Cell signalling pathways in mesothelial cells treated with mineral fibres

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by

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DEDICATION

For Graham Patrick

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Mum and dad, this thesis is for you, so you'd better read it cover to cover!

ABSTRACT

Cell signalling events in mesothelial cells elicited by asbestos

Malignant mesothelioma (MM) is a major health problem as it is an invariably fatal disease resulting from occupational exposure to asbestos. The long latency period of this disease means that death rates will continue to rise for 10-20 years before improved exposure regulations take effect.

The studies described here were designed to explore mechanisms by which asbestos exposure elicits this malignancy. Cell signalling events germane to malignant transformation were investigated in rat (4/4 RM4) and human (MET5A) mesothelial cells *in vitro* following exposure to asbestos. The activation state of the MAPK family and Akt were probed, because these pathways are pivotal in determining death or survival of the cell. The results suggest that extracellular signal regulated protein kinase (ERK), p38 and Akt are activated by asbestos exposure. For the former two, at least, this activation depended on oxidative stress. A selective inhibitor of EGFR tyrosine kinase, PKI166, inhibited asbestos-mediated Akt and ERK activation. Asbestos-mediated Akt activation was also inhibited by LY294002, an inhibitor of phosphatidylinositol 3-kinase (PI3K). The effect of events triggered by asbestos downstream of these kinases, on the transcription factors activator protein (AP) -1 and nuclear factor-κB (NF-κB) were investigated. Pharmacological inhibition of either the ERK pathway by UO126 or the p38 pathway by SB203580 ameliorated crocidolite-induced AP-1 activation. Inhibition of the Akt pathway by LY294002 or PKI166 reduced crocidolite-induced NF-κB translocation. The same panel of inhibitors were used to investigate the role of these pathways in defined endpoints characteristic of asbestos exposure *i.e.* cell death and survival/proliferation. In both cell lines the p38 pathway was involved in cell death and the Akt pathway in survival. In 4/4 RM4 cells activation of the ERK pathway also induced cell death. In contrast, in MET5A cells ERK appeared to be involved in survival.

Taken together these findings suggest that asbestos elicits a complex array of interacting cell signalling events, the balance of which may steer the cell onto the fateful route leading ultimately to MM.

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ABBREVIATIONS

Activating transcription factor (ATF)
Activator protein-1 (AP-1)
Adenosine 5'-triphosphate (ATP)
Apoptosis signal-regulated kinase 1 (ASK1)
Apoptotic protease activating factor-1 (Apaf-1)
Bcl-2 homology-3 domains (BH3)
Bovine serum albumin (BSA)
cAMP responsive element (CRE)
cAMP responsive element binding protein (CREB)
Cyclin-dependent kinase (CDK)
Death domains (DD)
Death-inducing signalling complexes (DISCs)
Dimethyl sulphoxide (DMSO)
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT)
Epidermal growth factor (EGF)
Epidermal growth factor receptor (EGFR)
Extracellular signal-regulated protein kinase (ERK)
Enhanced chemiluminescence (ECL)
Electrophoretic mobility shift assays (EMSA)
Glucose transporters (GLUT)
Glycogen synthase kinase 3 (GSK3)
Growth factor receptor binding protein 2 (Grb2)
Health and Safety Executive (HSE)
4-hydroxy-2-nonenal (4-HNE)
IκB kinases (IKKs)
Inhibitors of NF-κB (IκB)
Interleukin-1β (IL-1β)
c-jun amino terminal kinase (JNK)
Kinase assay buffer (KAB)
Malignant mesothelioma (MM)
Malondialdeyde (MDA)
Man made mineral fibres (MMMF)
MAPK-activated protein kinase-2 (MAPKAPK-2)
Mitogen activated protein kinases (MAPKs)
Mitogen activated protein kinase kinase (MAPKK/MEK)
Mitogen activated protein kinase kinase kinase (MAPKKK/MEKK)
National Institute for Environmental Health (NIEHS)
Nicotinamide adenine dehydrogenase (NADPH)
Nitric oxide (NO)
Nuclear factor-κB (NF-κB)
Phosphatase and tensin homologue (PTEN)
Phosphatidylinositol 3-kinase (PI3K)
Phosphatidylinositol (3,4)-bisphosphate [PtdIns(3,4)P_2]
Phosphatidylinositol (3,4,5)-trisphosphate [PtdIns(3,4,5)P_3]
Phospholipase C (PLC)
Phosphorylated heat and acid stable protein regulated by insulin (PHAS-I)

Phosphotyrosine binding (PTB)
Platelet-derived growth factor (PDGF)
Pleckstrin-homology (PH)
Polymorphonuclear leukocytes (PMN)
Polynucleotide kinase (PNK)
Protein kinase A (PKA)
Protein kinase B (PKB)
Protein phosphatases-1 (PP1)
Protein phosphatases-2A (PP2A)
Protein tyrosine phosphatases (PTPases)
PtdIns-dependent kinase-1 (PDK1)
Rat pleural mesothelial (RPM)
Reactive oxygen species (ROS)
Rel homology domain (RHD)
Retinoblastoma (Rb)
Room temperature (RT)
Serum response element (SRE)
Simian virus 40 (SV40)
Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)
Son of sevenless (SOS)
Src homology 2 (SH2)
Stress activated protein kinases (SAPKs)
T-antigen (Tag)
t-antigen (tag)
TBS-Tween 20 (TBS-T)
Ternary complex factor (TCF)
TGFβ-activated kinase (TAK-1)
TNF receptor associated death domain (TRADD)
Transforming growth factor β (TGFβ)
Tris buffered saline (TBS)
Tris-EDTA (TE)
Triton lysis buffer (TLB)
Tumour necrosis factor (TNF)
12-O-tetradecanoate-13-acetate (TPA)
TPA-responsive element (TRE)
Union Internationale Contre le Cancer (UICC)
Ultraviolet (UV)

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CHAPTER 1

INTRODUCTION

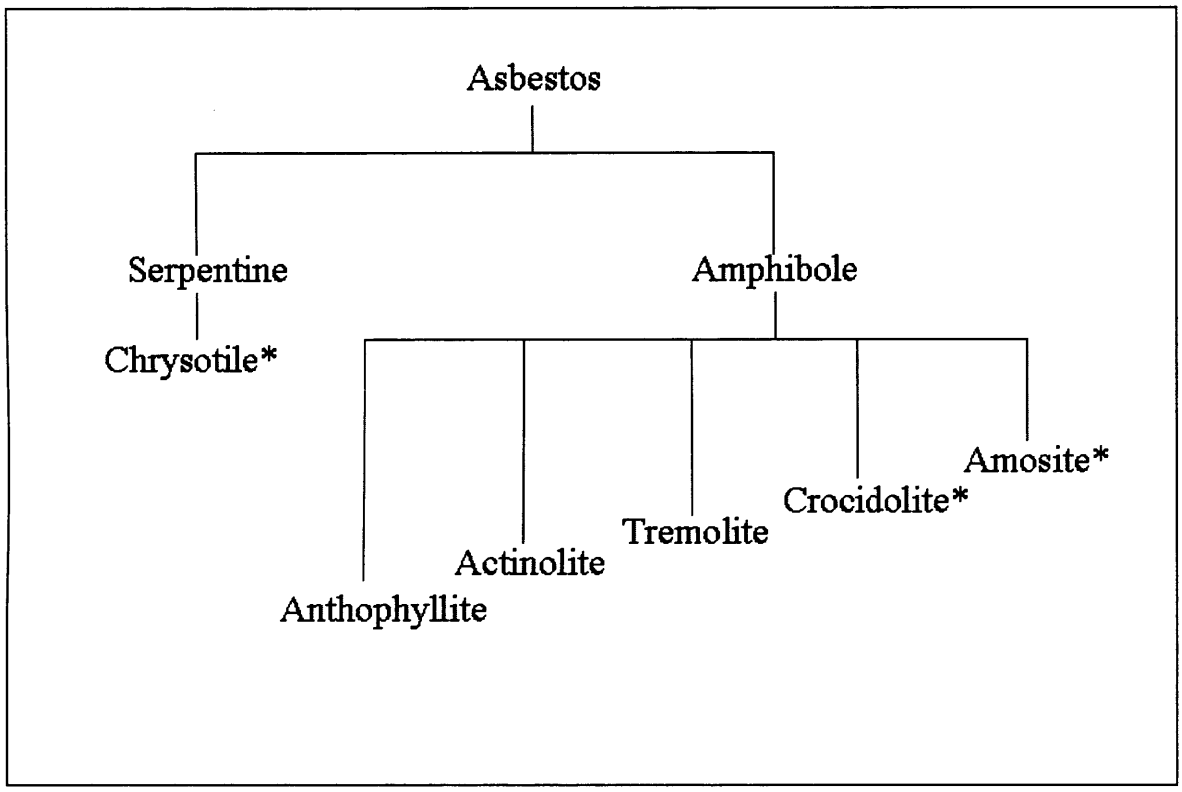
1.1.1 Asbestos background

The use of asbestos can be traced back to A.D. 50 when the Romans documented a material known as amianthus (this is derived from a Greek word meaning undefiled, incorruptable). In these ancient times the fibres were woven into sheets and used for oil wicks and cremation shrouds. Indeed, it is the use of asbestos in these lamps that gave it its present name because, the oil, if kept replenished, seemed to burn continuously. Thus the Greeks applied the term "ασβεστοζ" which means inextinguishable flame (Sinclair, 1959).

Marco Polo first described the methods used for the extraction of this group of minerals in 1250. Whilst travelling in what is now known as Siberia he came across a fire resistant textile that the inhabitants described as "salamander skin."
The full-scale exploitation of asbestos did not start until the latter part of the 19th century when the deposits at Thetford and Coleraine (Canada) opened up in 1877. Following this, further mines in Russia, Cyprus and Italy were commissioned and a world market was quickly established. Slightly later, at the beginning of the twentieth century, more mines were opened in Zimbabwe, South Africa and the USA. Initially these mines produced chrysotile (white asbestos) but in the Cape Province and Transvaal regions of South Africa other types of asbestos (amphibole) were discovered. These were called crocidolite so named because of its flaky appearance, and amosite-derived from the capital letters of the mining company responsible for its extraction- "Asbestos Mines of South Africa" (Sinclair, 1959; Jones, 1897).

1.1.2 Types of asbestos

Asbestos is broadly divided into two types: Amphibole and Serpentine. As shown in Figure 1.1 the only member of the serpentine group is chrysotile, which accounts for over 90% of mined asbestos.

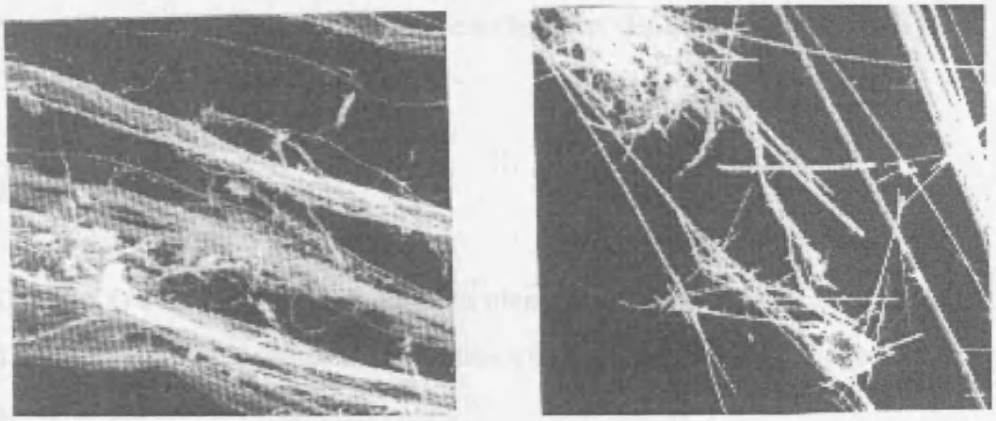


* Indicates types most commonly encountered commercially.

Figure 1.1 Asbestos subtypes

Chrysotile fibres have a curly and barbed appearance when viewed under an electron microscope (Figure 1.2a). Chrysotile possesses the ability to split longitudinally to form long, thin fibres that are readily respirable (Harrison et al., 1999). Due to the crystal lattice structure of chrysotile it is not as durable in the lung as its amphibole counterparts and its ability to induce mesothelioma is a matter for much debate (Landrigan et al., 1999; McDonald et al., 1997). However, it is widely accepted that chrysotile can cause lung cancer and asbestosis at high exposure levels over

protracted periods of time (Stayner et al., 1997). In contrast to serpentine asbestos, amphibole asbestos exhibits a more rod-like structure (Figure 1.2b).



a b

Figure 1.2 Electron micrograph of chrysotile (a) and crocidolite (b) asbestos

These fibres, of which, amosite (brown asbestos) and crocidolite (blue asbestos) are the most widely used commercially, are characterised as being more durable in the lung (Hesterberg et al., 1996), and are intimately linked to the causation of asbestos-induced mesothelioma (Wagner et al., 1960). The theoretical chemical composition of the above asbestos sub-types is listed in Table 1.1. They all share common silicate groups *e.g.* Si_8O_{22}, but each fibre type is defined by the presence of a range of cations including Mg^{2+} and $Fe^{2+/3+}$.

Group	Type	Composition
Serpentine	Chrysotile	$Mg_6Si_4O_{10}(OH)_8$
Amphibole	Crocidolite	$Na_2(Fe^{3+})_2(Fe^{2+})_3Si_8O_{22}(OH)_2$
	Amosite	$(Fe,Mg)_7Si_8O_{22}(OH)_2$
	Anthophyllite	$(Mg,Fe)_7Si_8O_{22}(OH)_2$
	Tremolite	$Ca_2Mg_5Si_8O_{22}(OH)_2$
	Actinolite	$Ca_2(Mg,Fe)_5Si_8O_{22}(OH)_2$

Table 1.1 Chemical characteristics of asbestos fibres.

It is important to note that these figures and compositions are approximate only; many samples are often contaminated with other asbestos types, and the exact composition of any fibre sample would depend on where it was mined. This fact has been the focus of much debate in the scientific community, with respect to the carcinogenicity of

chrysotile and will be discussed in more detail later. Also worthy of note is erionite. Whilst this is not a type of asbestos, it does occur in a fibrous habit and is more potent than any asbestos fibre in causing mesothelioma (Boutin, 1999).

1.1.3 Uses of asbestos

Over 3000 uses of asbestos have been identified (Liddell and Miller, 1991). Below is a list of some of the more common uses together with the percentage and type of asbestos used.

ASBESTOS CEMENT Tiles, drainage and rain water pipes, flues for boilers, water tanks and fire surrounds.	**10-15%** Chrysotile, some may contain Crocidolite.
INSULATION BOARDS Cladding for walls, ceilings and structural steelwork. Acoustic and thermal insulation.	**20-40%** usually Amosite but may also contain Chrysotile or Crocidolite.
LAGGING Hot water pipes, boilers etc.	**~90%** Chrysotile, Crocidolite or Amosite.
SPRAYED COATINGS Fire protection to structural steelwork. Thermal and acoustic insulation.	**~85%** Chrysotile, Crocidolite or Amosite.
ASBESTOS CLOTHS AND TEXTILES Fire blankets, fireproof clothing, oven gloves.	**65-100%** Chrysotile, but Crocidolite was also used.
GASKETS	**~70%** Chrysotile but may also be Crocidolite
FRICTON PRODUCTS Brake linings, clutch plates.	Previously **30-70%** Chrysotile but now much less.

(Taken from www.voelckerscience.co.uk.)

Table 1.2 Common uses of asbestos.

It is the physico-chemical properties of asbestos, which have led to its widespread usage in the applications listed above. Its high tensile strength, resistance to heat and chemical attack and low cost, relative to man-made alternatives, meant vast quantities of asbestos were mined from the locations listed earlier throughout the 19th and 20th

century. The production of asbestos reached its peak in the middle of the 20th century with 150,000 tonnes brought into the UK alone, despite concerns over possible detrimental health effects first voiced at the turn of the century. It wasn't until 1933 that the first set of regulations was introduced to try and curb exposure to asbestos in the UK. Elsewhere in the world this was to be much later.

In the 1960s the link between amphibole asbestos and mesothelioma was first suggested (Wagner et al., 1960), and consequently the types of asbestos that fell into this category were banned from commercial use. However, there has been much controversy over the carcinogenicity of the non-amphibole asbestos, chrysotile, which means that it is still used in some applications throughout the world (Health and Safety Commission UK, 1979). In 1999 all imports of asbestos of any type into the UK were banned (Harrison et al., 1999).

1.1.4 Other fibres

With the global realisation that asbestos was no longer suitable for use in many, if not all, applications mentioned above, it became important to find safer alternatives. At present these can be broadly divided into three categories: the man-made mineral fibres (MMMF), including glass fibres, mineral wools and ceramic fibres; synthetic organic fibres, including carbon fibre and natural organic fibres *e.g.* cellulose. It is beyond the scope of this thesis to explain in detail the uses of the individual fibre types, but suffice to say that none of them perform as well as asbestos over such a broad range of applications, and at such low cost. This is highlighted by the fact that some substitutes, such as aramid and carbon fibre may be ten times more expensive than asbestos on a mass-to-mass basis (Hodgson, 1989). Long-term toxicological studies have still not proved, beyond doubt, that these fibres are totally non-pathogenic and this represents a key area of research (Hesterberg et al., 1996; Luoto et al., 1997). Many researchers would argue that any fibre that is respirable and durable is potentially dangerous, and the issue is unlikely to be resolved in the near future. Additionally, health factors other than those involved with asbestos exposure may arise from substitute fibres. In particular would be the applications where asbestos was used in fireproofing as alternatives may give off toxic fumes under conditions of extreme heat. In the meantime, rigid guidelines defined by the Health and Safety

Executive (HSE) are designed to keep exposure levels well below what many deem as safe.

1.1.5 Diseases Associated with asbestos exposure

Whilst in general, diseases caused by exposure to any dust are collectively known as pneumoconiosis, asbestos is typically associated with the following lung-related illnesses.

1.1.5.1 Pulmonary fibrosis (asbestosis)

This disease was first described in 1903 and describes a condition of excessive collagen deposition by fibroblasts in the lung. This lesion is associated with high occupational exposure to asbestos (Craighead and Mossman, 1982) and the link was first realised following the death of an asbestos textile worker in 1928 who had no infective or other occupational cause for this disease. A subsequent study by the chief medical inspector of factories demonstrated that a quarter of 363 asbestos textile workers had pulmonary fibrosis considered to be due to asbestos exposure, furthermore the incidence of fibrosis correlated with intensity and duration of exposure (Liddell and Miller, 1991).

Studies *in vitro* have shown that fibroblasts secrete increased levels of collagen when treated with asbestos without modulations in collagen gene expression, which suggests that asbestos acts downstream of transcription possibly through stabilisation of collagen mRNA (Mossman et al., 1986). The presence of excessive collagen *in vivo* results in scar tissue that is less elastic than normal lung tissue and impairs gas exchange causing breathlessness and progressive disability. It is thought that high exposures to asbestos are required for asbestosis to develop due to the more intense and protracted inflammatory response under these conditions. A number of studies have implicated the involvement of a wide variety of cytokines and chemokines in the development of this disease. Central to this process is thought to be tumour necrosis factor-α (TNF-α) because transgenic mice overexpressing TNF-α in alveolar type II cells spontaneously develop fibrosis with similar features encountered under asbestos

exposed conditions (Miyazaki et al., 1995) and in a TNF-α receptor knockout model no fibroproliferative lesions were noted following exposure to chrysotile asbestos, in contrast to wild type animals (Liu et al., 1998).

Due to the early recognition that high levels of occupational exposure to asbestos were responsible for asbestosis, regulations were introduced in the early 1930s to monitor and curb exposure in the workplace. As a result, this disease is no longer a major health problem in the UK; however, the study of asbestosis pathogenesis is still considered to be worthwhile as it represents a model system of pulmonary fibrosis, which may be applied to other diseases.

1.1.5.2 Bronchogenic carcinoma (lung cancer)

Bronchogenic carcinomas arise from the epithelial lining of the bronchioles, trachea or alveoli. Following the realisation that asbestos was far more toxic than many people first thought, researchers sought to examine whether it may be responsible for further diseases. From 1934 a link between asbestos exposure and lung cancer was suspected as cohort studies demonstrated an increased risk of lung cancer in workers occupationally exposed to asbestos for 20 years or more, of about ten times that encountered in the general population (Doll, 1993). As with asbestosis the probability of developing this disease and the latency period between exposure and presentation of symptoms depends on the intensity and duration of exposure. Furthermore, the aetiology of bronchogenic carcinoma in asbestos exposed individuals is thought to involve additional factors. In particular, smoking plays an important role with asbestos-induced lung cancers only rarely appearing in non-smokers (Saracci, 1977; (Liddell, 2001).

Carcinogenesis is described as a multistage process including initiation and promotion. Initiation involves the alteration of DNA resulting in an inheritable change that may become malignant. Promotion involves the division and proliferation of initiated cells by a tumour-promoting agent that may not be genotoxic. As with many models of pathogenesis, there may be agents that do not fit neatly into this process and this is thought to be the case with asbestos. This is largely due to the interaction

of smoking and asbestos in causing lung cancer. A popular theory is that asbestos acts as a tumour promoter because it shares numerous cellular effects with classical tumour promoters such as phorbol ester compounds *e.g.* generation of oxidants and activation of cell signalling pathways (Mossman et al., 1985). The synergism of asbestos and cigarette smoke in causing lung cancer is believed to arise from a number of factors. Firstly, the damage caused by smoking allows increased uptake of fibres by tracheobronchial epithelial cells and also reduces their clearance from the lung (McFadden et al., 1986a; McFadden et al., 1986b). Additionally, fibres can act as carriers of the carcinogens in cigarette smoke by adsorbing them onto the their surface and this allows prolonged cellular exposure (Mossman, 1983).

1.1.5.3 Malignant mesothelioma

The diseases described above are not considered as major health concerns with respect to asbestos in the western world in the present day due to the high levels of exposure required for their initiation. The introduction of safety regulations in the last century means that these levels are no longer reached and the population of workers who were exposed prior to this are probably already deceased. However, in the case of malignant mesothelioma (MM) the exposure levels needed for pathogenesis are thought to be much lower, and due to the long latency period between first exposure and presentation of disease it is expected that the death rates from this disease will get worse before they get better (Peto et al., 1995).

