase suppressor of Ras. Journal of Biological Chemistry 2000; 275: 17276-17280.

Yaffe MB, Schutkowski M, Shen MH, Zhou XZ, Stukenberg PT, Rahfeld JU, Xu J, Kuang J, Kirschner MW, Fischer G, Cantley LC, Lu KP. Sequence-specific and phosphorylation-dependent proline isomerization: A potential mitotic regulatory mechanism. Science 1997; 278: 1957-1960.

Yang CS, Chen LS, Lee MJ, Balentine D, Kuo MC, Schantz SP. Blood and urine levels of tea catechins after ingestion of different amounts of green tea by human volunteers. Cancer Epidemiology Biomarkers and Prevention 1998; 7: 351-354.

Yang CS, Chung JY, Yang GY, Chhabra SK, Lee MJ. Tea and tea polyphenols in cancer prevention. Journal of Nutrition 2000; 130: 472S-478S.

Yang FJ, de Villers WJS, McClain CJ, Varilek GW. Green tea polyphenols block endotoxin-induced tumor necrosis factor-production and lethality in a murine model. Journal of Nutrition 1998; 128: 2334-2340.

Yang FJ, Oz HS, Barve S, De Villiers WJS, McClain CJ, Varilek GW. The green tea polyphenol (-)-epigallocatechin-3-gallate blocks nuclear factor-kappa B activation by inhibiting I kappa B kinase activity in the intestinal epithelial cell line IEC-6. Molecular Pharmacology 2001; 60: 528-533.

Yang GY, Liao J, Kim K, Yurkow EJ, Yang CS. Inhibition of growth and induction of apoptosis in human cancer cell lines by tea polyphenols. Carcinogenesis 1998; 19: 611-616.

Yang GY, Liao J, Li CC, Chung J, Yurkow EJ, Ho CT, Yang CS. Effect of black and green tea polyphenols on c-jun phosphorylation and H_2O_2 production in transformed and non-transformed human bronchial cell lines: possible mechanisms of cell growth inhibition and apoptosis induction. Carcinogenesis 2000; 21: 2035-2039.

Yang TTC, Koo MWL. Hypocholesterolemic effects of Chinese tea. Pharmacological Research 1997; 35: 505-512.

Yaron A, Hatzubai A, Davis M, Lavon I, Amit S, Manning AM, Andersen JS, Mann M, Mercurio F, Ben-Neriah Y. Identification of the receptor component of the I kappa B alpha-ubiquitin ligase. Nature 1998; 396: 590-594.

Yee WL, Wang Q, Agdinaoay T, Dang K, Chang HL, Grandinetti A, Franke AA, Theriault A. Green tea catechins decrease apolipoprotein B-100 secretion from HepG2 cells. Molecular and Cellular Biochemistry 2002; 229: 85-92.

Yet SF, Pellacani A, Patterson C, Tan L, Folta SC, Foster L, Lee WS, Hsieh CM, Perrella MA. Induction of heme oxygenase-1 expression in vascular smooth muscle cells - A link to endotoxic shock. Journal of Biological Chemistry 1997; 272: 4295-4301.

Yin YX, Terauchi Y, Solomon GG, Aizawa S, Rangarajan PN, Yazaki Y, Kadowaki T, Barrett JC. Involvement of p85 in p53-dependent apoptotic response to oxidative stress. Nature 1998; 391: 707-710.

Yokozawa T, Oura H, Sakanaka S, Ishigaki S, Kim M. Depressor effect of tannin in green tea on rats with renal hypertension. Bioscience Biotechnology and Biochemistry 1994; 58: 855-858.

You H, Zheng HW, Murray SA, Yu Q, Uchida T, Fan DM, Xiao ZXJ. IGF-1 induces Pin1 expression in promoting cell cycle S-phase entry. Journal of Cellular Biochemistry 2002; 84: 211-216.

You MJ, Ku PT, Hrdlickova R, Bose HR. ch-IAP1, a member of the inhibitor-of-apoptosis protein family, is a mediator of the antiapoptotic activity of the v-Rel oncoprotein. Molecular and Cellular Biology 1997; 17: 7328-7341.

Yu R, Chen C, Mo YY, Hebbar V, Owuor ED, Tann TH, Kong ANT. Activation of mitogen-activated protein kinase pathways induces antioxidant response element-mediated gene expression via a Nrf2-dependent mechanism. Journal of Biological Chemistry 2000; 275: 39907-39913.

Yu R, Lei W, Mandlekar S, Weber MJ, Der CJ, Wu J, Kong ANT. Role of a mitogen-activated protein kinase pathway in the induction of phase II detoxifying enzymes by chemicals. Journal of Biological Chemistry 1999; 274: 27545-27552.

Yu R, Mandlekar S, Lei W, Fahl WE, Tan TH, Kong ANT. p38 mitogen-activated protein kinase negatively regulates the induction of phase II drug-metabolizing enzymes that detoxify carcinogens. Journal of Biological Chemistry 2000b; 275: 2322-2327.

Yuhanna IS, MacRitchie AN, Lantin-Hermoso RL, Wells LB, Shaul PW. Nitric oxide (NO) upregulates NO synthase expression in fetal intrapulmonary artery endothelial cells. American Journal of Respiratory Cell and Molecular Biology 1999; 21: 629-636.

Yun J, Chae HD, Choy HE, Chung J, Yoo HS, Han MH, Shin DY. p53 negatively regulates cdc2 transcription via the CCAAT- binding NF-Y transcription factor. Journal of Biological Chemistry 1999; 274: 29677-29682.

Yusuf S, Dagenais G, Pogue J, Bosch J, Sleight P. Vitamin E supplementation and cardiovascular events in high-risk patients. New England Journal of Medicine 2000; 342: 154-160.

Zacchi P, Gostissa M, Uchida T, Salvagno C, Avolio F, Volinia S, Ronai Z, Blandino G, Schneider C, Del Sal G. The prolyl isomerase Pin1 reveals a mechanism to control p53 functions after genotoxic insults. Nature 2002; 419: 853-857.

Zbikowska HM, Olas B, Wachowicz B, Krajewski T. Response of blood platelets to resveratrol. Platelets 1999; 10: 247-252.

Zeldin DC. Epoxygenase pathways of arachidonic acid metabolism. Journal of Biological Chemistry 2001; 276: 36059-36062.

Zempo N, Kenagy RD, Au YPT Bendeck M, Clowes MM, Reidy MA, Clowes AW. Matrix metalloproteinases of vascular wall cells are increased in balloon-injured rat carotid-artery. Journal of Vascular Surgery 1994; 20: 209-217.

Zettler ME, Pierce GN. Cell cycle proteins and atherosclerosis. Herz 2000; 25: 100-107.

Zhang M, Chiang JYL. Transcriptional regulation of the human sterol 12 alpha-hydroxylase gene (CYP8B1) - Roles of hepatocyte nuclear factor 4 alpha in mediating bile acid repression. Journal of Biological Chemistry 2001; 276: 41690-41699.

Zhang WL, Lu Q, Xie ZJ, Mellgren RL. Inhibition of the growth of WI-38 fibroblasts by benzyloxycarbonyl-Leu-Leu-Tyr diazomethyl ketone: Evidence that cleavage of p53 by a calpain-like protease is necessary for G(1) to S-phase transition. Oncogene 1997; 14: 255-263.

Zhao Q, Lee FS. Mitogen-activated protein kinase ERK kinase kinases 2 and 3 activate nuclear factor-kappa B through I kappa B kinase-alpha and I kappa B kinase-beta. Journal of Biological Chemistry 1999; 274: 8355-8358.

Zheng HW, You H, Zhou XZ, Murray SA, Uchida T, Wulf G, Gu L, Tang XR, Lu KP, Xiao ZXJ. The prolyl isomerase Pin1 is a regulator of p53 in genotoxic response. Nature 2002; 419: 849-853.

Zhou P, Jiang W, Zhang YJ, Kahn SM, Schieren I, Santella RM, Weinstein IB. Antisense to cyclin D1 inhibits growth and reverses the transformed phenotype of human esophageal cancer-cells. Oncogene 1995; 11: 571-580.

Zhou XZ, Kops O, Werner A, Lu PJ, Shen MH, Stoller G, Kullertz G, Stark M, Fischer G, Lu KP. Pin1-dependent prolyl isomerization regulates dephosphorylation of Cdc25C and tau proteins. Molecular Cell 2000; 6: 873-883.

Zhou YF, Leon MB, Waclawiw MA, Popma JJ, Yu ZX, Finkel T, Epstein SE. Association between prior cytomegalovirus infection and the risk of restenosis after coronary atherectomy. New England Journal of Medicine 1996; 335: 624-630.

Ziegler SF, Ramsdell F, Alderson MR. The activation antigen CD69. Stem Cells 1994; 12: 456-465.

Zimmermann KC, Bonzon C, Green DR. The machinery of programmed cell death. Pharmacology and Therapeutics 2001; 92: 57-70.

Zohlnhofer D, Klein CA, Richter T Brandl R, Murr A, Nuhrenberg T, Schomig A, Baeuerle PA, Neumann FJ. Gene expression profiling of human stent-induced neointima by cDNA array analysis of microscopic specimens retrieved by helix cutter atherectomy - Detection of FK506-binding protein 12 upregulation. Circulation 2001; 103: 1396-1402.

Zong WX, Edelstein LC, Chen CL, Bash J, Gelinas C. The prosurvival Bcl-2 homolog Bfl-1/A1 is a direct transcriptional target of NF-kappa B that blocks TNF alpha-induced apoptosis. Genes and Development 1999; 13: 382-387.

Zou JG, Huang YZ, Chen Q, Wei EH, Hsieh TC, Wu JM. Resveratrol inhibits copper ion-induced and azo compound-initiated oxidative modification of human low density lipoprotein. Biochemistry and Molecular Biology International 1999; 47: 1089-1096.

Zwacka RM, Zhang YL, Zhou WH, Halldorson J, Engelhardt JF. Ischemia/reperfusion injury in the liver of BALB/c mice activates AP-1 and nuclear factor kappa B independently of I kappa B degradation. Hepatology 1998; 28: 1022-1030.

Publications

Published abstracts

Atherfold PA, Manson MM. The chemopreventive agent EGCG induces cell cycle arrest and apoptosis in HUVEC. Free Radical Biology and Medicine 2002; 33: 83 Suppl 2.

Posters presented

Atherfold PA and Manson MM. The chemopreventive agent EGCG induces cell cycle arrest and apoptosis in HUVEC. The Oxygen Society, The Society for Free Radical Biology and Medicine. November 2002, San Antonio, Texas, USA.

Atherfold PA, Fox LH, Howells LM, Hudson EA, Ruchatz H and Manson MM. Mechanisms of Epigallocatechin gallate in Human umbilical vein endothelial cells. American Association for Cancer Research (AACR), Annual Frontiers in Cancer Prevention meeting. October 2002, Boston, Massachusetts, USA.