The link between asbestos exposure and MM was not realised until 1960, when researchers in the north western cape province of South Africa noticed unusually high rates of this pleural tumour in patients, the first of which also had evidence of asbestosis (Wagner et al., 1960). Since then, further studies have documented excessive mesothelioma rates in a number of occupations including gas mask manufacturers (Acheson et al., 1982) and insulators (Elmes and Simpson, 1977). In the non-exposed population pleural mesothelioma is an extremely rare tumour, occurring at a rate of approximately 1 in 3000 (Peto et al., 1995). Epidemiological studies have shown that the latency period between initial exposure and diagnosis is commonly more than 30 years and may be as high as 60 years (Browne, 1983). Annual raw imports of asbestos into the European Union peaked in the first half of the

1970s and remained high until 1980 before dropping steadily towards the 1990s (European Commission, 1996). Despite increasingly stringent regulations governing exposure limits, users in many countries were exposed at uncontrolled levels during this time meaning that death rates will continue to rise until 2020 (Peto et al., 1999). Statistical analyses of the available data from the mesothelioma register suggest that western European individuals born between 1945 and 1950 who started their working lives in the 1960s and 1970s are most at risk (Peto et al., 1999). This cohort is expected to have 1 death in 150 from mesothelioma within the whole population, which represents a two-fold increase in death rates from 1990-1994 to 2015-2019. Figure 1.3 demonstrates the increase in mesothelioma death rates in men and women from the early 1970s to the mid 1990s. Due to the male domination in asbestos-related occupations, the death rates are far higher in this group and appear to rise almost exponentially during the time frame of this study. Despite the difficulty in predicting these rates due to either over- or underdiagnosing mesothelioma as the cause of death by physicians, these figures are proving quite accurate in the present day (Peto et al., 1999).

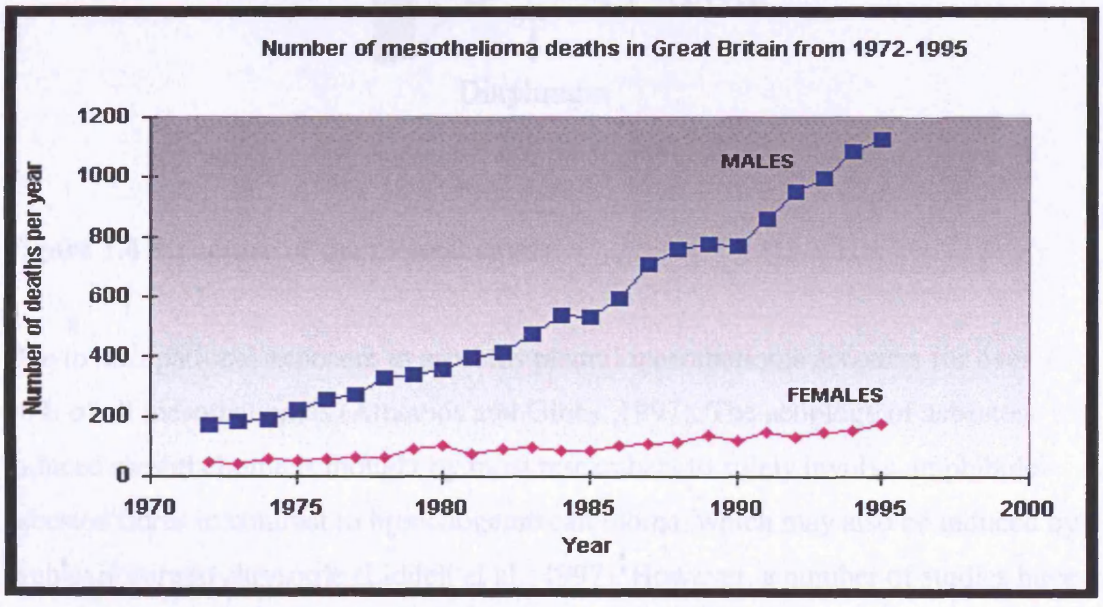


Figure 1.3 Death rates due to mesothelioma between 1972 and 1995 (Taken from Peto *et al.*, 1999).

Mesothelioma arises from the oncogenic transformation of mesothelial cells. Under normal circumstances mesothelial cells form a sheet over internal organs to allow

them to move freely against each other, the layer over the organ is referred to as visceral and that on the body wall parietal. A thin layer of fluid is present between these layers to reduce friction. In the case of the pleural cavity, mesothelial cells envelop the lung (visceral pleura) and line the inside of the chest wall (parietal pleura). Malignant pleural mesothelioma arises from the parietal layer (Boutin et al., 1991). This structure is demonstrated in figure 1.4.

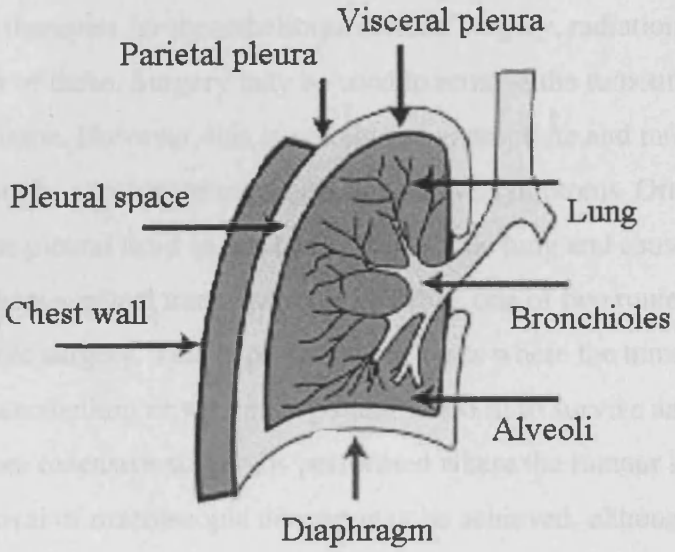


Figure 1.4 Structure of the pleural cavity

Due to occupational exposure to asbestos pleural mesothelioma accounts for over 90% of all mesotheliomas (Attanoos and Gibbs, 1997). The aetiology of asbestos-induced mesothelioma is thought by most researchers to solely involve amphibole asbestos fibres in contrast to bronchogenic carcinoma, which may also be induced by high exposure to chrysotile (Liddell et al., 1997). However, a number of studies have proposed that infection with Simian virus 40 (SV40) from contaminated polio vaccines may contribute to the increased rate of mesothelioma noted in the second half of the 20th century because remnants from SV40 infection have been found in mesothelioma tumour samples (Carbone et al., 1994; Carbone et al., 2000). SV40 binds to and inactivates the tumour suppressors p53 and retinblastoma (Rb) protein

(Carbone et al., 1997; Herzig et al., 1999) and may therefore allow the unregulated proliferation of infected cells. Geographical differences do exist in the presence of SV40 in mesothelioma samples ranging from none detected in samples from Turkey (Emri et al., 2000; De Rienzo et al., 2002) to 60% in samples from the US (Carbone et al., 1999). This theory is highly contentious and at present there is no general agreement on the importance of these findings.

1.1.5.4 Existing therapies for malignant mesothelioma

Conventional therapies for mesothelioma include surgery, radiation, chemotherapy or a combination of these. Surgery may be used to remove the tumour and some of the surrounding tissue. However, this is not always appropriate and more non-invasive techniques may be required to minimise and relieve symptoms. Often this involves drainage of the pleural fluid as this can compress the lung and cause difficulties in breathing. Where surgical intervention is possible, one of two routes is followed. The first is palliative surgery. This is performed in cases where the tumour has spread beyond the mesothelium or where the patient is too ill to survive an extensive operation. More extensive surgery is performed where the tumour is well localised and total removal of macroscopic disease may be achieved, although it is highly likely that microscopic groups of tumour cells do remain and therefore long term prognosis is poor. Radiation therapy involves targeting the tumour with high-energy X-rays to kill cancer cells and is often used as an adjuvant therapy following surgery to remove remaining tumour deposits. Chemotherapy is designed to specifically target cancer cells with orally or systemically administered drugs, but the benefits of this treatment are limited. Despite this variety of treatment options, the prognosis for mesothelioma sufferers is very poor, currently the median survival time from diagnosis to death is around 6 to 9 months, which may be improved to 12 months with chemotherapy and, in selected patients, to 22-37 months with confined modality treaments (Kindler and Vogelzang, 2002).

1.1.5.5 Other diseases associated with asbestos exposure

A number of other diseases are also associated with exposure to asbestos. These include benign lesions such as pleural plaques. Plaques usually develop on the parietal

pleura or diaphragm (Boutin et al., 1996) and are caused by the accumulation of acellular collagen. Whilst these changes may indicate previous exposure to asbestos, they are not thought to progress to malignant disease (Mossman and Sesko, 1990). Some studies have also shown increased levels of other tumours (*e.g.* gastrointestinal) in asbestos exposed cohorts, however, these findings have not been confirmed in other groups and it may be that a number of other factors are responsible for these effects (Selikoff, 1974; Kang et al., 1997; Levine, 1985).

1.1.6 Characteristics of fibres affecting pathogenesis

Certain properties of asbestos, most commonly found in the amphibole subgroup are thought to be crucial to their pathogenicity. The most important are thought to be dimension, durability and surface properties.

1.1.6.1 Fibre dimension

A fibre is a structure longer than 5μm, less than 3μm in diameter and with an aspect ratio (length: diameter) greater than 3:1. Fibre dimension is thought to be critical in the carcinogenicity of asbestos at a number of stages. The first concerns respirability. In order for fibres to penetrate to the lower regions of the lung they must have high aspect ratios. The laws of physics state that longer, thinner fibres direct themselves along the direction of the airflow thus decreasing their drag force and will therefore tend to be deposited at sites where sudden changes in airflow direction take place. In the lung this occurs at branches in the respiratory system, and studies have shown that these bifurcations are major sites for deposition in rat inhalation models (Brody et al., 1981). In reality, diameter is the most important of these factors; only fibres of less than 3μm diameter can reach the alveolar region of the lung and subsequently fibres as long as 30-40μm may be deposited there (Donaldson et al., 1993). High aspect ratio is also crucial for pathogenic events following deposition in the lung. Pioneering studies by Stanton and Lanyard (1978) showed that fibres below 5μm are low in pathogenicity and that above 8-10μm there is strong correlation between fibre length and pathogenicity in a system of pleural cavity fibre instillation. This mode of fibre delivery avoids the respiratory system enabling researchers to gauge more accurately

what the pleura is being exposed to. In this case dimension is important because fibres that are too large (>8μm) to be phagocytosed by pulmonary macrophages can cause frustrated phagocytosis (Stanton and Layard, 1978). This phenomenon is part of the defence system of macrophages and involves a respiratory burst that produces reactive oxygen species (ROS), which can trigger a plethora of cellular responses involved in combating oxidative stress. The effects of oxidative stress on biological processes are discussed more fully below.

1.1.6.2 Fibre durability

Whilst fibre dimension influences entry and deposition in the lung, durability determines their persistence and continued bioreactivity (Sebastien, 1991). The properties of asbestos that led to its widespread usage include resistance to chemical attack and mechanical strength. These properties are also thought to be crucial for the deleterious effects of asbestos in the pulmonary system. The importance of durability has been investigated by comparing fibres that have been leached with hydrochloric acid (HCl) with native fibres. Following this treatment chrysotile fibres lose magnesium from their crystal lattice and become shorter and thicker (Morgan and Holmes, 1986). Quantitatively the dissolutions of chrysotile, crocidolite or amosite in 4M HCl for 30 minutes are 60%, 6% and 8% respectively (Hardy and Aust, 1995). The ability of both chrysotile and crocidolite asbestos to induce mesotheliomas in intrapleurally injected rats was reduced by this leaching treatment (Monchaux et al., 1981). In a series of further *in vitro* studies the toxicity of chrysotile was reduced more significantly by leaching than crocidolite (Reiss et al., 1980; Yano et al., 1984), (Jaurand et al., 1984) and is believed to be the basis of why chrysotile is less able to induce mesothelioma than crocidolite (Mossman and Sesko, 1990). Following deposition in the lung, chrysotile splits longitudinally (Jones et al., 1989; McDonald et al., 1989; Roggli and Brody, 1984) and magnesium is lost through a similar mechanism to that recreated following dissolution studies *in vitro* (Thomassin et al., 1980; Langer and Nolan, 1994). This means that over time the population of chrysotile fibres in the lung will have decreasing length and be more susceptible to clearance mechanisms (Wagner et al., 1974). In contrast, amphibole fibres are more

durable in the lung and may persist for decades after exposure compared to months for chrysotile (Mossman and Churg, 1998).

1.1.6.3 Surface properties of fibres

The importance of factors other than fibre dimension and durability in pathogenesis is demonstrated by erionite. This fibre is found naturally occurring in the Cappadocian region of Turkey and is thought to be responsible for the unusually high rate of malignant mesothelioma in villagers from that area, in the apparent absence of other triggers (Metintas et al., 1999). Rat inhalation studies comparing asbestos and erionite fibres of comparable size show that with erionite exposure the latency period is decreased and the overall incidence of mesothelioma is increased (Wagner et al., 1985). Other studies have shown that erionite is also less toxic *in vitro* when compared to amphibole asbestos fibres on a mass-to-mass basis, however, this may be due to the smaller proportion of fibrous material in erionite samples (Pelin et al., 1992). In addition, a number of laboratories have modified the surface of fibres with a variety of agents in attempts to elucidate the importance of surface characteristics. Studies by Timblin *et al* (1998) focused on substituting cations on the surface of erionite, which is enabled by the channelled structure of the fibre. The results from this study showed that c-*fos* expression was similar irrespective of cation present, but Na substituted fibres were most potent in their ability to induce c-*jun* expression, additionally these fibres were not capable of inducing significant increases in apoptosis (Timblin et al., 1998b).

The largest body of evidence points to the ability of asbestos fibres to catalyse the production of ROS as being crucial for their toxicity. Increases in ROS by asbestos are thought to arise from two mechanisms. The first is due to the inherent properties of fibres, in particular the iron that is found on their surface. Whereas all asbestos fibres have iron in their structure, crocidolite and amosite have higher levels than chrysotile (Hardy and Aust, 1995), which may be a factor in the difference in pathogenicity between these fibres. Due to the non-solubility of asbestos fibres in biological fluids, any chemical reactions are expected to take place at the solid-liquid interface. These include the Fenton reaction, which involves the oxidation of ferrous iron (Fe^{2+}) to ferric iron (Fe^{3+}) by H_2O_2 resulting in the formation of hydroxyl radical

($HO^{\bullet}$) species (Kamp et al., 1992; Hardy and Aust, 1995; Weitzman and Graceffa, 1984):

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^- + HO^{\bullet}$$ (Weitzman and Graceffa, 1984)

The more redox active Fe^{2+} is regenerated by the reaction of Fe^{3+} with superoxide (O_2^-) or other biological reducing agents:

$$Fe^{3+} + O_2^- \rightarrow Fe^{2+} + O_2$$

Iron can also cause increases in $HO^{\bullet}$ species via the Haber-Weiss equation:

$$O_2^- + H_2O_2 \rightarrow \text{iron} \rightarrow HO^- + HO^{\bullet} + O_2$$

Ferrous iron can also induce the formation of other highly reactive free radicals from species containing an –OOH group, again through Fenton chemistry:

$$Fe^{2+} + ROOH \rightarrow Fe^{3+} + HO^- + RO^{\bullet}$$

The final reaction is particularly important in biological systems, where R may represent part of a lipid or protein and so this reaction would produce lipid (lipid peroxides) or protein radicals. A number of studies have examined the effects of asbestos on lipid peroxidation and the role of iron in this process. Weitzman *et al.* (1985) showed that the formation of lipid peroxide species, particularly malondialdehyde and its precursors, were significantly increased by crocidolite, amosite or chrysotile in a cell-free system and that this could be inhibited by iron chelation of the fibres by deferoxamine (Weitzman and Weitberg, 1985). The production of these lipid peroxide species may have functional consequences *in vivo* through modulation of signalling pathways (Uchida et al., 1999) or alterations to DNA (Howden and Faux, 1996).

The second mechanism whereby asbestos induces ROS formation is indirect and requires cellular participation during the attempted phagocytosis of asbestos fibres.

The key to this mechanism is the ability of phagocytic cells such as polymorphonuclear leukocytes (PMN) or alveolar macrophages to produce ROS upon activation by foreign matter, in this case, asbestos. Ishizaki *et al.* (1997) showed that PMN require calcium influx and nicotinamide adenine dehydrogenase (NAD) oxidase activity to generate ROS following asbestos exposure. Blockade of phagocytosis by cytochalasin B (CB) could potentiate ROS production probably through elevation of calcium influx (Ishizaki et al., 1997). This indicates that PMN cells utilise a common method of dealing with particulate matter. Whilst these studies did not directly assess these effects in mesothelial cells, it may be expected that they behave in the same way due to their ability to phagocytose foreign particles. Furthermore, researchers have shown that fibre length is not critical for this response because short (75%$\leq$1.0μm) crocidolite fibres were equally potent in eliciting H_2O_2 release from peritoneal macrophages as a longer (83%>1.1μm) fibre preparation (Goodglick and Kane, 1990). The consequences of ROS overproduction are discussed in more detail in a later section.

1.2.1 Cell signalling

Due to the complex nature of multicellular organisms, there is a necessity for systems that enable cells to communicate with each other and then pass the signal from the cell surface to the nucleus. In addition to normal tissue homeostasis, cell signalling mechanisms enable organisms to adapt to changing environmental conditions, which may otherwise be harmful. Whilst there are a huge number of signal transduction pathways in mammalian cells, researchers have elucidated the function of many of these in both normal and diseased states. In carcinogenesis the homeostasis of tissues is clearly disrupted and the improper regulation of cell proliferation and apoptosis by cell signalling modules is crucial to this process. Therefore, the study of this balance is vital in formulating strategies that both prevent and treat cancer.

1.2.2 Mitogen activated protein kinase signalling pathways

The mitogen activated protein kinases (MAPKs) are a super family of protein kinases that are intimately involved in signal transduction and are expressed ubiquitously in

mammalian cells (Cobb and Schafer, 1996). MAPK signalling pathways are especially important in cellular processes such as apoptosis, proliferation and differentiation and are therefore of particular interest when studying carcinogenesis.

The MAPK pathways may be loosely defined into two categories: i) those that are activated by stress and ii) those activated by growth factor-bound transmembrane receptors with intrinsic tyrosine kinase activity. The former is represented by the stress activated protein kinases (SAPKs), which encompasses the c-jun amino terminal kinase (JNK) and p38 pathways, whereas the latter is known as the extracellular signal-regulated protein kinase (ERK) pathway (Seger and Krebs, 1995). MAPK pathways share a common mode of signal transduction that involves a module of three sequential protein kinases. The first is the mitogen activated protein kinase kinase kinase (MAPKKK/MEKK), which dually phosphorylates and activates the second member, mitogen activated protein kinase kinase (MAPKK/MEK) and this enzyme then activates MAPK by dual phosphorylation (Cobb and Schafer, 1996). Researchers have show that scaffold proteins bind these proteins in close proximity thus allowing efficient transduction of the signal through these cascades (Herskowitz, 1995).

1.2.2.1 The ERK signalling pathway

To explain the processes involved in the regulation of these pathways it is useful to employ a well-studied example. In the case of the ERK pathway this is best served by epidermal growth factor (EGF) signalling. This growth factor is recognised at the plasma membrane by the epidermal growth factor receptor (EGFR), a member of the c-erb family. Upon ligand recognition the receptor dimerises with other members of the c-erb family. This dimerisation activates the receptor's intrinsic tyrosine kinase activity resulting in autophosphorylation of specific tyrosine residues present on the intracellular domain. Certain phosphorylated tyrosine residues are recognised by proteins that have Src homology 2 (SH2) motifs *e.g.* growth factor receptor binding protein 2 (Grb2) or phospholipase C (PLC)-γ. Grb2 is an adapter protein and couples the signal from receptor to a membrane limited intracellular signalling protein such as p21ras (p21ras is posttranslationally modified to include a lipid moiety, ensuring that

it stays in the plane of the plasma membrane) via a Ras guanine nucleotide exchange factor *e.g.* son of sevenless (SOS) (Cobb and Schafer, 1996). p21ras is regulated in a similar way to G proteins *i.e.* it is a guanine nucleotide binding protein which is active when bound to GTP but inactive when bound to GDP. In this case, SOS catalyses the exchange of GDP for GTP to activate p21ras and its intrinsic GTPase activity causes its own inactivation over a period of time. A role for p21ras in cancer is now widely accepted, indeed, it is estimated that 30% of all human tumours contain an activating mutation in this protein (Votjek and Der, 1998). Following this adaptation of the signal at the plasma membrane it enters the classical three-enzyme MAPK module. p21ras binds directly, and activates Raf, a typical example of a MAPKKK/MEKK. To date, three isoforms of Raf have been characterised: c-Raf-1, A-Raf and B-Raf and each of these is thought to play a specific role in cell signalling both in times of health and disease (Votjek and Der, 1998). Raf is a serine/threonine protein kinase, which activates MAPKK/MEK1/2 by phosphorylating two serine or threonine residues found in a particular motif (Cobb and Schafer, 1996):

-Ser-x-x-x-Ser/Thr-

MEK1/2 are dual specificity kinases, which specifically phosphorylate threonine and tyrosine residues at set locations on ERK1/2 (p44 and p42 respectively). Other kinases that bear similar phosphorylation sites have been shown to be poor *in vitro* substrates of MEK1/2 and this demonstrates the specificity of signalling within this pathway (Lin et al., 1995). Again these locations bear a signature amino acid sequence, in this case it is:

-Thr-Glu-Tyr-

Pharmacological manipulation of the ERK pathway is achieved through inhibition of MEK1/2. In these studies UO126 was used, which non-competitively inhibits active MEK1/2 (Favata et al., 1998). Dual phosphorylation of ERK1/2 represents a key area of regulation. When only one of the residues is phosphorylated, the enzyme is not active. Therefore this may be a method of "priming" the enzyme for activation (Blumer and Johnson, 1994). Upon activation, ERK1/2 may translocate to the nucleus

(Lenormand et al., 1993; Lenormand et al., 1998) depending on the longevity of the signal and the activity of factors involved in switching off the signal (Lenormand et al., 1993; Traverse et al., 1992). Therefore, spatial and temporal differences in ERK1/2 activation may result in quite diverse responses, dictated at least in part by whether active ERK1/2 species persist long enough to enter the nucleus and phosphorylate nuclear targets. ERKs are proline directed kinases and phosphorylate proteins with a –PX(S/T)P- sequence. These include phospholipase A_2 (Nemenoff et al., 1993) and EGFR (Takishima et al., 1991). In cases where active ERKs enter the nucleus a number of transcription factors may be affected, the best studied are c-Myc (discussed below) and Elk-1 (Seger and Krebs, 1995; Treisman, 1995). Elk-1 is a ternary complex factor (TCF) that forms a complex with the serum response factor (SRF) upon phosphorylation to increase transcription of genes that contain the serum response element (SRE), including c-*fos* (Gille et al., 1992). In addition to receptors with intrinsic tyrosine kinase activity, ERKs may be activated by other receptors and Ras independent mechanisms (Della Rocca et al., 1997; Dikic et al., 1996).

1.2.2.2 The SAPK pathways

The SAPK pathway encompasses the p38 and JNK signalling pathways. The regulation and functions of these pathways are not as well understood as the ERK pathway, which is in part due to the more complex nature of the mechanisms involved. SAPKs are activated in the response to ultraviolet (UV) light, cytokines, osmotic shock, inhibitors of DNA, RNA and protein synthesis and, to a lesser extent, growth factors (Derijard et al., 1994; Karin, 1995a; Paul et al., 1997; Kyriakis et al., 1994). This spectrum of regulators clearly indicates that these enzymes are important in transducing a variety of stress responses.

p38 was first discovered following sequence analysis of ERK1/2. These studies showed that ERK1/2 shared striking sequence homology with a number of *S. cerevisiae* protein kinases, including HOG1 (Boulton et al., 1991; Boulton and Cobb, 1991), which is vital in the response to osmotic stress in yeast (Brewster et al., 1993). Kyriakis *et al.* (1990) initially discovered JNK2 as a 54kDa kinase that became phosphorylated on serine/threonine and tyrosine residues following treatment with cycloheximide in rat liver (Kyriakis and Avruch, 1990). Sequence analysis of this

protein revealed that it was closely related to a number of other stress activated protein kinases (Kyriakis et al., 1994), including a 46kDa kinase that had recently been shown to phosphorylate c-Jun at Ser^{63} and Ser^{73} following *UV* radiation: JNK1 (Derijard et al., 1994).

The SAPK pathways follow the same three enzyme module mode of activation as the ERK pathway. In the case of the JNK pathway, MEKK1 and 2 fulfil the role of MAPKKK (Blank et al., 1996). Signalling events upstream of MEKK activation are complex and shall not be discussed in this thesis. Whilst MEKK1 has additional downstream targets (Karin and Delhase, 1998), including MEK1/2 in some experimental systems (Minden et al., 1994a) it is not thought to activate p38, which led to the search for a p38 pathway MAPKKK. This is now thought to be the MEKK like protein transforming growth factor β (TGFβ)-activated kinase (TAK-1) (Moriguchi et al., 1996a). The next level of SAPK pathway activation has some interesting features due to the cross-talk that exists between the p38 and JNK pathways. To date 4 MAPKK isoforms have been identified for the SAPKs. Early studies showed that one of these, MEK/MKK4, could serve as a dual function kinase, activating both p38 and JNK (Derijard et al., 1995), (Lin et al., 1995; Sanchez et al., 1994). Another isoform, MKK3 could be activated independently of MKK4 and this enzyme was specific for p38 (Derijard et al., 1995). Further studies have shown additional members to this subfamily, one of these was discovered in osmotic stress experiments and is another p38-specific activator, termed MKK6 (Meier et al., 1996; Moriguchi et al., 1996b). The presence of a JNK activator other than MKK4 had been suspected following the observation that $MKK4^{(-/-)}$ cells were not completely devoid of JNK activity (Yang et al., 1997; Nishina et al., 1997) and this was confirmed with the discovery of MKK7, a SAPKK that specifically activates JNK (Tournier et al., 1997).

As with ERK1/2, p38 and JNK1/2 rely on dual Thr/Tyr phosphorylation for activation. Again, the residues involved are separated by one amino acid:

JNK1/2	-Thr-Pro-Tyr-
p38	-Thr-Gly-Tyr-

A variety of intracellular targets of SAPKs have been identified, and these include a number of transcription factors *e.g.* c-Jun, ATF-2, Elk-1 and cAMP responsive element binding protein (CREB) (Pulverer et al., 1991; van Dam et al., 1995; (Gupta et al., 1995; Whitmarsh et al., 1995). Of particular interest to these studies is the involvement of SAPKs in the formation of stable and active activator protein-1 (AP-1) complexes.

1.2.2.3 Inactivation of MAPKs

The multifaceted effects of MAPK activation dictate that there must be some form of negative regulation, otherwise inappropriate, prolonged signalling would occur. This is largely achieved through protein phosphatases, which inactivate MAPKs by dephosphorylation of one or other of the residues required for activity. A group of these, the MAPK phosphatases (MKPs), are dual specificity phosphatases that are thought to act on MAPK family members *in vivo* (Sun et al., 1993). MKPs can dephosphorylate both threonine and tyrosine residues (hence dual specificity) and previous studies have shown that particular members of the MKP family are selective toward certain MAPKs by up to two orders of magnitude (Groom et al., 1996; Muda et al., 1996). A second group of phosphatases are also able to inactivate MAPKs. These are the protein tyrosine phosphatases (PTPases) and are differentially expressed in different cell types. As with MKPs, PTPases have distinct specificities determined by protein-protein interactions between themselves and the MAPK substrate (Keyse, 2000). In addition to the action of phosphatases, indirect regulation of ERKs may be achieved through phosphorylation of c-Raf-1 at Ser^{43} by protein kinase A (PKA), which decreases its ability to associate with and activate Ras (Wu et al., 1993).

1.2.3 Structure, regulation and function of AP-1

AP-1 is a collective term referring to a dimeric transcription factor composed of Jun (c-Jun, Jun B, Jun D), Fos (c-Fos, Fos B, Fra1, Fra2) or activating transcription factor (ATF-2, ATF-3, B-ATF) family members (Angel and Karin, 1991). Fos and ATF proteins can form stable heterodimers with Jun, additionally ATF and Jun can form stable homodimers (Ziff, 1990) and these interactions are formed through 'leucine-zipper' motifs. These motifs consist of an α-helix, in which every seventh amino acid

is a leucine that protrudes from the side of the structure providing a hydrophobic surface to mediate dimerisation (Landschulz et al., 1988). The exact makeup of AP-1 may drive the transcription of particular subset of genes through slightly different affinities for particular promoter sites. Jun:Jun and Fos:Jun preferentially bind to the 12-O-tetradecanoate-13-acetate (TPA)-responsive element (TRE), whereas dimers containing ATF-2 are targeted to cAMP responsive element (CRE) sites (Hai and Curran, 1991). This feature means that the convergence of heterologous signals on AP-1 does not always produce the same result. The regulation of AP-1 relies on both translational and post-translational regulation of Jun, Fos and ATF-2. Most of the genes involved in encoding AP-1 components are 'immediate early' genes, because their transcription is rapidly induced following extracellular stimuli and is performed independently of *de novo* protein synthesis (Karin, 1996). The regulation of c-Fos synthesis through the SRE has been described above. In the case of Jun family members a specialised TRE is used that is recognised by Jun:ATF-2 heterodimers. Therefore c-*jun* can positively autoregulate its own expression (van Dam et al., 1993). In contrast, ATF-2 is expressed constitutively and is regulated post-translationally by phosphorylation as described below (Karin, 1995b).

Phosphorylation of c-Jun, c-Fos and ATF-2 by upstream kinases is crucial for AP-1 regulation as this can both alter their transcriptional activity (Smeal et al., 1994) and increase their stability (Musti et al., 1997), enabling the potentiation of a transcription mediated response without *de novo* protein synthesis. c-Jun can be phosphorylated at a number of sites with varying consequences. The best understood is the phosphorylation of Ser^{63} and Ser^{73} by JNK (Derijard et al., 1994), which enhances its ability for transcription as either homo- or heterodimers (Pulverer et al., 1991). In contrast, phosphorylation of c-Jun by ERK1/2 occurs at C-terminal inhibitory sites that decrease the DNA binding affinity of AP-1 (Minden et al., 1994b). c-Fos may be phosphorylated at Thr^{232} by FRK, a proline directed kinase stimulated by growth factors. As with c-Jun this event potentiates the transcriptional activity of the factor (Deng and Karin, 1994). ATF-2 can be phosphorylated by either JNKs or p38 at Thr^{69} and Thr^{71}, which as with c-Fos and c-Jun has a positive effect on gene transcription (Karin et al., 1997).

As mentioned above, the complexity of signals upstream of AP-1 allow it to mediate a wide array of responses. A number of transgenic knockout mice models have enabled the individual study of some of the components involved in AP-1 formation in terms of impact on the whole organism. The majority of cell types in homozygous c-*fos*$^{-/-}$ mice are viable and can differentiate and proliferate, which surprised ressearchers due to the striking increases noted in c-*fos* expression following growth factor stimulation. However, in this model there were complications with bone formation, gametogenesis and neuronal functions (Johnson et al., 1992). It is therefore thought that in the absence of c-*fos*, other Fos family members may act as efficient substitutes. In contrast, c-*jun* is essential for normal development as c-*jun*$^{-/-}$ mouse embryos die during mid-to-late gestation and show many signs of abnormal development (Johnson et al., 1993), (Hilberg et al., 1993). Further studies have shown that ATF-2 is also crucial for normal development as mice lacking this gene display a wide range of abnormalities especially of the central nervous system (Reimold et al., 1996). On a cellular level the effects of AP-1 are equally complex, and appear to depend on both cell type and the context in which the signal is transmitted. The involvement of AP-1 in apoptosis, in particular, has received much attention. Researchers have shown that in certain stressful environments c-*fos* and c-*jun* expression are increased prior to apoptosis (Colotta et al., 1992; Smeyne et al., 1993) suggesting a pivotal role for AP-1 under these conditions. However, TNF- and Fas-induced apoptosis do not require AP-1 (Lenczowski et al., 1997; Liu et al., 1996). To complicate matters further, many studies have shown that AP-1 is also important for proliferation and differentiation. This was first shown by the ability of c-Jun to transform cells alone (Bos et al., 1990). Further investigations in a number of model systems have added to this observation and suggest that this effect is mediated through direct inhibition of p53 expression and progression through the cell cycle (Schreiber et al., 1999). A diagrammatic representation of how MAPK pathways lead to AP-1 is shown below:

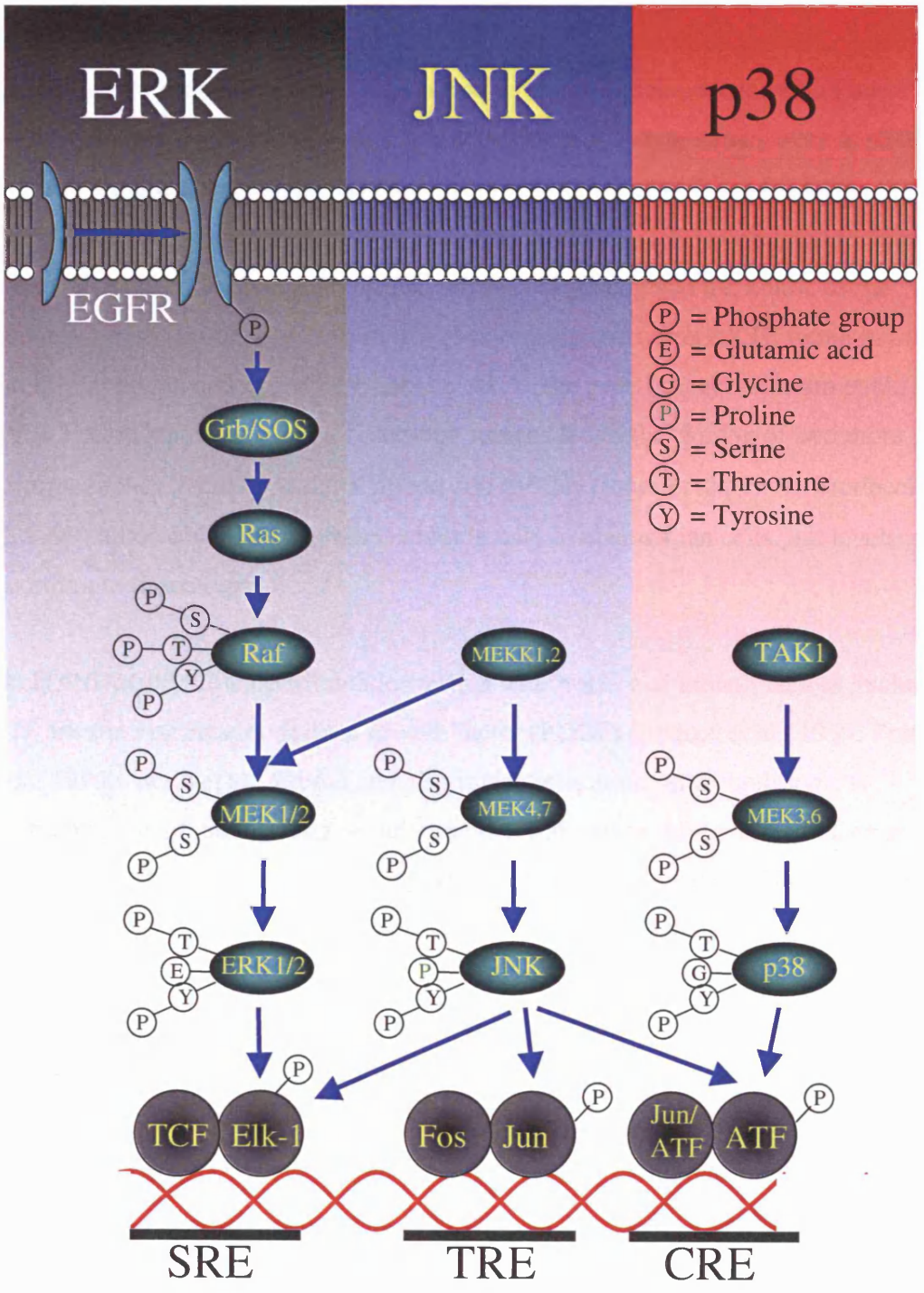


Figure 1.5 MAPK signalling pathways leading to AP-1 activation

1.2.4 Akt/protein kinase B signalling

Three different groups discovered the serine/threonine kinase, Akt/protein kinase B (PKB), simultaneously in 1991; this meant that three different names were applied to the same protein. Two groups had used its homology with PKC and PKA for identification and so called it PKB (Coffer and Woodgett, 1991) or related to A and C kinases (RAC) (Jones et al., 1991). The third group discovered the kinase as the product of the v-akt oncogene from the transforming retrovirus AKT8 found in rodent T-cell lymphoma, and applied the name c-Akt to the gene product (Haslam et al., 1993). Following these studies, homology screens led to the cloning of two more isoforms of Akt, namely Akt2/PKBβand Akt3/PKBγ (Jones et al., 1991; Brodbeck et al., 1999) all of which are expressed ubiquitously in mammalian cells, but levels vary according to tissue type.

Akt is activated following stimulation with a wide variety of growth factors, including EGF, insulin and platelet-derived growth factor (PDGF) (Peruzzi et al., 1999; Franke et al., 1995; Alessi et al., 1996a) and also under certain stressful conditions. A common theme of Akt activation under these conditions involves oxidative stress. This may be caused by a number of agents, the best described being UV radiation and H_2O_2, which as described below, may activate EGFR and initiate downstream signalling components (Wang et al., 2000a; Wan et al., 2001; Huang et al., 2001). Ligand recognition by these receptors increases tyrosine phosphorylation on their cytoplasmic domains allowing the recruitment of SH2-containing proteins. One such protein is phosphatidylinositol 3-kinase (PI3K), which has a SH2 domain in its p85 regulatory subunit. Association with growth factor receptors results in its activation (Heldin, 1995). An alternative mode of PI3K activation has also been described whereby Ras can activate the p110 subunit directly (Rodriguez-Viciana et al., 1994). Activation of PI3K localises the p110 catalytic domain of PI3K to the plasma membrane where it increases phosphorylation of phosphatidylinositol species at the 3' position of the inositol ring. These species *e.g.* phosphatidylinositol (3,4)-bisphosphate [PtdIns(3,4)P_2] and phosphatidylinositol (3,4,5)-trisphosphate [PtdIns(3,4,5)P_3] can bind proteins with a pleckstrin-homology (PH) domain such as Akt thus recruiting them to the plasma membrane. Whilst binding of these lipid

species is not sufficient for Akt activation it is thought that the resultant localisation of Akt at the plasma membrane enables its phosphorylation at Thr^{308} and Ser^{473} by two upstream kinases. The first of these was shown also to be dependent on PtdIns(3,4)P_2 or PtdIns(3,4,5)P_3, again as a result of containing a PH domain and was discovered by Alessi et al. (1997). This enzyme was later shown to phosphorylate Akt at Thr^{308} and due to its dependence on PtdIns was termed PtdIns-dependent kinase-1 (PDK1) (Stokoe et al., 1997). Whilst the enzyme responsible for phosphorylation of Akt at Ser^{473} is also PtdIns dependent, it has not yet been isolated. This putative enzyme has been called PDK-2. Recent studies suggest that, in some cell types at least, PDK-2 may be MAPK-activated protein kinase-2 (MAPKAPK-2), a downstream target of p38 (Rane et al., 2001). In addition to phosphorylation, Akt is also regulated by multimerisation through binding via its PH domains (Datta et al., 1995). A more recent finding is that Akt may also require tyrosine phosphorylation for efficient activation. This theory was proposed following studies demonstrating tyrosine phosphorylation of two residues close to the activation loop (Tyr^{315}/Tyr^{326}), the mutation of which to phenylalanine residues abolished the kinase activity of Akt following growth factor stimulation (Chen et al., 2001). The following schematic diagram (Figure 1.6) outlines the sequence of events leading to Akt activation:

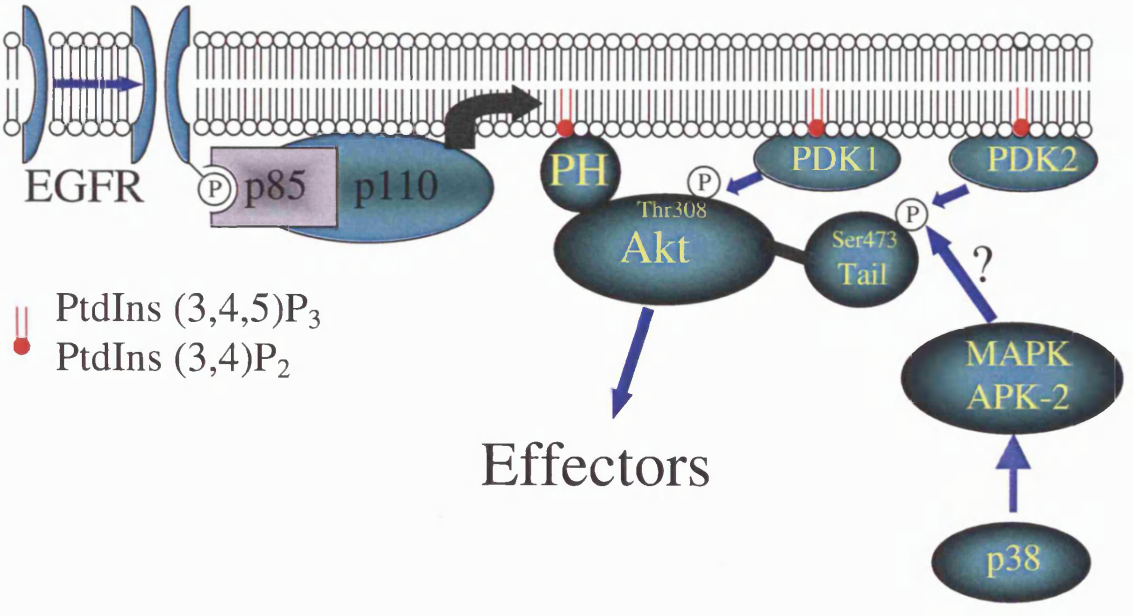


Figure 1.6 Regulation of Akt by EGFR/PI3K

Following activation Akt may translocate to the nucleus, in a manner similar to the MAPKs, where it can exert effects directly on nuclear substrates (Meier et al., 1997; Andjelkovic et al., 1997). The downstream targets of Akt are diverse and may be loosely categorised into those having metabolic and cell survival functions. On a molecular level, research by Alessi *et al.* (1996a), showed that the optimum sequence for efficient phosphorylation by Akt was -Arg-X-Arg-Y-Z-Ser/Thr-Hyd-, where X is any amino acid, Y and Z are small amino acids other than glycine and Hyd is an amino acid with a hydrophobic side chain (Alessi et al., 1996b). Despite there being a large number of proteins that fall into this category, the exact position of this sequence of residues within the tertiary structure of the protein will determine if it is a viable substrate for Akt. The involvement of Akt in metabolic functions is best described by insulin signalling to insulin-responsive tissues. Briefly, recognition of insulin by its cognate receptor leads to Akt activation via the mechanism outlined above, Akt then exerts its metabolic effects through a number of downstream targets. Some of these are the glucose transporters (GLUT), which are responsible for glucose uptake. Akt can upregulate expression of GLUT1 and GLUT3 (Barthel et al., 1999) and increase the membrane translocation of GLUT4 (Kohn et al., 1996). One of the best-described roles for Akt is the phosphorylation and inactivation of glycogen synthase kinase 3 (GSK3), which enables glycogen synthase to remain in a relatively dephosphorylated and active state resulting in enhanced glycogen synthesis (Cross et al., 1995). Furthermore, the metabolic effects of Akt may be mediated through a number of additional mediators such as those involved in protein synthesis *e.g.* eIF-4E (Gingras et al., 1999).

The second role for Akt has become the focus of intense research over recent years. A huge body of evidence has been gathered that demonstrates Akt is crucial for growth factor-mediated cell survival and the blockade of apoptosis. It is thought that Akt exerts its effects on these processes through a variety of mechanisms. Studies have shown that the promotion of cell survival by IGF-1 in stimulated cerebellar granule cells (Dudek et al., 1997) and fibroblasts is largely dependent on Akt (Kennedy et al., 1997). The ectopic expression of dominant negative forms of Akt under these conditions diminishes cell survival. The blockade of apoptosis in stressful environments by Akt is achieved through the modulation of both pro- and antiapoptotic factors.

In order to maintain normal cellular function, Akt is negatively controlled by phosphatase and tensin homologue (PTEN). This phosphatase shares sequence identity with PTPases and is deleted or mutated in a number of human cancers and tumour cell lines (Steck et al., 1997; Li et al., 1997). A series of studies led to the conclusion that PTEN was not a protein phosphatase but had highest substrate affinity for phosphoinositides, especially PtdIns(3,4,5)P_3 (Maehama and Dixon, 1998). Therefore, active PTEN antagonises PI3K and functions as a tumour suppressor gene, with some researchers now believing that it may be at least as important as p53 in resistance to oncogenesis.

1.2.5 Nuclear Factor-κB

Nuclear factor-κB (NF-κB) was initially discovered by immunologists as a transcription factor that bound to the immunoglobulin kappa light chain enhancer in B cells stimulated with lipopolysaccharide (Sen and Baltimore, 1986). Like AP-1, NF-κB is a dimeric transcription factor. It is composed of homo- or heterodimers of Rel family members p65 (RelA), p50 (NF-κB1), p52 (NF-κB2), c-Rel and RelB (Mercurio and Manning, 1999). All of these proteins contain a 300 amino acid aminoterminal Rel homology domain (RHD), that is responsible for DNA binding, nuclear localisation and dimerisation (Kunsch et al., 1992). p50 and p52 are synthesised as precursors, p105 and p100 respectively, from which active transcription factors are released by proteolytic cleavage, whereas p65, RelB and c-Rel contain a transactivation domain, which is primarily responsible for transcriptional activation capacity of the complex (Zhong et al., 1997). In unstimulated cells NF-κB is retained in the cytoplasm through its interaction with members of the inhibitors of NF-κB (IκB) family. IκBs bind to the RHD and therefore mask the nuclear localisation signal. Different members of this family appear to have varying affinities for Rel family members, which may enable specific activation of a particular NF-κB dimer through selective regulation of upstream IκBs (Karin and Ben Neriah, 2000). Upstream of IκBs are the IκB kinases (IKKs). These are found in a 'signalsome' complex consisting of IKKα, IKKβ and two regulatory IKKγ subunits. The former two subunits are responsible for IκB phosphorylation,

which leads to its dissociation from NF-κB and subsequent ubiquitin dependent degradation, allowing NF-κB to enter the nucleus (Lee et al., 1998; Zandi et al., 1998). Whilst IKKα and β share many structural similarities, they perform distinct tasks as both $IKK\alpha^{-/-}$ and $IKK\beta^{-/-}$ transgenic mice die either during development or immediately after birth (Li et al., 1999b; Li et al., 1999a). Factors that regulate the function of IKK are quite complex, as this is the point of convergence of a number of upstream signals originating from extracellular stimuli, especially cytokines. Of particular interest to this work is the involvement of Akt, which is thought to phosphorylate IKK and trigger the eventual translocation of NF-κB to the nucleus (Kane et al., 1999). In addition to this traditional view of NF-κB regulation, more recent studies have shown that events post translocation may also be important. This type of IκB-independent NF-κB regulation was first shown when it was demonstrated that the p38 inhibitor, SB203580, could inhibit NF-κB dependent gene expression without altering its translocation or DNA binding (Vanden Berghe et al., 1998).

As with AP-1, the complexity of signals upstream of NF-κB allow this transcription factor to be involved in a large number of cellular events including immune and stress responses, inflammation and apoptosis (Li and Karin, 1999). This is possible through the sheer number of genes containing NF-κB binding sites in their regulatory elements. More than 150 having been identified to date (Pahl, 1999). These genes fall into a number of categories but are largely linked as being important during tissue injury and repair. As a result NF-κB activation is consistently reported in many models of disease, especially cancer and those involving chronic immune activation. Many researchers have alluded to the importance of NF-κB in cancer, high levels of nuclear NF-κB have been shown in breast cancer (Nakshatri et al., 1997), Hodgkin's lymphoma (Sovak et al., 1997) and many other solid tumour derived cell lines (Visconti et al., 1997). In this setting it is thought that constitutive activation of NF-κB confers resistance to apoptosis, which may be especially important in allowing tumour cells to resist chemotherapy and radiotherapy (Van Antwerp et al., 1996), (Beg and Baltimore, 1996). Therefore, inhibition of NF-κB in combination with traditional cytotoxic drugs may prove to be extremely useful in future cancer therapies.

1.2.6 Redox regulation of cell signalling

The catalysis of ROS production by asbestos fibres is widely regarded as being crucial in asbestos related disease. However, ROS production has also been demonstrated during normal signalling events including receptor recognition of PDGF, EGF and many cytokines (Bae et al., 1997; Sundaresan et al., 1995). Furthermore, ROS may be produced by NADPH oxidase in phagocytic cells as part of their host defence repertoire, a function that can be performed to a lesser degree by non-phagocytic cells for signalling purposes (Griendling et al., 1997; Sorescu et al., 2001).

The modulation of receptor tyrosine kinase function by oxidants means that all downstream effectors may be affected in some way. Therefore the list of signalling molecules regulated by redox is long and will not necessarily reflect a direct effect. Activation of ERK1/2 signalling by oxidative stress may occur through a number of possible mechanisms. At the level of the receptor, inhibition of tyrosine phosphatases that dephosphorylate EGFR may be critical in initiating signal transduction pathways. Researchers have shown that the production of ROS during normal signalling is crucial for the propagation of a response (Bae et al., 1997). This may be because the activity of PTPases is 10-1000 times greater than protein tyrosine kinases, and therefore the signal generated from ligand binding alone is insufficient without concurrent phosphatase inhibition (Fischer et al., 1991). On a molecular level this effect is mediated by reversible covalent modification of active site reactive cysteine residues (-R-S$^-$) to a sulfenic ion (-R-S-O-H) and subsequent loss of catalytic activity (Finkel, 2000). An alternative point of ERK pathway regulation by oxidants is found in Ras, in this case nitric oxide (NO) binds to a cysteine residue found on the surface of this protein resulting in its activation (Lander et al., 1995). Another mechanism has been described whereby thioredoxin, an antioxidant protein, which is usually bound to and inhibits apoptosis signal-regulated kinase 1 (ASK1), dissociates under oxidative stress-inducing conditions allowing free ASK1 to activate the SAPK pathways and possibly trigger apoptosis (Gotoh and Cooper, 1998). ROS formation can also increase the production of lipid peroxide species that are highly reactive and can form both protein and DNA adducts resulting in loss of function and inheritable mutations respectively. One of these lipid peroxide species, 4-hydroxy-2-nonenal (4-HNE), has been shown to activate the SAPK pathways (Uchida et al., 1999). Activation of Akt

by oxidative stress has been well described (Wang et al., 2000a; Peus et al., 1999) and may occur through one of the mechanisms outlined above, or possibly through upstream activation of MAPKAPK-2, however, whether this is relevant *in vivo* remains to be seen (Freshney et al., 1994). Modulation of these signalling pathways will then have downstream consequences on transcription factors but researchers have also shown more direct effects. The respective subunits of both AP-1 and NF-κB contain cysteine residues in their DNA binding domains that may be modified as described above. Studies have shown that treatment with reducing agents increases the DNA binding of these factors, which suggests the opposite would be true with oxidising agents (Toledano and Leonard, 1991; Hayashi et al., 1993; Abate et al., 1990). Due to these seemingly contradictory effects on transcription factors it seems likely that the eventual outcome is specific on cellular context.

1.2.7 Phenotypic endpoints of cell signalling systems

Cell death and proliferation are vital but opposite phenotypic endpoints that may follow activation of cell signalling pathways. The balance between these two processes is crucial throughout the lifetime of multicellular organisms. At early stages in life these processes control tissue growth and development and later on they are required to maintain tissue homeostasis. In cancer, this balance is disrupted allowing the survival and unregulated proliferation of a population of mutated cells.

1.2.7.1 Apoptosis

Cell death in these conditions is not merely a spontaneous occurrence, but a carefully controlled, energy requiring process called apoptosis. Apoptosis was first described in 1972 as a series of morphological changes prior to cell death under certain physiological and pathological environmental conditions (Kerr et al., 1972). The fundamental role of apoptosis is to remove potentially harmful cells that are damaged in any way without further damage to neighbouring cells. Improper regulation of apoptosis may be involved in many aspects of tumour cell biology. These range from simply increasing cell survival time meaning that the chance of gaining sufficient

genetic lesions for carcinogenesis is increased, to allowing the survival of cells that have detached from the extracellular matrix during metastasis.

Since its initial discovery, the mechanism behind apoptosis has been extensively studied. There are thought to be two pathways leading to apoptosis. These are commonly referred to as the 'intrinsic' and 'extrinsic' pathways. 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 the resting state, and rely on proteolytic cleavage and subsequent molecular reorganisation to become active (Stennicke and Salvesen, 1999). The extrinsic pathway is activated from a signal at the cell surface, for example the binding of TNF-α to its cognate receptor (Wallach et al., 1999). Ligand recognition leads to aggregation of the cytoplasmic death domains (DD) of the receptors, and the recruitment of adaptor proteins that also bear this motif *e.g.* TNF receptor associated death domain (TRADD) and MORT1/FADD (Hsu et al., 1995). The recruitment of these adapter molecules to the protein complex allows the binding of initiator caspases, such as caspase 8 and 10 (Boldin et al., 1996), (Fernandes-Alnemri et al., 1996). The pro-form of these enzymes possesses mild proteolytic activity. Aggregation in these death-inducing signalling complexes (DISCs) may allow sufficient intramolecular processing as to release active caspase (Muzio et al., 1998). Active caspase 8 is able to cleave a number of downstream proteins, notably the executioner caspases (caspases 3, 6 and 7) (Grutter, 2000). In turn, the executioner caspases cleave a number of proteins primarily involved in DNA repair and the structural integrity of the cytoskeleton (Nicholson, 1999), leading to the characteristic changes in morphology as first described by Kerr *et al.* (1972).

The intrinsic pathway is dependent on the mitochondria and is activated following genotoxic injury, growth factor deprivation and hypoxia (Green and Reed, 1998). These stimuli cause the outer membrane of the mitochondria to become leaky, allowing cytochrome *c* and other metabolic enzymes to escape. Cytosolic cytochrome *c* binds to a caspase activating protein, apoptotic protease activating factor-1 (Apaf-1), which in turn binds pro-caspase 9 allowing its proteolytic processing in a manner similar to that hypothesised for caspase 8 (Srinivasula et al., 1998). However, active

caspase 9 has to remain in this complex, known as the apoptosome, to retain its activity (Zimmermann et al., 2001). In common with the other initiator caspases, caspase 9 exerts its effects through activation of the executioner caspases.

In addition to caspases, the Bcl-2 family of proteins is known to be critical in the regulation of apoptosis. This family has both antiapoptotic members, which include Bcl-2, Bcl-X_L, Bcl-W, Bfl-1 and Bcl-B as well as proapoptotic members, which include Bax, Bad, Bid, Bim and Nix (Reed, 1997). It is thought that the antiapoptotic effects of Bcl-2 family proteins such as Bcl-2 and Bcl-X_L may result from their structural similarity to pore-forming domains of certain bacterial toxins, and may therefore serve in maintaining integrity of the mitochondrial membrane thus preventing cytochrome *c* release (Zimmermann et al., 2001). Furthermore, these proteins have at least one hydrophobic pocket on their surface, which suggests that they interact with other proteins. One of these is known to be Apaf-1 as has been shown in co-precipitation studies (Pan et al., 1998). The proapoptotic members of the Bcl-2 family display greater structural diversity suggesting they act through differing mechanisms. Surprisingly some of these proteins are also able to form channels and increase cytochrome c release, which raises interesting questions as to how such seemingly similar properties can have such opposing effects (Schendel et al., 1999; Narita et al., 1998). Other proteins in the proapoptotic subgroup of Bcl-2 proteins have Bcl-2 homology-3 domains (BH3), which are thought to bind the hydrophobic pocket present in the antiapoptotic proteins and thus directly antagonise their effects (Cheng et al., 2001b).

The above factors are all, at least in part, under the control of signal transduction cascades. The role of ERK activation in growth factor mediated cell proliferation has already been discussed. However, recent studies have shown that ERK can also inhibit apoptosis. These centred on the ability of ERK to inhibit cytosolic caspase activation following cytochrome *c* release in a B-Raf dependent manner (Erhardt et al., 1999). Another antiapoptotic target of ERK has been identified as Bad. Phosphorylation of this protein at Ser^{112} and Ser^{136} by ERK increases its affinity for 14-3-3 protein and thus sequesters it away from dimerisation with Bcl-2 and Bcl-X_L (Zha et al., 1996). This event has been shown both in conditions of cytokine induced cell survival in haemopoietic cells (Scheid and Duronio, 1998) and also under hypoxic

conditions in cortical neurons (Jin et al., 2002). Furthermore, ERK activation can lead to modulations in the expression of antiapoptotic factors such as Bcl-X_L, which has been shown following both fibroblast growth factor-2 (FGF-2) (Pardo et al., 2002) and EGF stimulation (Jost et al., 2001). The targets of SAPK pathway activation in apoptosis are less well understood, but SAPK activation prior to effector caspases and apoptosis under many stressful conditions is well described (Cheng et al., 2001a; Chen et al., 1996; Zou et al., 2002). SAPKs may mediate these effects through changes in gene expression, for example, upregulation of Fas ligand expression has been demonstrated to be JNK dependent in neuronal cells following growth factor deprivation (Le Niculescu et al., 1999)

Akt has a more direct effect in the prevention of apoptosis, it has been shown to directly phosphorylate and inhibit caspase 9 *in vitro* (Cardone et al., 1998). Akt may also down modulate Bad by phosphorylation through the same mechanism as described for ERK (Datta et al., 1997). In addition to these direct effects on the cell death machinery, Akt may also have influences through the direction of gene expression. In particular, the activation of NF-κB by Akt can lead to increases in the expression of antiapoptotic Bcl-2 family members and proinflammatory cytokines involved in cell survival (Catz and Johnson, 2001; Kurland et al., 2001).

The p53 tumour suppressor gene plays a central role in the regulation of many of the events described above. Whilst a comprehensive review is beyond the scope of this thesis, several key points are worth mentioning. p53 can induce growth arrest to prevent the replication of damaged DNA and allow DNA repair, or can cause apoptosis in grossly damaged cells. The transcriptional activity of p53 is important in regulating two families of genes. The first encodes proteins that act at the level of receptor signalling for apoptosis. A classical example is the increased expression of the Fas death receptor following DNA damage. In this model active p53 is required to induce apoptosis thus allowing the removal of cells with potentially pathogenic gene alterations (Owen-Schaub et al., 1995). The second group includes Bax, a proapoptotic member of the Bcl-2 family. As with Fas, Bax expression is positively regulated by p53. This facilitates cytochrome *c* release and consequent triggering of the intrinsic caspase cascade (Narita et al., 1998). Whilst these functions of p53 have largely been determined from *in vitro* experiments, its *in vivo* importance is

unquestionable. This is supported by the fact that about half of all human tumours have some kind of p53 mutation, including MM (Appasani et al., 1999), with yet more being unable to induce or respond to p53 (Ryan et al., 2001).

1.2.7.2 Proliferation

The control of proliferation is achieved through regulation of the cell cycle. The cell cycle represents a series of stages that must be passed through in order for a cell to divide. In the resting state cells are in G_0, so called quiescence. In order to pass into the next stage, G_1, a stimulus from growth factors is required. This stimulus may activate Ras dependent signalling cascades such as those described above resulting in the activation of genes required for transition through G_1 (Downward, 1998b). Central to cell cycle progression are a family of proteins called cyclins, of which there are four types, D, E, A and B, that are produced sequentially through the cycle (Sherr, 1996). In the initiation of the cell cycle, the expression and stabilisation of D-type cyclins is increased through activation of signal transduction cascades, which enables its assembly with catalytic cyclin-dependent kinase (CDK) partners, Cdk4 and Cdk6 (Sherr, 1996). Raf, ERK and the PI3K/Akt pathway can all positively regulate Cdk4 to promote progression (Marshall, 1999). Cyclin-D CDKs phosphorylate the retinoblastoma tumour suppressor protein thereby inactivating its capacity to repress E2F family-dependent transcription factors (Dyson, 1998). E2F accumulation activates the transcription of a variety of genes involved in DNA replication and progression through the cell cycle to S phase. These include the E-type cyclins, factors that inhibit p53-dependent apoptosis and c-*myc* (Dyson, 1998). Myc plays a pivotal role in directing the cell cycle due to its ability to regulate cyclin D/cdk4 and E2F. Therefore it is a suitable target for regulation itself. Again ERK and Akt are important for this. ERK phosphorylates Ser^{62} and thus stabilises Myc protein. Akt phosphorylates and inactivates GSK-3 (GSK-3 phosphorylates Myc at Thr^{58}, targeting it for ubiquitination and degradation). Therefore Akt, like ERK improves Myc stability (Sears et al., 2000). Between G_1 and S phase there is a checkpoint that is crucial in deciding whether or not a cell is committed to division, which cannot be passed until DNA damage has been repaired. Following this point the stimulus provided by growth factors is no longer required (Levine, 1997). Dysregulation at this point, allowing the division of cells with genomic instability, is crucial in oncogenesis

(Pardee, 1989). During S phase the cycle relies upon A-type cyclins with associated CDKs for efficient DNA synthesis. The degradation of D- and E-type cyclins and E2F is also required to prevent apoptosis (Lees and Weinberg, 1999). The accumulation of cyclin B begins towards the end of S phase and continues through G_2. This cyclin binds with its associated CDK, cdc2, but this complex is kept in an inactive state through phosphorylation at key regulatory sites on cdc2 (Sherr, 1996). As the cell passes from G_2 to M phase, the level of cdc2 phosphorylation is decreased. This activates the complex and locates it to the nuclear membrane where it phosphorylates several targets, including laminin, which causes the nuclear membrane to break down allowing mitosis to ensue (Peter et al., 1990). The SAPK pathways are thought to have somewhat complex interactions with the cell cycle. Under non-stressed conditions JNK is required for Cdk2-Cdc2 activity and concomitant transition through G_2 (Potapova et al., 2000), but overexpression of a downstream target, ATF-2, inhibits progression through the G1-S checkpoint and decreases proliferation (Crowe and Shemirani, 2000). Furthermore, activation of p38γ by γ-radiation is important in preventing transition through the G_2-M checkpoint thus providing a means of linking a stress response to prevention of genotoxic inheritance (Wang et al., 2000b).

1.3 AIMS

1) Main aim.

Elucidation of the activation state of signal transduction pathways in mesothelial cells following exposure to crocidolite asbestos, including investigations into the mechanism(s) involved and downstream consequences. This is important in unravelling the sequence of events that occur in mesothelial cells following exposure to asbestos, which may show how the disease process is initiated. The mechanisms involved may provide information regarding asbestos associated mesothelial cell carcinogenesis and to identify targets for chemoprevention.

2) Objectives.

The following cell signalling elements were chosen due to their importance in maintaining normal tissue homeostasis under healthy conditions, but that are also implicated in well-defined disease states. These pathways have been implicated in many diseases, including those of chronic inflammation, which may or may not be directly involved in carcinogenesis. With the current availability of selective inhibitors to specific factors in these pathways, the impact of each pathway on defined endpoints, including transcription factor activation and cell death/proliferation, may be established.

i) Analysis of ERK pathway activation in 4/4 RM4 and MET5A cells under asbestos exposed conditions.

Comparing the ERK pathway response between human and rat cells may verify the suitability of using a rat model versus SV40 transformed human cells and provide insights into how asbestos treatment may interact with SV40 infection.

ii) Analysis of SAPK pathway activation in 4/4 RM4 and MET5A cells under asbestos exposed conditions.

The elucidation of the activation state of these pathways may provide a mechanism explaining the altered phenotype of asbestos exposed mesothelial cells *i.e.* apoptosis and heightened inflammatory state.

iii) Analysis of Akt activation in MET5A cells under asbestos exposed conditions.

Determination of a means whereby mesothelial cells can escape cell death under potentially genotoxic conditions may be useful in outlining the early events in asbestos-induced mesothelioma.

iv) Derivisation of mechanistic factors involved in asbestos-induced signal transduction.

Determination of the physical/chemical interactions between fibres and cells that lead to the activation of signalling pathways. This could lead to the identification of possible targets to decrease the harmful effects of asbestos

v) Downstream consequences of signal pathway activation.

Determination of the effect of the signal generated following asbestos exposure in relation to transcription factor activation may prove the effect is not an artefact but has a functional consequence.

vi) Phenotypic consequences of signal pathway activation.

Determination of the effects of the signal generated on cell viability and surrogate markers of apoptosis. These investigations will further expand knowledge of the functional consequence of asbestos-induced signal transduction.

CHAPTER 2

MATERIALS AND METHODS

CHAPTER 2

2.1 MATERIALS.

2.1.1 General reagents and chemicals

Unless specified otherwise, all reagents and chemicals were purchased from Sigma-Aldrich Company Ltd (Poole, UK).

2.1.2 Crocidolite asbestos

Reference samples of crocidolite were from either the Union Internationale Contre le Cancer (UICC) or the National Institute for Environmental Health (NIEHS). Due to the way in which these samples are prepared they represent 2 distinct populations of fibres with respect to their physical dimensions, thus enabling crude comparisons of fibre geometry upon cellular effects (see table 2.1 below). Unfortunately the NIEHS sample was in limited supply therefore the range of experiments over which they could be used was restricted. Milled crocidolite was generated by milling UICC crocidolite in an agate ball mill for 8 hours. To ensure no fibrous particles were present, a sample was viewed under the electron microscope. In the interests of safety, waste involving milled crocidolite was treated as if it were fibrous.

	MEAN LENGTH(μm)	*MEAN WIDTH(μm)*
UICC	2.63	0.22
NIEHS	11.4	0.27

Table 2.1 Physical characteristics of crocidolite fibres.

2.1.3 Cell lines

The 4/4 RM-4 cell line was purchased from the European Collection of Animal Cell Cultures (Porton Down, Wiltshire, UK) in frozen vials. This cell line is derived from spontaneously transformed rat mesothelial cells. They were obtained at passage 8 and

were routinely cultured as outlined below until they had reached 20 divisions, following this they were discarded and a fresh culture was initiated.
The human mesothelial cell line, MET-5A, was a kind gift from Dr B. Gerwin (Laboratory of Human Carcinogenesis, National Cancer Institute, National Institutes of Health, Bethesda, USA). This line was derived from normal human mesothelial cells that were transfected with a plasmid containing SV40 early region DNA. These cells have one copy of SV40 early region DNA in their genome and express SV40 large T antigen. They continue to exhibit features of normal mesothelial cells including sensitivity to the cytotoxic effects of asbestos fibres. One year after injection subcutaneously or intraperitoneally in athymic nude mice, these cells remain non-tumorigenic, and therefore provide a good model system for the *in vitro* study of fibre carcinogenesis (Ke et al., 1989).

2.1.4 Cell culture media

4/4 RM-4 cells were grown in Hams F12 (Gibco BRL, Paisley, UK) containing 15% foetal calf serum (FCS) Gibco). MET5A cells were grown in DMEM/F12 (Gibco) containing 10% FCS, 50 units/ml penicillin, 100mg/ml streptomycin, 100ng/ml hydrocortisone, 2.5mg/ml insulin, 2.5mg/ml transferrin and 2.5ng/ml selenium.

2.1.5 Antibodies

EGFR (1005) rabbit polyclonal, α-tubulin mouse monoclonal, ERK1/2 rabbit polyclonal, JNK1 rabbit polyclonal and Akt1 goat polyclonal antibodies were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA); anti-p38 rabbit polyclonal was from Sigma; anti-phospho-ERK1/2 (Thr^{202}/Tyr^{204}) (E10) mouse monoclonal, -JNK1 (Thr^{183}/Tyr^{185}) (G9) mouse monoclonal, -p38 (Thr^{180}/Tyr^{182}) (S8B10) mouse monoclonal and -Akt (Ser^{473}) rabbit polyclonal were from New England Biolabs (NEB), Beverly, MA). The donkey anti-rabbit secondary antibody used in western blotting (horseradish peroxidase (HRP) conjugate) was obtained from Amersham (Amersham, UK), the anti-goat was from Santa Cruz and the anti-mouse from Sigma. Caspase 3 mouse monoclonal antibody was a kind gift from Dr Grant Dewson (Leicester University, UK).

2.1.6 Oligonucleotides

AP-1 and NF-κB oligonucleotides and reagents necessary for ^{32}P labelling *i.e.* T4 polynucleotide kinase and kinase buffer were from Promega (Southampton, UK).

Oligonucleotide sequences:
AP-1

5'-CGC TTG ATG AGT CAG CCG GAA-3'
3'-GCG AAC TAC TCA GTC GGC CTT-5'

NF-κB

5'-AGT TGA GGG GAC TTT CCC AGG C-3'
3'-TCA ACT CCC CTG AAA GGG TCC G-5'

2.1.7 Radioisotopes

$\gamma^{32}P$ adenosine 5'-triphosphate ($\gamma^{32}P$-ATP) was purchased in 9.25MBq aliquots from Amersham Life Science Ltd (Amersham, UK).

2.1.8 Peptide Substrates

Phosphorylated heat and acid stable protein regulated by insulin (PHAS-I) was obtained from Calbiochem (La Jolla, CA). Histone H2B was from Roche (Indianapolis, IN). The DEVD.AFC substrate for caspase 3 activity assays was from Bio-Source (Camarillo, CA).

2.1.9 Buffers

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

10x Tris/glycine buffer	*final concentration*
30.3g Tris base	250mM
144g glycine	2M

The components were dissolved in 1000ml distilled water and stored at room temperature.

SDS Running Buffer	*final concentration*
100ml 10x Tris/glycine buffer	25mM Tris base 200mM glycine
10ml 10% w/v sodium dodecylsulphate	0.1% w/v

This was made up to 1000ml with distilled water.

10x Western blot lysis buffer	*final concentration*
2ml 1M Tris (pH7.5)	200mM
3ml 5M NaCl	1.5M
200µl 0.5M EDTA	10mM
200µl 0.5M EGTA	10mM
1ml Triton X-100	10% v/v
250µl 1M sodium pyrophosphate	25mM
200µl 0.5M β-Glycerophosphate	10mM
1ml 100mM sodium orthovanadate	10mM

20µl 5mg/ml leupeptin	10µg/ml

This was made up to 10ml with distilled water and diluted to 1x as necessary with water.
1mM PMSF was added immediately prior to use.

4x Western blot sample buffer	*final concentration*
1.52g Tris base	12.5mM
30ml glycerol	60%
4g SDS	8%
100mg bromophenol blue	0.02% w/v

This was made up to 50ml with distilled water.
5% β-mercaptoethanol was added immediately prior to use.

Transfer buffer	*final concentration*
200ml methanol	20%
100ml 10x Tris/glycine buffer	48mM Tris
	38mM glycine

This was made up to 1000ml with distilled water.

10x Tris buffered saline (TBS)	*final concentration*
24.2g Tris base	20mM
80g NaCl	137mM

This was made up to 1000ml with distilled water and the pH adjusted to 7.6.

1x TBS-Tween 20 (TBS-T)	*final concentration*

100ml TBS	
1ml Tween 20	0.1% v/v

This was made up to 1000ml with distilled water.

2x Triton lysis buffer (TLB)	*final concentration*
10ml 1M Tris base, pH 7.4	20mM
4g NaCl	137mM
1.575g β-glycerophosphate	25mM
0.45g sodium pyrophosphate	2mM
2ml 0.5M EDTA	2mM
50ml glycerol	10% v/v
0.156g benzamidine	2mM

This was made up to 250ml with distilled water, stored at 4°C and diluted 1:1 before use.

For 10ml 1x TLB the following were added immediately prior to use:

	final volume
100mM sodium orthovanadate	100µl
100mM phenylmethylsulphonyl fluoride	100µl
5mg/ml pepstatin	10µl
2mg/ml aprotonin	40µl
5mg/ml leupeptin	10µl
Triton X-100	100µl
1M DTT	5µl

Kinase assay buffer (KAB)	*final concentration*
5ml 500mM Hepes, pH 7.4	25mM
5ml 500mM β-glycerophosphate	25mM
5ml 500mM $MgCl_2$	25mM
100µl 500mM EDTA	0.5mM

The following were added immediately prior to use:

	final concentration
1M DTT	0.5mM
100mM sodium orthovanadate	0.5mM

5x Kinase assay sample buffer	*final concentration*
4.8ml 10% w/v SDS	2.4% v/v
12ml glycerol	60% v/v
0.202g Tris base	250mM

This was made up to 20ml with distilled water.

For 1ml 5x buffer the following were added immediately prior to use:

	final volume
1M DTT	1ml
Bromophenol blue	1mg

Buffer A	*final concentration*
50µl 1M Hepes	10mM
25µl 2M KCl	10mM
10µl 1M $MgCl_2$	2mM
1µl 0.5M EDTA	0.1mM
300µl 5mg/ml leupeptin	0.3mg/ml
10µl 100mM sodium orthovanadate	0.2mM
5µl 1M DTT	1mM
5µl 0.4M PMSF	0.4mM

This was made up to 5ml with distilled water.

Buffer B	*final concentration*
100µl Triton x-100	10% v/v

This was made up to 1ml with distilled water.

Buffer C	*final concentration*
50µl 1M Hepes	50mM
25µl 2M KCl	50mM
100µl 3M NaCl	300mM
0.2µl 0.5M EDTA	0.1mM
1µl 1M DTT	1mM
2µl 0.4M PMSF	0.8mM
100µl glycerol	10% v/v
0.4µl 0.5M NaF	0.2mM
2µl 100mM sodium orthovanadate	0.2mM

This was made up to 1ml with distilled water.

Tris-EDTA (TE) buffer	*final concentration*
100ml 1M Tris base, pH 8.0	1M
3.722g EDTA	0.1M

2x Binding buffer	*final concentration*
0.8g Ficoll	8% w/v
0.4ml 1M Hepes, pH 7.5	20mM

This was made up to 10ml with distilled water.

Binding conditions	*final concentration*
200µl 2x binding buffer	1x
20µl 1µg/µl poly[dI-dC]	0.05µg/µl
4µl 10mM $MgCl_2$	100µM

4µl 10mM DTT	100µM
20µl ^{32}P labelled oligo	
4µl Sample	
72µl water	

0.25x TBE Buffer	*final concentration*
50ml 1.78M Tris-borate/40mM EDTA	89mM Tris-borate/2mM EDTA

This was made up to 1000ml with distilled water.

Caspase-3 assay buffer	*final concentration*
11.9g Hepes	100mM
50g Sucrose	10% w/v
0.5g CHAPS	0.1% w/v

This was made up to 500ml with distilled water and NaOH was used to pH the solution to 7.0.
Immediately prior to use DTT was added to reach a final concentration of 10mM.

2.2 METHODS

2.2.1 Cell Culture

2.2.1.1 Resuscitation from liquid nitrogen

Cells retrieved from a liquid nitrogen dewer were placed in an incubator at 37°C/5%CO_2 until the vial had completely thawed. They were then added to 5ml of warmed media in a 25cm^2 flask. After approximately 8 hrs the media was removed and replaced with fresh so as to eradicate dimethyl sulphoxide (DMSO) present in the cryomix. Both of the cell lines used were tested for mycoplasma contamination

(Mycoplasma Experience, UK), in both cases the cell lines were found to be free of any infection of this kind. Following attainment of confluency, cells were passaged as below to a $75cm^2$, then $150cm^2$ flask.

NB. All reagents and equipment were prepared and kept under strictly aseptic conditions.

2.2.1.2 Routine subculture of cell lines

Once cells had reached confluency in $150cm^2$ flasks they were passaged. A 1x trypsin/EDTA (1xTE) solution was prepared in PBS. 5mls of this was used to wash the cells following removal of the medium. A further 5mls of 1xTE was used to detach the cells from the base of the flask, usually 5 mins at 37°C was sufficient. Following this the cell suspension was added to an equal volume of warmed media in a universal and spun for 5 mins at 300g. The cell pellet was then resuspended in fresh medium, and the resultant suspension used to seed cell culture plates (90/140mm) for experimentation, usually 1:2 to 1:5.

2.2.1.3 Freezing down cells for storage

Cells were harvested as per routine subculture protocol. They were then resuspended in 90% FCS/10% DMSO at around 4x 10^6 cells per ml. 1ml of this suspension was then aliquoted into each ampoule. Cells were frozen to –80°C at a rate of 1-3°C/min overnight then stored in liquid nitrogen until required.

2.2.2 Treatment of cells in culture

Due to the solid nature of asbestos fibres they cannot be simply added to cells in a solution. Fibres were aliquoted into glass vials in approximately 10-50mg units. This procedure was carried out in an inhalation chamber, which is completely sealed from the outside environment to prevent inhalation by workers. Once the weight of fibre in each vial had been determined the samples were autoclaved for 30 mins to destroy

any endotoxin present in the sample. For use in exposing cells the fibres were suspended at 2mg/ml in warmed medium. Suspension was achieved by 5 mins of sonication followed by trituration through a 21-gauge needle. Once this had been completed the fibres were added as quickly as possible because they have a propensity to settle out of the medium. Excess fibre and equipment used *e.g.* needles and syringes were double bagged and disposed of via certified asbestos handlers.

2.2.3 Biorad Protein concentration assay

Following treatment of cells it is necessary to calculate the concentration of protein within each individual sample, so variations can be taken into account. This is achieved by using the Biorad assay, which is a colorimetric means of measuring protein levels developed by Bradford in 1976. The first step was to construct a standard of curve using a series of solutions containing known protein concentrations. Table 2.2 Shows the range of concentrations formulated and the requisite volumes of a 1mg/ml bovine serum albumin (BSA) standard and water to reach the desired concentration in a 1ml cuvette.

[protein] (mg/ml)	BSA standard (µl)	water (µl)
0	0	800
2	1.6	798.4
4	3.2	796.8
6	4.8	795.2
8	6.4	793.6
10	8.0	792.0
12	9.6	790.4

Table 2.2 Volumes to make standard BSA solutions

To these 200µl of Biorad reagent was added and the solution mixed by inversion with parafilm. Each concentration was duplicated and the absorbance at 595nm was read on a spectrophotometer using 0mg/ml BSA as a blank.

4µl of each sample was then added to 796µl water in a 1ml cuvette and again 200µl of Biorad reagent was added prior to mixing. As with the standards, each sample was duplicated to correct for minor pipetting errors. The concentration in each sample was calculated from the standard curve.

2.2.4 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

These gels were used for both western blotting and the *in vitro* kinase assays. A range of acrylamide concentrations within the gel were used according to the molecular weight of the protein under investigation. As a general rule, a 10% gel was used for western blotting and 12% for the *in vitro* kinase assays. To prepare these gels a set of standard solutions are required; these and the relative volumes (ml) required are shown in table 2.3.

	10%	12%	5% (stack)
H2O	5.9	4.9	3.4
30% w/v acrylamide and bis-acrylamide	5.0	6.0	0.83
1.5M Tris-HCl, pH8.8	3.8	3.8	-
0.5M Tris-HCl, pH 6.8	-	-	0.63
10% SDS	0.15	0.15	0.05
10% ammonium persulphate (APS)	0.15	0.15	0.05
N,N,N',N'-tetramethylenediamine (TEMED)	0.006	0.006	0.005

Table 2.3 Recipes for SDS-PAGE

2.2.4.1 Gel pouring

The Biorad mini protean III kit was used for SDS-PAGE and was assembled according to the manufacturer's instructions. To ensure there were no leaks, the assembly was first filled with water. To prepare the gel, the reagents listed above were mixed, with APS and TEMED being added last. The assembly was filled using a 5ml pipette to a line approximately 7-8mm below the level of the bottom of the wells. 1ml of water was then overlaid to exclude air and ensure a level gel surface. Following polymerisation (30 mins) the water was poured off and the stacking gel was added, finally a 10-tooth comb was inserted.

2.2.4.2 Gel running conditions

Firstly 500mls of SDS running buffer was prepared with ~100mls of this used to fill the inner electrode assembly after removal of the combs from the gel cassette. The wells were then washed out using a 21-gauge needle and syringe to remove any acrylamide occluding the wells. 5µl of Biorad kaleidoscope markers were added to each gel immediately prior to the addition of sample using round gel loading pipette tips. The cassette was placed in the gel tank along with the remainder of the running buffer. Gels were run at 150V until the dye front had just run off the bottom of the gel. Following this the gel was ready for transfer onto nitrocellulose membrane (western blotting) or drying down (kinase assay).

2.2.5 Western blotting

2.2.5.1 Sample preparation

Cells were grown to 90% confluency on 140mm dishes. The media was removed and the cells were briefly washed with warmed PBS. The cells were then incubated in serum free media for 24h. Following treatment of the cells with the desired test agents the media was removed and the cells washed once with PBS. 150µl of lysis buffer

was then added and the cells were scraped from the plate into 1.5ml microtubes and incubated on ice for 20 mins. The samples were then spun at 1,000G at 4°C for 20 mins to remove cellular debris. 12µl of supernatant was kept for the Biorad protein concentration assay. 200µl of the supernatant was then removed and added to 67µl of 4x sample buffer. Samples were frozen at –20°C and denatured for 5 mins at 100°C prior to gel loading.

2.2.5.2 Protein Transfer

Nitrocellulose membrane was cut to the same size as the gel and soaked in cold transfer buffer for 1h. Following completion of running, the gel was soaked in cold transfer buffer for 15 mins along with 4 pieces of 3MM blotting paper and 2 fibre pads. These were then assembled in a Biorad wet blotting apparatus as in figure 2.1 and run overnight at 30V (room temperature) or for 90 mins at 100V (4°C).

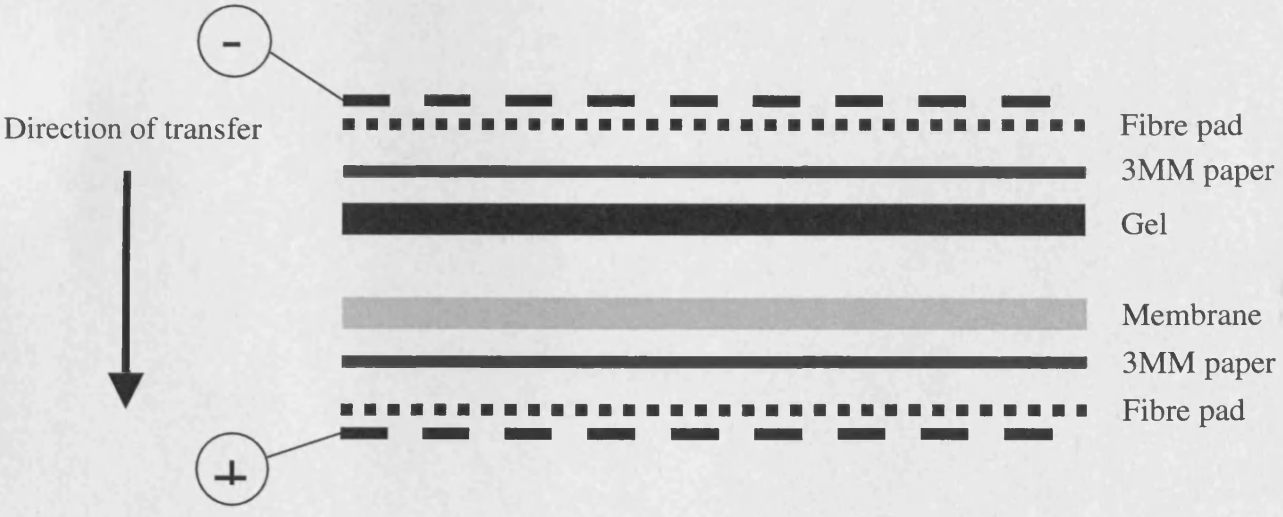


Figure 2.1 Assembly of wet blotting apparatus

2.2.5.3 Detection of proteins

Following transfer, membranes were blocked for 1-2h at room temperature (RT) in 5% marvel (w/v)/TBS-T. Blots were rinsed in TBS-T and incubated for either 2-3h at RT or overnight at 4°C in primary antibody. Blots were then washed 3 times for 5 mins in TBS-T and incubated in HRP-conjugated secondary antibody for 45-60 mins at RT. Both anti-goat and anti-rabbit secondary antibodies were diluted 1:2000 in TBS-T/5% marvel. Another 3x 5 min washes preceded exposure of the blot to 1ml enhanced chemiluminescence (ECL) reagent (Amersham) for 1min. Excess reagent was blotted off and the blot was developed under safelight conditions using Amersham Hyperfilm. Band density was measured with a Kodak imaging system, in all experiments values were divided by the intensity of the untreated control band so that the control value was always one.

2.2.6 *In vitro* complex kinase assays

2.2.6.1 Sample preparation

Cells were grown and treated as above. Following a PBS wash, cells were lysed in 300µl TLB then incubated on ice for 15 mins before storage at –80°C if required. Immediately prior to usage lysates were centrifuged at 1,000G for 15 min to remove cellular debris.

2.2.6.2 Immunoprecipitation and kinase reaction

For immunoprecipitation, 5µl of antibody was added to 20µl protein-A agarose beads and then incubated at room temperature for 1h with occasional flicking, after this time the beads were washed with PBS to remove unbound antibody. The whole cell lysates were added to the bead/antibody complex and tumbled for 3-4 h at 4 °C.
The immune complexes were washed twice in Triton lysis buffer then once in kinase buffer and then incubated with 30µl kinase buffer, 2µCi of [^{32}P]ATP, 3µl of 835µM

cold ATP (to reach final [ATP] of 50μM) and 5μg PHAS-I substrate for 30 min at 30 °C. Reactions were stopped by the addition of 5x sample buffer, then boiled for 5 min. Samples were spun for 30 seconds and the resultant supernatant was resolved by 12% SDS-PAGE. Finally, gels were dried and exposed to a PhosphorImager screen (Amersham) overnight. Incorporation of ^{32}P to the substrate was measured using a β–imaging system (Molecular Dynamics). Data was treated in the same way as described in the western blot to keep control values standard.

2.2.7 Electrophoretic mobility shift assays (EMSA)

2.2.7.1 Nuclear protein extractions

This procedure involves isolating and then lysing nuclei, due to low protein yields each treatment was duplicated in 140mm dishes, the samples were then pooled. Following treatment with test agents for desired times; the media was aspirated from the dishes. Cells were washed once and then scraped into approximately 5ml PBS. After a spin at 1,000G for 5 mins they were resuspended in 1 ml PBS. After a second spin for 30 secs they were incubated in 400μl buffer A for 15 mins on ice. The cells were then lysed by the addition of 25μl buffer B. After 15 secs agitation in a whirlimixer, nuclei were collected by centrifugation at 1,000G for 30 secs at 4 °C. The supernatant was removed and the pellet resuspended in 30μl buffer C to lyse the nuclei. Samples were then sonicated for 1 min and left to incubate on a rocking platform at 4 °C for 20 mins prior to a 5 min spin at 1,000g. An aliquot of the resultant supernatant was kept for Biorad protein assays and the remainder was stored at -80 °C.

2.2.7.2 ^{32}P labelling of oligonucleotide

5μl (8.75pmol) consensus oligonucleotide (Promega, Southampton, UK) was incubated with 1μl T4 polynucleotide kinase (PNK), 2μl T4 PNK buffer (10x), 5μl [^{32}P]ATP (3000Ci/mmol) and 7μl water at 37 °C for 30 mins. 80μl TE buffer was

added to stop the reaction and the resultant mixture was then loaded onto a NAP '5' sephadex G-25 column (Amersham). The purified radiolabeled oligonucleotide was eluted by subsequent addition of 100µl of TE buffer. The radioactivity was counted in each fraction and the 2 or 3 hottest fractions combined. Radiolabeled oligo was precipitated overnight at -20 °C with 2.5x the volume of ethanol and 0.1x the volume of 5M NaCl. The following day the oligo was collected by a 30 min spin at 1,000g. Finally the pellet was resuspended in 50µl water.

2.2.7.3 Binding of nuclear proteins to oligonucleotide and PAGE

4µg (volume kept constant throughout by addition of buffer C) of nuclear protein extract was incubated with 16µl binding conditions buffer, for each sample duplicate lanes were run to correct for any errors in protein loading. Additionally a competitive and non-competitive sample was prepared. For the competitive sample, 2µl (40 fold molar excess) of 'cold' oligonucleotide was added to compete out the band of interest. For the non-competitive lane a second 'cold' oligonucleotide with unrelated nucleotide sequence to the first was added (2µl), if the binding of the protein to DNA was specific the presence of this oligonucleotide should have no effect. All samples were incubated at room temperature for 20 mins prior to addition of 2µl 10% w/v bromophenol blue and separation by 4% PAGE in a Gibco V15.17 gel electrophoresis tank with 0.25x TBE buffer. The gel was run at 240V for 10 mins and then at 120V until the dye front was approximately 1.5 cm from the bottom. It was then dried onto 3MM blotting paper and exposed to a PhosphorImager screen for the required period of time. Band intensity was examined using a β–imaging system.

2.2.8 MTT cell viability assay

$3x10^5$ cells were plated onto a 96 well plate and allowed to settle for 24 hrs prior to removal of medium and replacement with serum free media for a further 24 hrs. Following treatment with test agents for required time periods, 20µl of 5mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was added to each

well and incubated for 1 hr at 37°C to allow development of blue formazan product. The media was then removed and 100µl of DMSO was added for approximately 30 mins to solubilise the dye. The absorbance of each well was read on a plate reader at 540nm. Where crocidolite was used duplicate wells were prepared, for these the same procedure was followed with the omission of MTT. The absorbance of these samples was subtracted from those where MTT was added to correct for absorbance of the asbestos fibres at 540nm (Mossman, 1983).

2.2.9 Lipid peroxidation assay

This assay was performed with a kit from Calbiochem. It enables the measurement of 4-HNE and malondialdeyde (MDA) that are end products of lipid peroxidation. The kit is based upon the reaction between a chromogenic reagent (N-methyl-2-phenylindole) and either MDA or 4-HNE to form a stable chromophore. This assay allows the measurement of either [MDA + 4-HNE] or [MDA], to calculate [4-HNE] therefore, [MDA] is subtracted from [MDA + 4-HNE]. Briefly, 3 x 10^6 cells were plated out and treated as required. They were then scraped into 400µl milli-Q H_20 and lysed by repetitive freeze/thawing in dry ice/37 °C water bath. 200µl of this sample was then added to 650µl 10.3mM N-methyl-2-phenylindole and mixed thoroughly. For measuring [MDA + 4-HNE] 150µl of 15.4M methanesulphonic acid was added, to measure [MDA] this was replaced by 150µl of 12N HCl. The tubes were tightly sealed and incubated at 45°C for 40 mins for [MDA + 4-HNE], or 60 mins to measure [MDA]. Samples were cooled on ice and then spun at 15,000G for 10 mins. Sample absorbance was then read at 586nm. To gauge the levels of MDA/4-HNE standard curves were prepared using standard solutions of 4-HNE and MDA between 0 and 20µM. Each sample was also assayed for protein after lysis so that lipid peroxidation could be approximated to cell number. The concentration of MDA + 4-HNE/MDA alone, was calculated using the following equation:

$[MDA + 4\text{-}HNE] = (A - A_o) \times 5/\varepsilon$

$[MDA] = (A - A_o) \times 5/\varepsilon$

Where:

A is the absorbance at 586nm for the sample.

A_o is the absorbance of the blank.

5 is the sample dilution factor (200µl sample in a total volume of 1ml).

ε is the apparent molar extinction coefficient obtained from the gradient of the standard curve.

The same value of ε may be used for calculating [MDA + 4-HNE] because under the conditions of that assay the molar extinction coefficients for those compounds are not significantly different. Under the conditions for measuring MDA concentration alone, 4-HNE does not absorb significantly and can therefore be omitted.

2.2.10 Chelation of $Fe^{2+/3+}$ from fibrous/milled crocidolite

Samples were suspended by sonication and trituration at 2mg/ml in 2mM 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine (Ferrozine) and mixed on a rotating platform for 24 hrs at room temperature to remove Fe3+ from the fibre surface. Following a 1,000g spin for 10 mins, fibres were washed in sterile water and then spun again at 1,000g for 10 mins. Samples were then resuspended at 2mg/ml in 2mM deferoxamine mesylate as above and mixed for a further 24 hrs. Samples were washed as before in sterile water. Finally, samples were resuspended in pre-warmed media and treated as above prior to cell treatment. Supernatants generated at the end of each 24 hr period were reserved. Duplicate particulate aliquots were incubated in milli-Q water and otherwise treated as the samples to be chelated. This was to correct for any possible loss of material during the chelation process.

To calculate concentrations of $Fe^{2+/3+}$ in these solutions, a series of $FeSO_4$ (Fe^{2+} containing) in 2mM Ferrozine and $FeCl_3$ (Fe^{3+} containing) in 2mM deferoxamine mesylate solutions were prepared. The absorbance (430nm for $FeCl_3$/deferoxamine or 562nm for $FeSO_4$/Ferrozine) of each solution was recorded and used to plot standard curves. The equation of each curve was used to calculate the respective amounts of

$Fe^{2+/3+}$ chelated from each of the samples. To correct for varying mass of sample, iron content was expressed as nmol/mg.

2.2.11 Measurement of caspase-3 activity

Cells were grown in T150 flasks until they had reached 90% confluency. Following treatment, the media was removed and retained. Cells were removed by 5mins incubation with 2xT/E buffer and ensuing washes to detach any remaining cells. The cell suspension was added to the media and spun for 5mins at 1,000G. The pellet was washed twice with ice cold PBS before resuspension in 200µl western blot lysis buffer. To ensure complete lysis, cells were frozen in liquid nitrogen and thawed in a 37°C water bath three times. Lysates were spun for 30mins at 1,000G (4 °C), following this, the supernatants were spun at 10,000G (4 °C) for a further 45mins to remove the membrane-containing fraction. With the samples kept on ice an aliquot was removed for protein concentration determination (which, if necessary, was diluted to bring the absorbance within the range of the assay). To measure the activity of caspase-3 within the sample, 100µl of lysate was mixed with 10µl Z-DEVD.AFC substrate and 1.25mls caspase-3 assay buffer. Fluorescence of the reaction product was measure at 505nm following excitation at 400nm over a period of 150secs. This data enabled the calculation of enzyme activity in pmol/min, which could then be corrected for protein concentration to reach a final figure measured in pmol/min/mg protein.

2.3 Statistical analyses

Data were normalised to control values and the significance of findings was determined using a two-tailed Students *t*-test. A p value of less than 0.05 was taken to be statistically significant.

CHAPTER 3

EFFECTS OF CROCIDOLITE ASBESTOS ON THE ERK PATHWAY IN MESOTHELIAL CELLS

CHAPTER 3

3.1 INTRODUCTION

The ERK MAPK pathway has been extensively studied in a vast array of cellular systems. It is responsible for transmitting extracellular signals from the plasma membrane to the nucleus where it can modulate the transcriptional activity of many transcription factors. The overriding role of the ERK pathway lies in mitogenic and proliferative responses and was first discovered as a protein that was phosphorylated on tyrosine and threonine residues in response to treatment with insulin and other growth factors (Ray and Sturgill, 1988). This is discussed more fully in chapter 1.

Recent studies have shown activation of the ERK pathway is mediated by oxidative stress (Guyton et al., 1996; Wang et al., 1998) and it appears to arise from changes in the activation state of growth factor receptors induced by ROS, in particular, H_2O_2 (Goldkorn et al., 1998; Peus et al., 1999). The mechanism behind this is thought to rely on the ability of ROS to inactivate intracellular phosphatases that usually terminate the signal emanating from the receptor, as opposed to actually activating receptors by mimicking their ligands in some way (Zwick et al., 1999; Ostman and Bohmer, 2001). Put simply, this means that ROS prevent the receptors from being switched off rather than switching them on, although the end effect is the same.
In keeping with its ability to induce oxidative stress, crocidolite asbestos has also been shown to activate EGFR and initiate signalling through the ERK cascade in primary rat pleural mesothelial (RPM) cells (Zanella et al., 1996). This was the first evidence to describe activation of a MAPK signalling pathway by asbestos and as such is the benchmark for further work in this area. Of key importance to this work is the demonstration that fibre geometry is crucial to the ERK response. This is because in the field of fibre research it is necessary to prove that any effects seen are fibre-specific and not just a non-specific response to solid particle exposure. For this reason milled crocidolite or riebeckite (essentially non-fibrous analogues of crocidolite) are often used as control particles, so that the difference in response may be attributed to the physical nature of the fibre *i.e.* high aspect ratio, as it is this feature of fibres which appears to be largely responsible for their carcinogenicity (Donaldson et al., 1993). In addition, the activation of ERK by crocidolite was protracted, in contrast to the effects seen with EGF, and because of the biopersistence of crocidolite fibres in

the lung it is postulated that this signalling pathway is consistently activated in the lung. It is possible that this prolonged response may be responsible, in part, for the carcinogenicity of crocidolite. The work of Zanella *et al.* (1996) goes on to present compelling evidence for involvement of EGFR activation in eliciting this response, with agents that inhibit EGFR activation also blocking the ERK response to crocidolite.

Subsequent studies revealed that the mechanism of crocidolite-induced ERK activation was shown to be oxidative stress dependent as pre-treatment with NAC for 18hrs prior to asbestos exposure inhibited the activation of ERK (Jimenez et al., 1997). This finding was in agreement with previous work, which had shown that increased expression of *c-fos* and *c-jun* proto-oncogenes by asbestos could be modulated by NAC (Janssen et al., 1995). These genes encode Fos and Jun proteins respectively, which may dimerise via the interaction of basic leucine zipper motifs to form DNA-binding AP-1 transcription factors (Angel and Karin, 1991). Whilst previous studies have demonstrated induction of c-*fos* by asbestos-induced ERK pathway activation in mesothelial cells (Timblin et al., 1998a; Zanella et al., 1999) none have investigated the importance of this activity in forming AP-1 complexes that can bind to their consensus DNA binding sites.

Downstream consequences of crocidolite-induced ERK activation were investigated with the selective MEK1 inhibitor PD98059. This compound was capable of reducing the level of apoptosis associated with asbestos exposure, however, levels were not reduced as far as untreated controls, indicating that other factors are involved (Jimenez et al., 1997).

In the present studies we aimed to investigate the activation of ERK by crocidolite in a human-derived cell line, MET 5A, to ensure that the rat model described above held in human cells. The MET 5A cell line is derived from normal human mesothelial cells that were transfected with a plasmid containing SV40 early region DNA. MET 5A cells have been shown to carry a single integrated copy of the SV40 early region DNA in their genome and express SV40 large T antigen. Crucially, these cells display features similar to those of normal human mesothelial cells in their responses to

asbestos (Ke et al., 1989). Experiments were carried out in parallel to those in 4/4 RM4 cells (a rat mesothelial cell line).

3.2 RESULTS

3.2.1 Effect of crocidolite on EGFR expression in 4/4 RM4 and MET 5A cells

The effect of crocidolite upon EGFR expression has been previously illustrated by western blot and immunohistochemical analyses (Pache et al., 1998; Zanella et al., 1996; Faux and Houghton, 2000). Given that our aim was to probe the activation of the ERK pathway it was important to show this result under our experimental conditions. The blots in figure 3.1 demonstrate the time-dependent upregulation in total EGFR expression in both cell lines. Quite clearly, the effect is transient with the peak at around 6hrs, and by 24hrs levels have returned to normal. Attempts to probe the phosphorylation status of the receptor under these conditions proved unsuccessful. Levels of α-tubulin were used to check for protein loading of the samples and these blots showed consistency throughout.

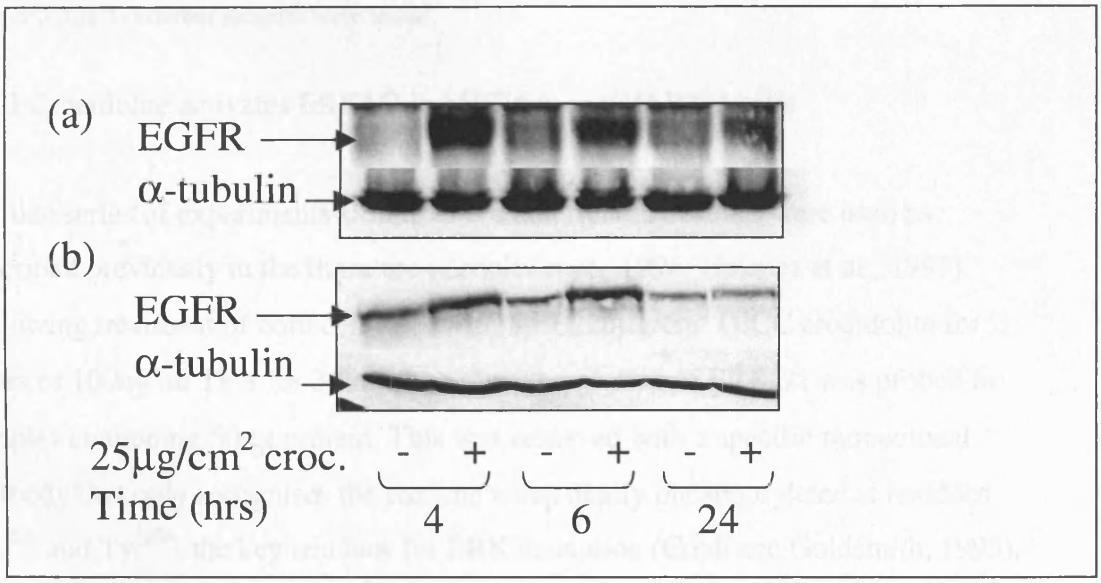


Figure 3.1 Upregulation of EGFR by UICC crocidolite in MET5A and 4/4 RM4 cells. Serum starved 4/4 RM4 (a) or MET 5A (b) cells were treated with 25μg/cm^2 UICC crocidolite for the times indicated, they were then lysed and proteins separated by SDS PAGE. Following transfer onto nitrocellulose membrane the resultant filters were immunoblotted with anti-EGFR antibody. Blots were then stripped and reprobed for α-tubulin. Bands were visualised by chemiluminescence. Experiments were repeated in duplicate.

3.2.2 ERK1/2 levels in 4/4 RM4 and MET 5A cell lines

ERK1/2 expression levels were checked by western blotting in untreated cells (Figure 3.2). Results shown are indicative from 3 individual samples from each cell line at different passages. It is obvious that expression of ERK1/2 represented by bands at 44 and 42kDa respectively, is very similar in these cell lines.

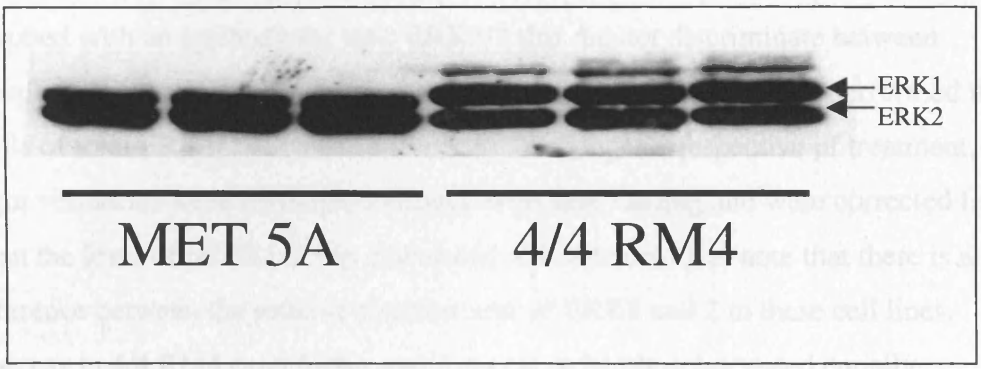


Figure 3.2 Levels of ERK1/2 in MET5A and 4/4 RM4 cells. Serum starved cells were lysed and proteins separated by SDS PAGE. Following transfer onto nitrocellulose membrane the resultant filters were immunoblotted with anti-ERK1/2 antibody. Bands were visualised by chemiluminescence. For each cell line 3 different samples were tested.

3.2.3 Crocidolite activates ERK1/2 in MET 5A and 4/4 RM4 cells

For this series of experiments similar doses and treatment times were used as described previously in the literature (Zanella et al., 1996; Jimenez et al., 1997). Following treatment of both cell lines with 5, 10, 25μg/cm^2 UICC crocidolite for 24hrs or 100ng/ml TPA for 30mins the phosphorylation of ERK1/2 was probed in samples containing 50μg protein. This was achieved with a specific monoclonal antibody that only recognises the enzyme when dually phosphorylated at residues Thr^{202} and Tyr^{204}, the key residues for ERK activation (Cobb and Goldsmith, 1995). TPA induced between 1.5 and 2 fold increases in pERK compared to untreated controls. At 5μg/cm^2, UICC crocidolite had no significant effect upon ERK1/2 phosphorylation in either cell line (Figure 3.3a and b), which was in contrast to results presented in previous studies (Zanella et al., 1996; Jimenez et al., 1997). However, this finding may be explained by differences in technique; in these experiments we

had used shorter fibres than Zanella *et al.* (1996) due to difficulties in obtaining samples of NIEHS fibres. Additionally, an established cell line was used that may be more resistant to the effects of asbestos fibres than primary cultures of RPM cells. However, at 10 and 25μg/cm^2 the effects of crocidolite upon ERK1/2 activation were significantly higher than in untreated control cultures ($p<0.05$), this was true in both of the cell lines tested. However, there were subtle differences between the two, the 4/4 RM4 cells appeared to be slightly more sensitive to the increasing doses of UICC crocidolite as they exhibited a slightly steeper dose-response curve. The difference was not, however, significant (Figure 3.4). In all cases blots were stripped and reprobed with an antibody for total ERK1/2 that did not discriminate between phosphorylated and non-phosphorylated protein. In each experiment performed the levels of total ERK1/2 remained essentially unchanged irrespective of treatment, slight variations were attributed to errors in protein loading and were corrected for when the level of pERK1/2 was calculated. It is interesting to note that there is a slight difference between the relative contributions of ERK1 and 2 in these cell lines. Whereas in 4/4 RM4 cells ERK1 and 2 appear to be phosphorylated equally throughout (Figure 3.3a), in MET 5A cells ERK2 appears to be in a higher phosphorylation state than ERK1, although the relative difference between ERK1 and 2 did not change under stimulated conditions (Figure 3.3b). Whether this is due to preferential signalling through ERK2 in this cell line or that the antibody did not recognised the epitope in pERK1 as well is unclear.

To ensure that the effects noted on ERK1/2 phosphorylation were indicative of actual kinase activity, *in vitro* complex kinase assays were performed with samples extracted from cells treated in the same way as for the western blot experiments. This technique involves immunoprecipitating the kinase of interest and then measuring its ability to radiolabel a peptide substrate under non-denaturing conditions. For our experiments we chose PHAS-I substrate as this was suitable for all MAPK family members. In these experiments we were able to make a crude investigation into the importance of fibre geometry on kinase activity by comparing the effects of UICC and NIEHS crocidolite, the relative physical characteristics of UICC and NIEHS crocidolite are described in chapter 2.

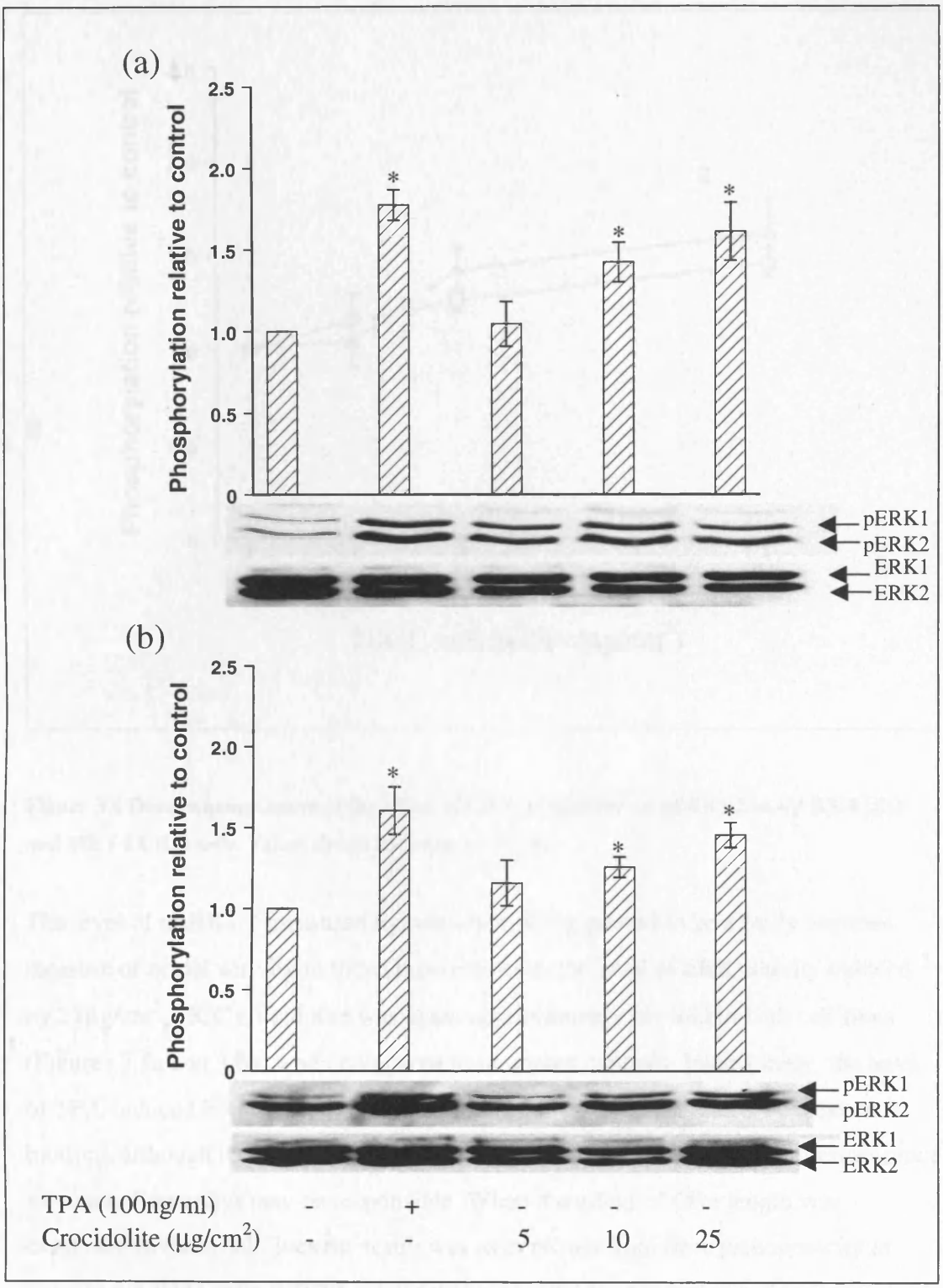


Figure 3.3 Effect of UICC crocidolite on ERK1/2 phosphorylation as determined by western blotting. 4/4 RM-4 (a) or MET5A cells (b) were serum starved for 24 hrs prior to treatment for 24 hrs with the indicated concentrations of UICC crocidolite. In each case a 30 minute treatment with TPA (100ng/ml) was included as a positive control. Cells were then lysed and the proteins separated by SDS PAGE. The proteins were transferred onto a nitrocellulose membrane and the resultant filters immunoblotted with anti-pERK1/2 antibody. Bands were visualised by chemiluminescence. Blots were then stripped and reprobed for native ERK1/2. Blots shown are representative of three experiments for each cell line. The data from three separate experiments were quantified by densitometry and the mean +/- S.E.M. plotted. * $p<0.05$.

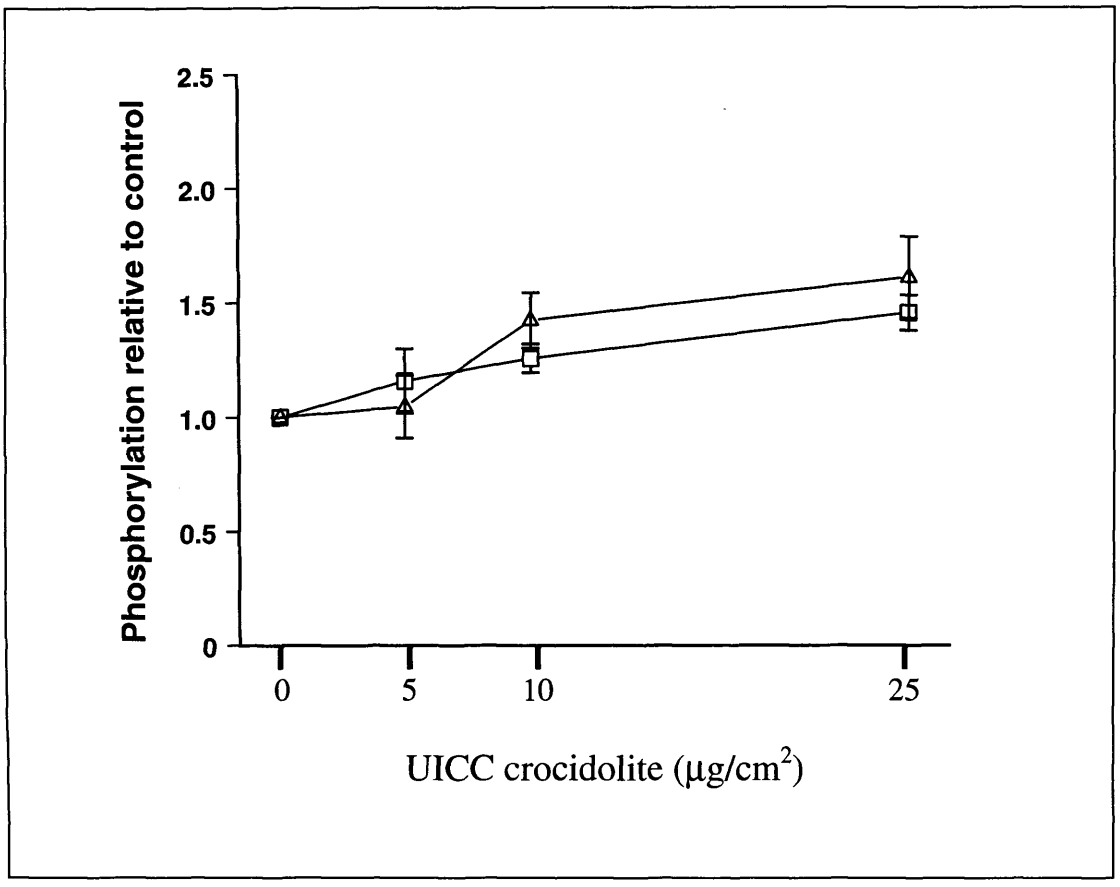


Figure 3.4 Dose-response curve of the effect of UICC crocidolite on pERK1/2 in 4/4 RM4 (△) and MET 5A (□) cells. Values shown are mean +/- S.E.M.

The level of pERK1/2 measured by western blotting proved to be a fairly accurate measure of actual activity in these experiments as the level of ERK activity induced by 25μg/cm^2 UICC crocidolite was again approximately twofold in both cell lines (Figures 3.5a and 3.6a) when compared to untreated controls. Interestingly, the level of TPA-induced ERK1/2 activity seems to have been underestimated by western blotting, although the differences in the way the result is detected (chemiluminescence *vs.* autoradiography) may be responsible. When the effect of fibre length was examined in these cell lines the result was as expected from fibre pathogenicity *in vivo*. In 4/4 RM4 cells, NIEHS crocidolite was capable of inducing a significant increase in ERK1/2 activity at all doses tested and the dose-response is much steeper in comparison to that observed with UICC exposure (Figure 3.7a).

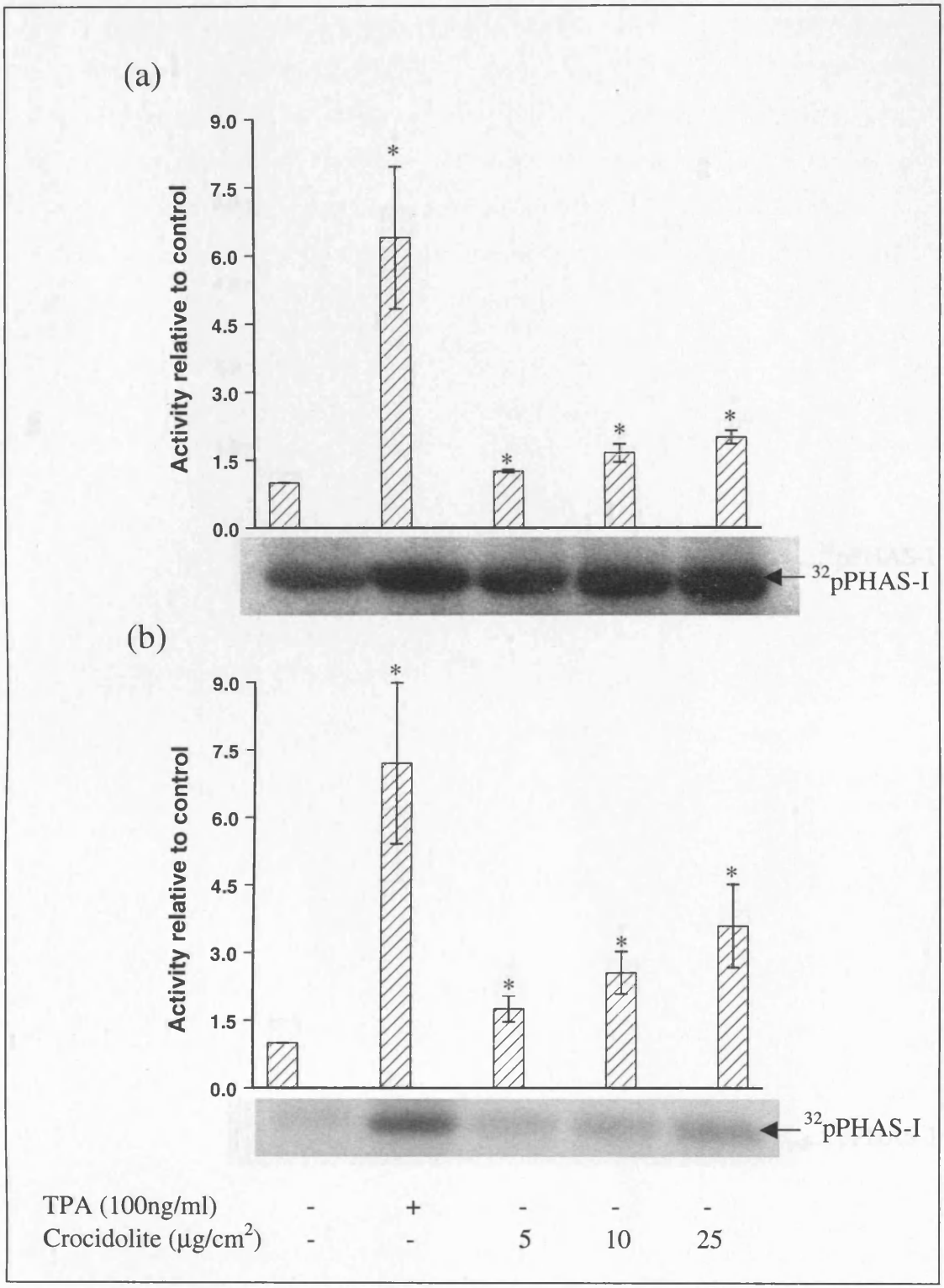


Figure 3.5 Effect of UICC and NIEHS crocidolite on ERK1/2 activity as determined by *in vitro* kinase assays in 4/4 RM-4 cells. Cells were serum starved for 24 hrs prior to treatment for 24 hrs with the indicated concentrations of (a) UICC crocidolite or (b) NIEHS crocidolite. In each case a 30 minute treatment with TPA (100ng/ml) was included as a positive control. Cells were then lysed and ERK1/2 was immunoprecipitated from equal amounts of protein of each sample. ERK1/2 activity was measured by the ability of the purified kinase to phosphorylate PHAS-I substrate in an *in vitro* kinase assay. Blots shown are representative of three experiments. They were quantified by densitometry and the mean +/- S.E.M. is shown. * $p<0.05$.

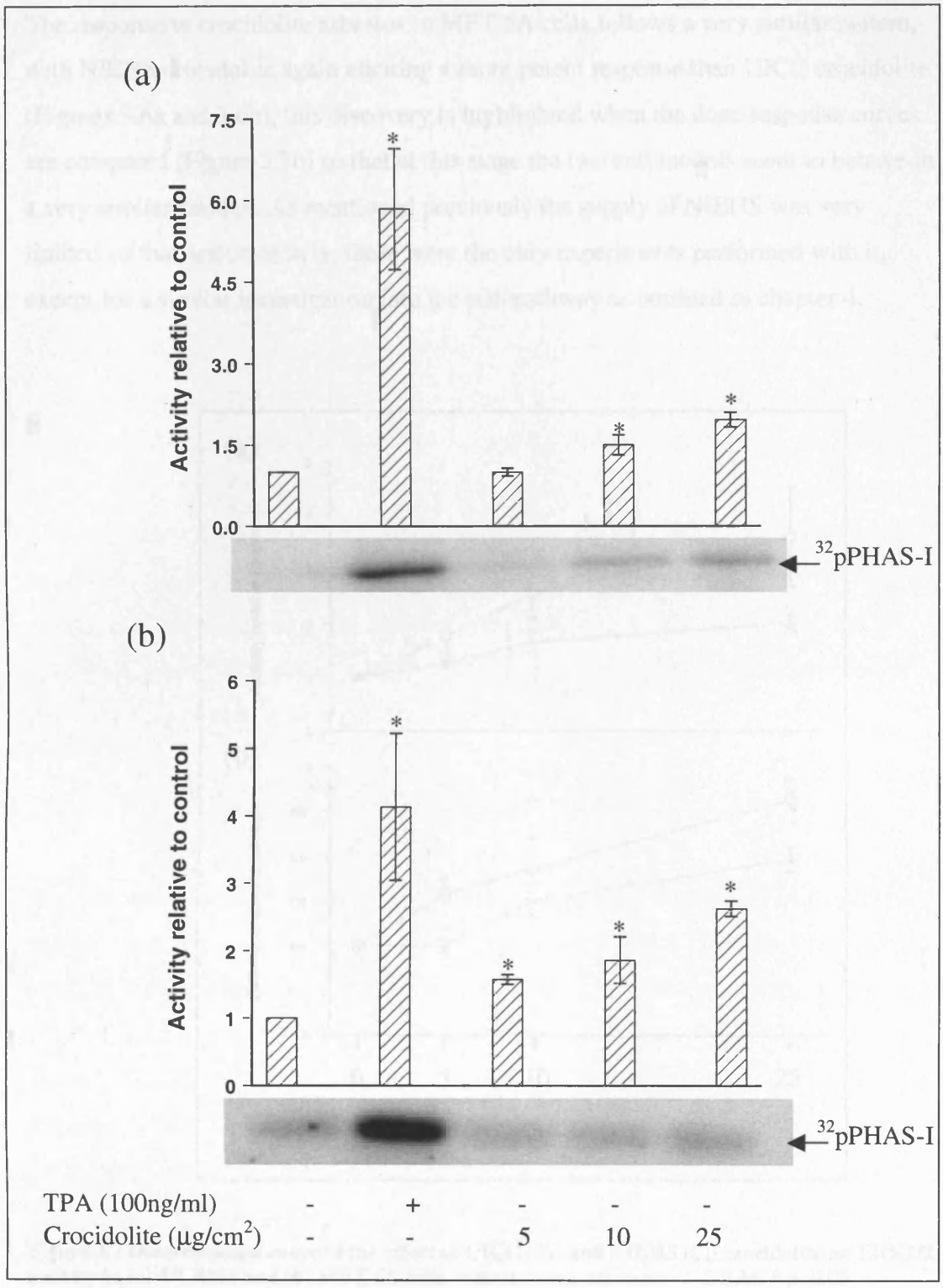


Figure 3.6 Effect of UICC and NIEHS crocidolite on ERK1/2 activity as determined by *in vitro* kinase assay in MET5A cells. Cells were serum starved for 24 hrs prior to treatment for 24 hrs with the indicated concentrations of (a) UICC crocidolite or (b) NIEHS crocidolite. In each case a 30 minute treatment with TPA (100ng/ml) was included as a positive control. Cells were then lysed and ERK1/2 was immunoprecipitated from equal amounts of protein of each sample. ERK1/2 activity was measured by the ability of the purified kinase to phosphorylate PHAS-I substrate in an *in vitro* kinase assay. Blots shown are representative of three experiments. They were quantified by densitometry and the mean +/- S.E.M. is shown. * $p<0.05$.

The response to crocidolite asbestos in MET 5A cells follows a very similar pattern, with NIEHS crocidolite again eliciting a more potent response than UICC crocidolite (Figures 3.6a and 3.6b), this discovery is highlighted when the dose-response curves are compared (Figure 3.7b) so that at this stage the two cell models seem to behave in a very similar fashion. As mentioned previously the supply of NIEHS was very limited so that, unfortunately, these were the only experiments performed with it, except for a similar investigation into the p38 pathway as outlined in chapter 4.

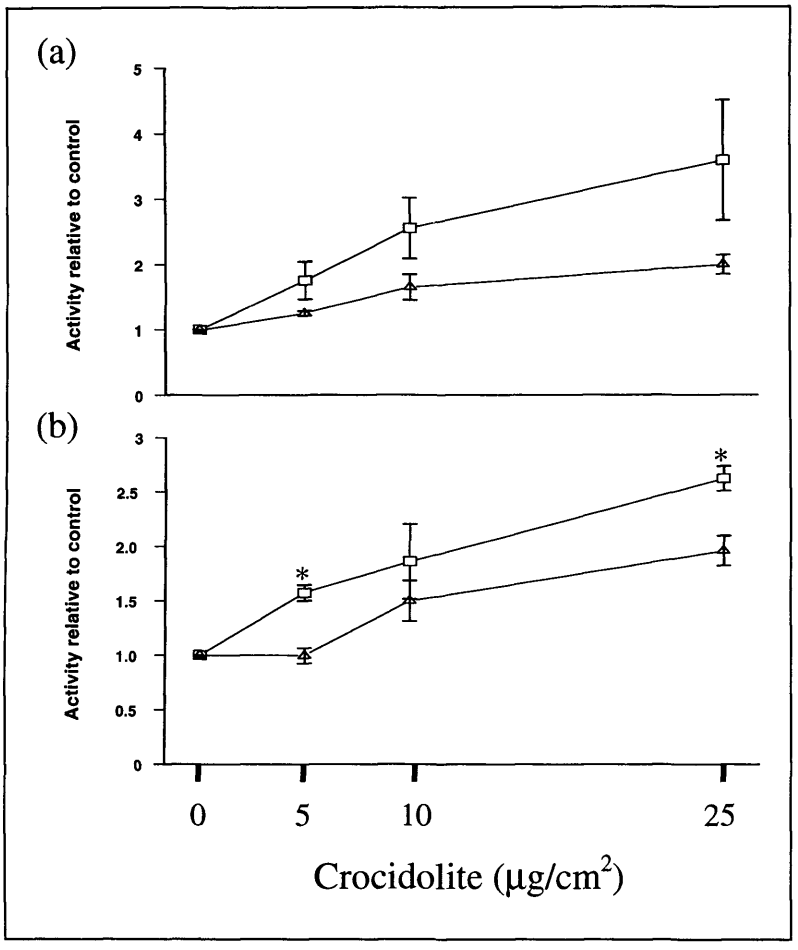


Figure 3.7 Dose-response curve of the effect of UICC (△) and NIEHS (□) crocidolite on ERK1/2 activity in (a) 4/4 RM4 and (b) MET 5A cells. Values shown are mean +/- S.E.M. * $p<0.05$.

Having established that western blotting provided an adequate means of assessing the level of active ERK in this system it was decided to use this methodology for the remainder of experiments instead of the *in vitro* kinase assay.

The time course of ERK1/2 phosphorylation by crocidolite in mesothelial cells was then investigated at 4, 8 and 24hrs. For each experiment cells were treated with 25μg/cm^2 UICC crocidolite as this elicited the greatest response without causing cytotoxicity. At each time point, an additional untreated control sample was prepared so that fluctuations in background pERK1/2 could be accounted for. At each time point the intensity value of the band was related to the concomitant control so that at each time the control value was 1. As previously, the levels of native ERK1/2 were also accounted for and on the whole these were similar. The effects of crocidolite have fairly large lag phases in each cell line with no increases at 4hrs in MET 5A cells although a small, but significant ($p<0.05$) increase in 4/4 RM4 cells was observed and may be accounted for by the time it takes the fibres to settle on the cells, which may be up to 2hrs in the case of long, thin fibres (Zanella et al., 1996). At 8 and 24hrs a steady-state increase in pERK1/2 levels was noted in both cell lines (Figure 3.8). Attempts at probing time points beyond 24hrs proved quite unsuccessful as this involved keeping the cells in serum free conditions for a period of time that was, in itself, causing oxidative stress (Moran et al., 2002).

3.2.4 Mechanism of crocidolite-induced ERK1/2 activation

The activation of the ERK pathway by crocidolite has previously been shown to be dependent on oxidative stress. The present studies we designed to further investigate this, and to determine if lipid peroxidation was involved by preincubating cells for 18hrs with either 1 or 5mM NAC before crocidolite exposure. Again TPA was included as a positive control, however, time restraints meant that parallel tests to see the effect of NAC on this response could not be performed. Both 1 and 5mM NAC were capable of significantly ($p<0.05$) reducing the crocidolite-induced response in MET 5A cells (Figure 3.9b). In the case of the 4/4 RM4 cells, the response was lowered to below untreated control levels, and was reflected where NAC was added in the absence of crocidolite (Figure 3.9a). This result suggests that these cells experience a background oxidative stress accounting for some of the basal activity of the ERK pathway.

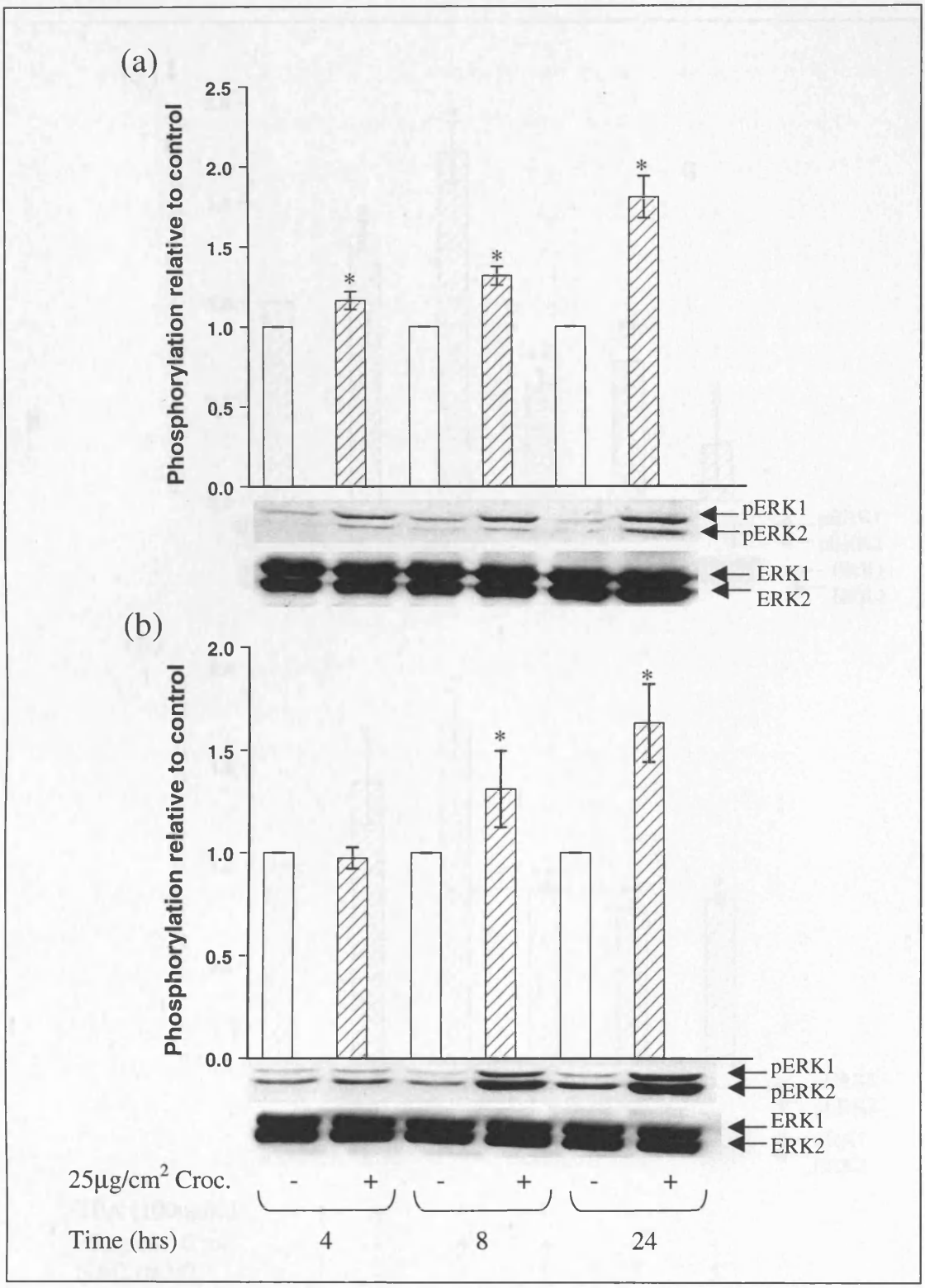


Figure 3.8 Time course of ERK1/2 phosphorylation by crocidolite as determined by western blotting. 4/4 RM-4 (a) or MET5A cells (b) were serum starved for 24 hrs prior to treatment with 25µg/cm^2 UICC crocidolite for times indicated, they were then lysed and the proteins separated by SDS PAGE. The proteins were transferred onto a nitrocellulose membrane and the resultant filters immunoblotted with anti-pERK1/2 antibody. Bands were visualised by chemiluminescence. Blots were then stripped and reprobed for native ERK1/2. Blots shown are representative of at least two experiments for each cell line. The data from each experiment was quantified by densitometry and the mean +/- S.E.M. plotted. * $p<0.05$.

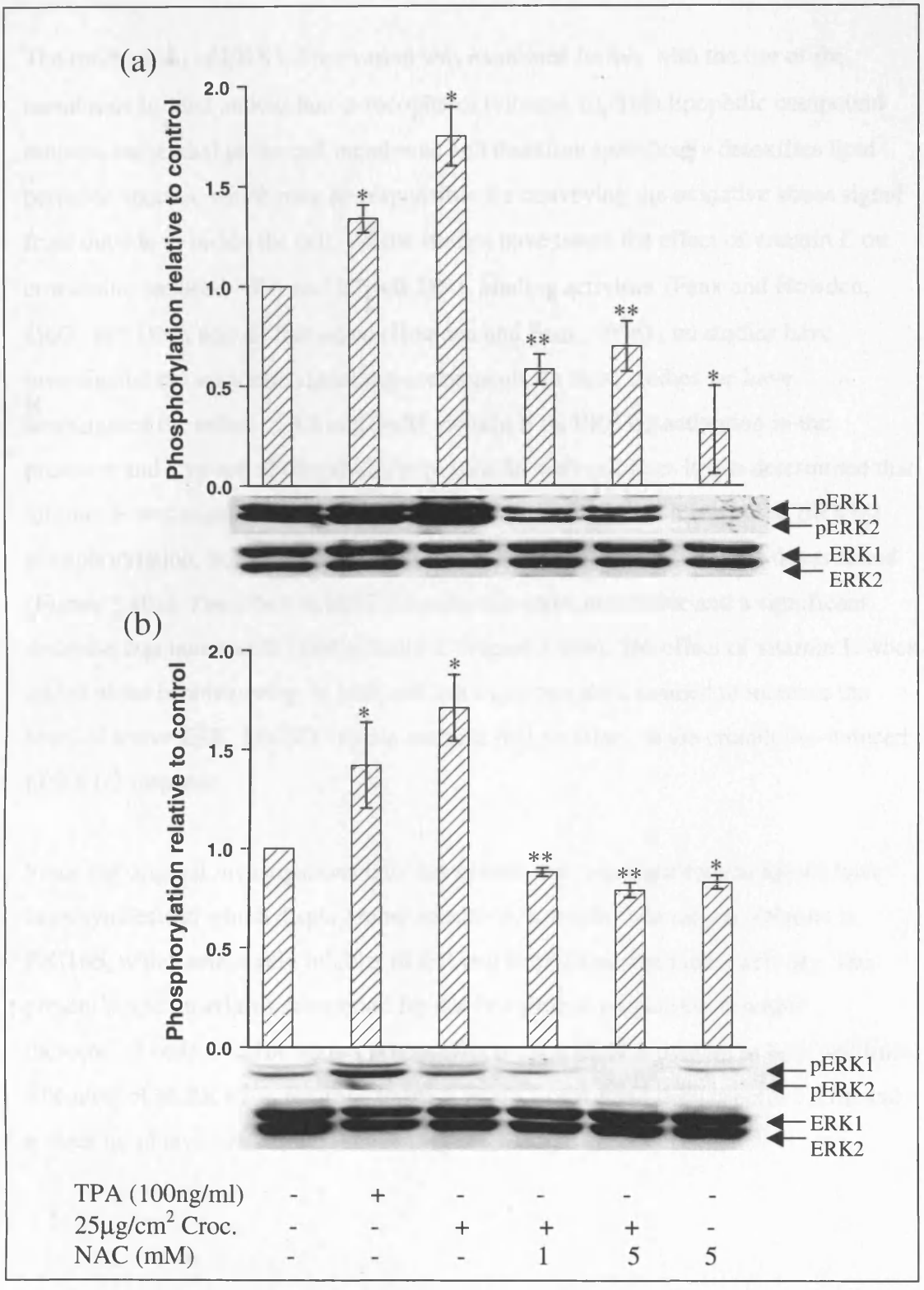


Figure 3.9 Effect of NAC pretreatment on the ability of UICC crocidolite to induce phosphorylation of ERK1/2 as determined by western blotting. 4/4 RM-4 (a) or MET5A cells (b) were serum starved for 6 hrs before 18 hrs incubation with doses of NAC indicated, then 25µg/cm^2 UICC crocidolite for 24 hrs, plus a TPA positive control as above. Samples were probed as above. Blots shown are representative of three experiments for each cell line. The data from three separate experiments were quantified by densitometry and the mean +/- S.E.M. plotted. * $p<0.05$ with respect to control. ** $p<0.05$ with respect to UICC crocidolite alone.

The mechanism of ERK1/2 activation was examined further with the use of the membrane limited antioxidant α-tocopherol (vitamin E). This lipophilic compound remains embedded in the cell membrane and therefore specifically detoxifies lipid peroxide species, which may be responsible for conveying the oxidative stress signal from outside to inside the cell. Whilst studies have tested the effect of vitamin E on crocidolite-induced AP-1 and NF-κB DNA binding activities (Faux and Howden, 1997) and DNA adduct formation (Howden and Faux, 1996) , no studies have investigated the upstream signalling components. In these studies we have investigated the effect of 0.5 and 1mM vitamin E on ERK1/2 activation in the presence and absence of crocidolite exposure. In both cell lines it was determined that vitamin E was capable of reducing the effect of crocidolite with respect to ERK1/2 phosphorylation, however, in 4/4 RM4 cells it was not significant at the doses tested (Figure 3.10a). The effect in MET 5A cells was more noticeable and a significant decrease was noted with 1mM vitamin E (Figure 3.10b). The effect of vitamin E when added alone is noteworthy, in both cell lines this treatment seemed to increase the level of active ERK. DMSO vehicle controls had no effect on the crocidolite-induced pERK1/2 response.

Since the original investigations into this system new pharmacological agents have been synthesised which display more selectivity towards their targets. Of note is PKI166, which selectively inhibits EGFR and ErbB2 tyrosine kinase activity. The present studies used this compound for the first time in crocidolite exposed mesothelial cells. PKI166 shows potent effects upon ERK activation in both cell lines. The level of pERK1/2 is returned to basal levels in 4/4 RM4 cells (Figure 3.11a) and to near basal levels in MET 5A cells (Figure 3.11b).

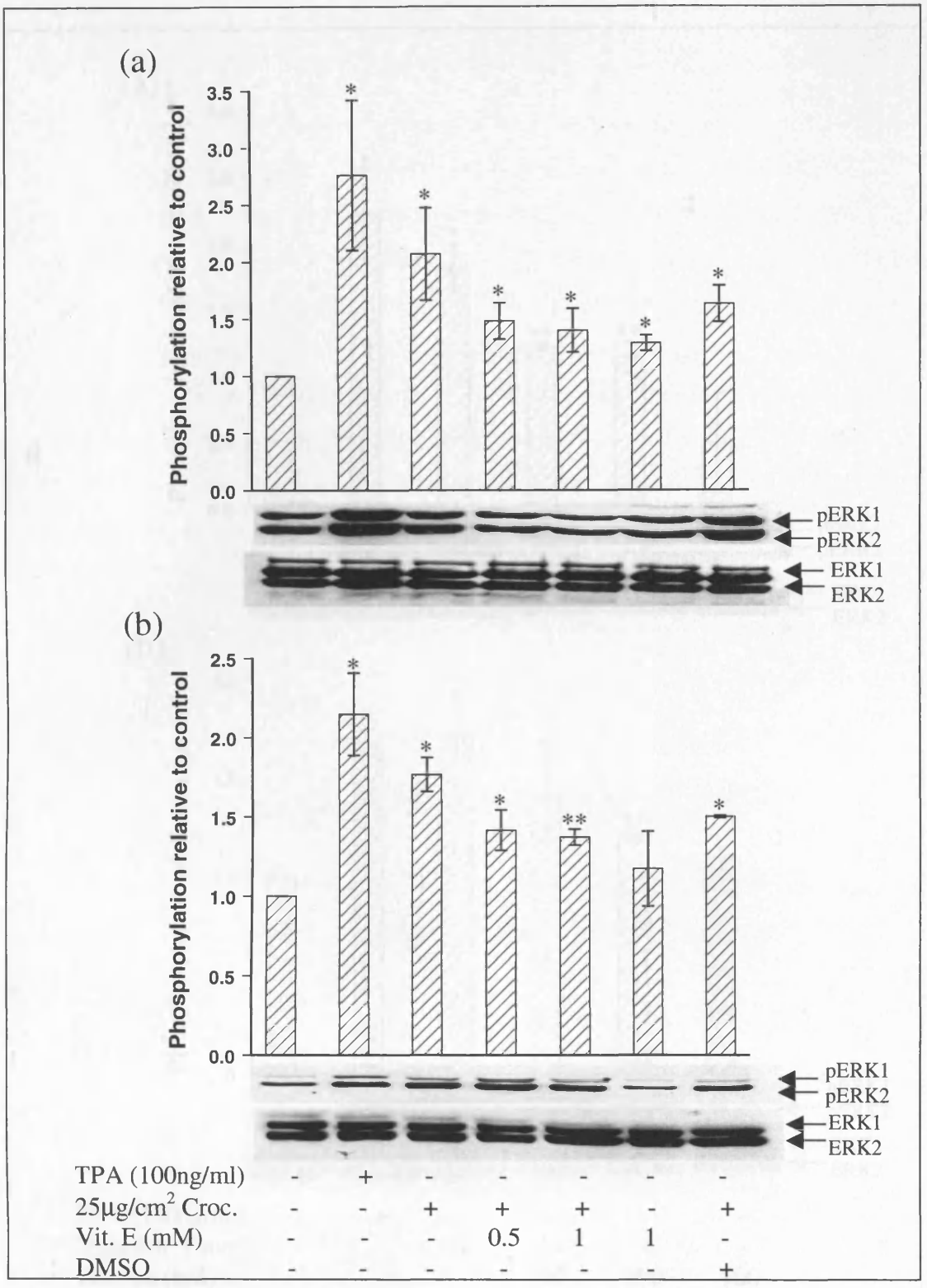


Figure 3.10 Effect of vitamin E pretreatment on the ability of UICC crocidolite to induce phosphorylation of ERK1/2 as determined by western blotting. 4/4 RM-4 (a) or MET5A cells (b) were serum starved for 24 hrs before 2 hrs incubation with vit. E or DMSO vehicle control, then 25µg/cm^2 UICC crocidolite for 24 hrs, plus a TPA positive control. Cells were lysed and the proteins separated by SDS PAGE. Following transfer onto a nitrocellulose membrane the resultant filters were immunoblotted with anti-pERK1/2 antibody. Bands were visualised by chemiluminescence. Blots were then stripped and reprobed for native ERK1/2. Blots shown are representative of three experiments for each cell line. The data from three separate experiments were quantified by densitometry and the mean +/- S.E.M. plotted. * $p<0.05$ with respect to control. ** $p<0.05$ with respect to UICC crocidolite alone.

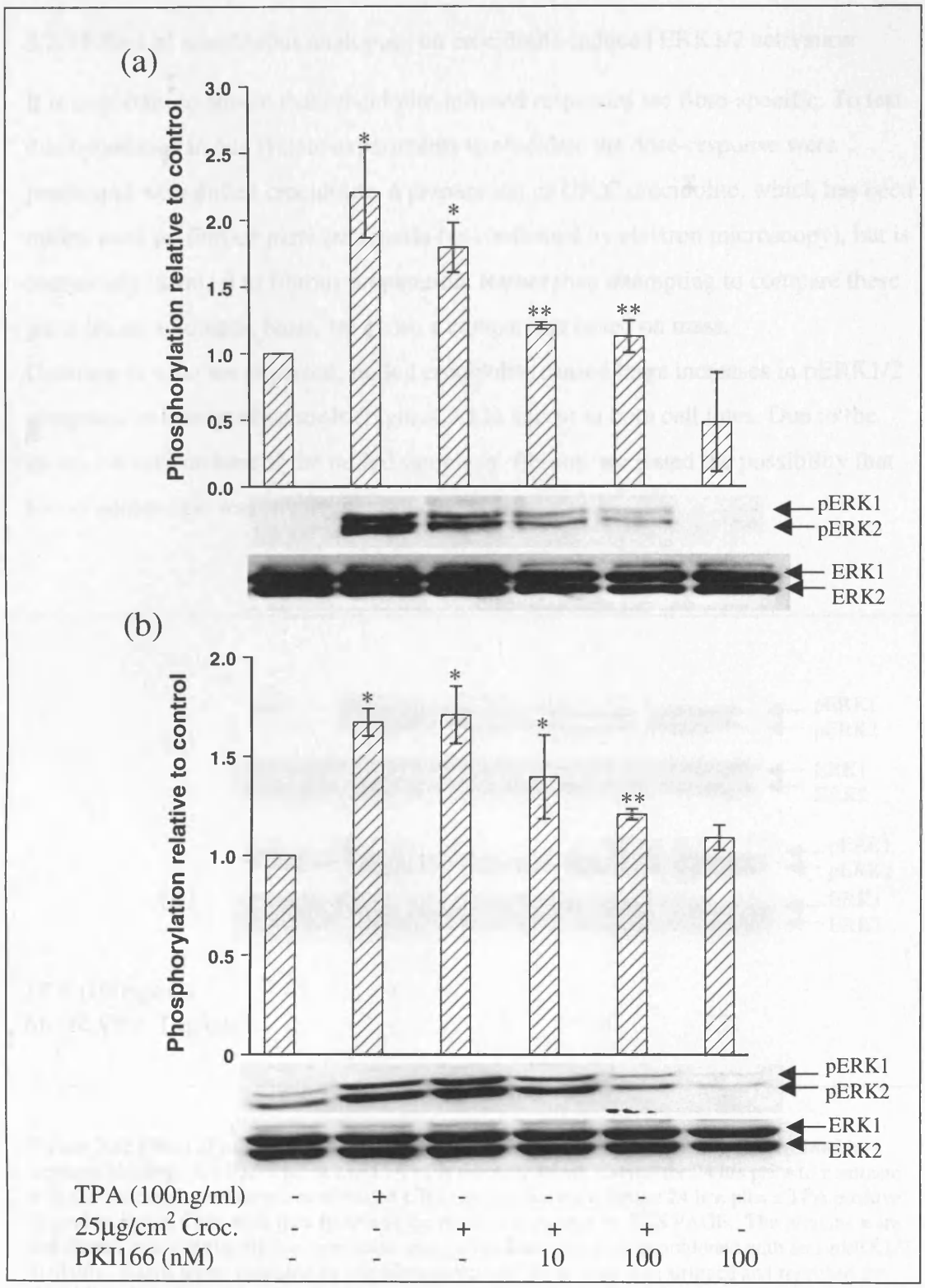


Figure 3.11 Effect of PKI166 pretreatment on the ability of UICC crocidolite to induce phosphorylation of ERK1/2 as determined by western blotting. 4/4 RM-4 (a) or MET5A cells (b) were serum starved for 24 hrs before 2 hrs incubation with PKI166, then 25µg/cm^2 UICC crocidolite for 24 hrs, plus a TPA positive control. Cells were lysed and the proteins separated by SDS PAGE. Following transfer onto a nitrocellulose membrane the resultant filters were immunoblotted with anti-pERK1/2 antibody. Bands were visualised by chemiluminescence. Blots were then stripped and reprobed for ERK1/2. Blots shown are representative of three experiments for each cell line. The data from three separate experiments were quantified by densitometry and the mean +/- S.E.M. plotted. * $p<0.05$ with respect to control. ** $p<0.05$ with respect to UICC crocidolite alone.

3.2.5 Effect of non-fibrous analogues on crocidolite-induced ERK1/2 activation

It is important to ensure that crocidolite-induced responses are fibre-specific. To test this hypothesis in our system experiments to elucidate the dose-response were performed with milled crocidolite, a preparation of UICC crocidolite, which has been milled until no fibrous particles remain (as confirmed by electron microscopy), but is chemically identical to fibrous preparation. Rather than attempting to compare these particles on a numeric basis, we chose a comparison based on mass.

Contrary to what we expected, milled crocidolite caused large increases in pERK1/2 compared to untreated controls (Figures 3.12a and b) in both cell lines. Due to the increased surface area of the milled sample *cf.* fibrous, we tested the possibility that bio-available iron was involved.

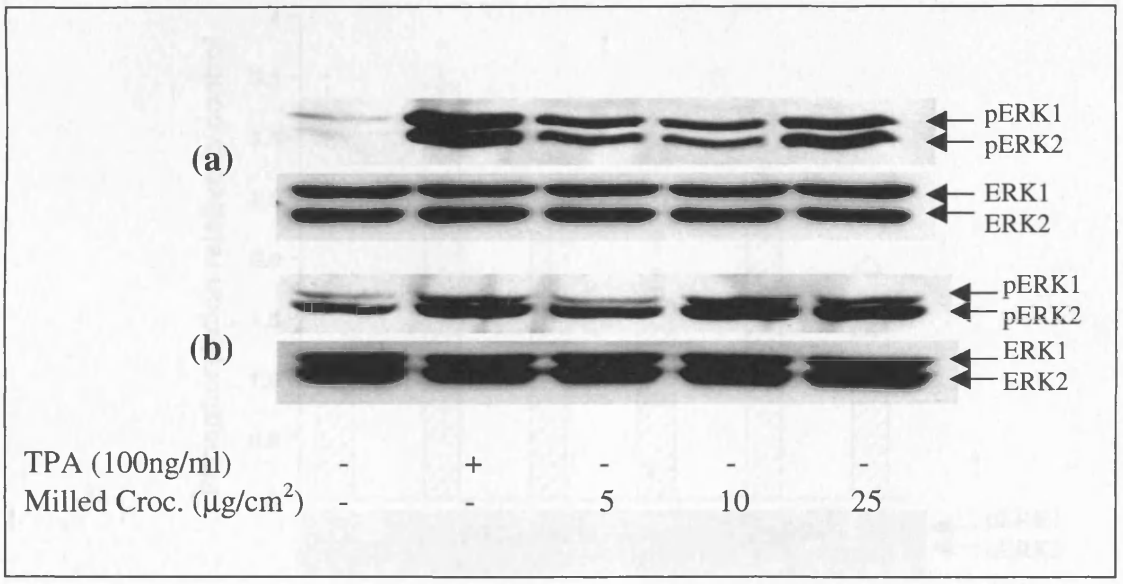


Figure 3.12 Effect of milled UICC crocidolite on ERK1/2 phosphorylation as determined by western blotting. 4/4 RM-4 (a) or MET5A cells (b) were serum starved for 24 hrs prior to treatment with the indicated concentrations of milled UICC crocidolite for a further 24 hrs, plus a TPA positive control as above. Cells were then lysed and the proteins separated by SDS PAGE. The proteins were transferred onto a nitrocellulose membrane and the resultant filters immunoblotted with anti-pERK1/2 antibody. Bands were visualised by chemiluminescence. Blots were then stripped and reprobed for ERK1/2. Blots shown are representative of one experiment for each cell line.

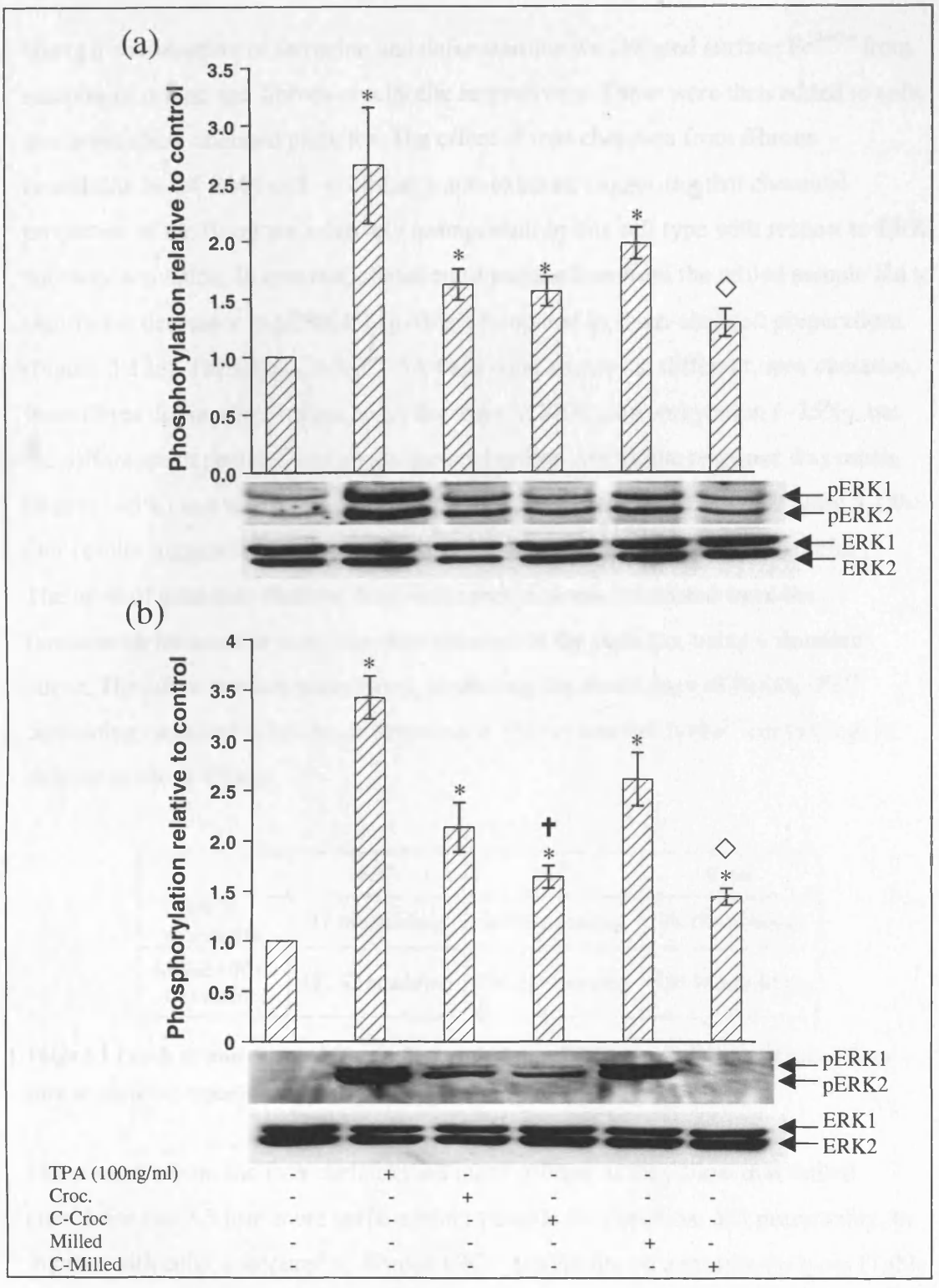


Figure 3.13 Effect of iron chelation (C) on the ability of fibrous and milled UICC crocidolite to induce phosphorylation of ERK1/2 as determined by western blotting. 4/4 RM-4 (a) or MET5A cells (b) were serum starved for 24 hrs prior to treatment with 25μg/cm^2 of chelated/sham-chelated particle for 24 hrs, plus a TPA positive control as above. Cells were then lysed and the proteins separated by SDS PAGE. Following transfer onto a nitrocellulose membrane the resultant filters were immunoblotted with anti-pERK1/2 antibody. Bands were visualised by chemiluminescence. Blots were then stripped and reprobed for ERK1/2. Blots shown are representative of three experiments for each cell line. The data from three separate experiments were quantified by densitometry and the mean +/- S.E.M. plotted. * $p<0.05$ with respect to untreated control. † $p<0.05$ with respect to sham-chelated crocidolite. ◇ $p<0.05$ with respect to sham-chelated milled crocidolite.

Using a combination of ferrozine and deferoxamine we chelated surface $Fe^{2+/3+}$ from samples of milled and fibrous crocidolite respectively. These were then added to cells alongside sham-chelated particles. The effect of iron chelation from fibrous crocidolite in 4/4 RM4 cells is virtually non-existent, suggesting that chemical properties of the fibres are relatively unimportant in this cell type with respect to ERK pathway activation. In contrast, chelation of surface iron from the milled sample led to significant decreases in pERK1/2 ($p<0.05$) compared to sham-chelated preparations (Figure 3.13a). The results in MET 5A cells were somewhat different; iron chelation from fibres did produce a significant decrease in ERK phosphorylation (~25%), but the difference in chelated and sham-chelated milled crocidolite response was much larger (~45%) and was still significantly larger than untreated controls (Figure 3.13b). Our results suggest that surface iron has a slightly different effect in these cells.
The level of total iron chelated from these particles was calculated from the ferrozine/deferoxamine solutions after removal of the particles, using a standard curve. The curve was calculated from measuring the absorbance of $FeSO_4$ (Fe^{2+} containing) standard solutions in ferrozine at 562nm and $FeCl_3$ (Fe^{3+} containing) in deferoxamine at 430nm.

	Fe^{2+}	Fe^{3+}	Total
UICC crocidolite	47.78nmols/mg	46.49 nmols/mg	94.27 nmols/mg
Milled UICC crocidolite	132.92 nmols/mg	196.21 nmols/mg	329.14 nmols/mg

Table 3.1 Levels of iron chelated from milled and fibrous UICC crocidolite. Mean values from three independent experiments are shown.

These results from the iron chelation are quite striking as they show that milled crocidolite has 3.5 fold more surface iron available for chelation, and presumably, to interact with cells, compared to fibrous UICC crocidolite on a mass/mass basis (Table 3.1).

3.2.6 Role of the ERK pathway in crocidolite-induced AP-1 DNA binding

Having established activation of the ERK pathway under crocidolite exposed conditions and also the mechanism behind this activation, downstream consequences of ERK1/2 activation were investigated.

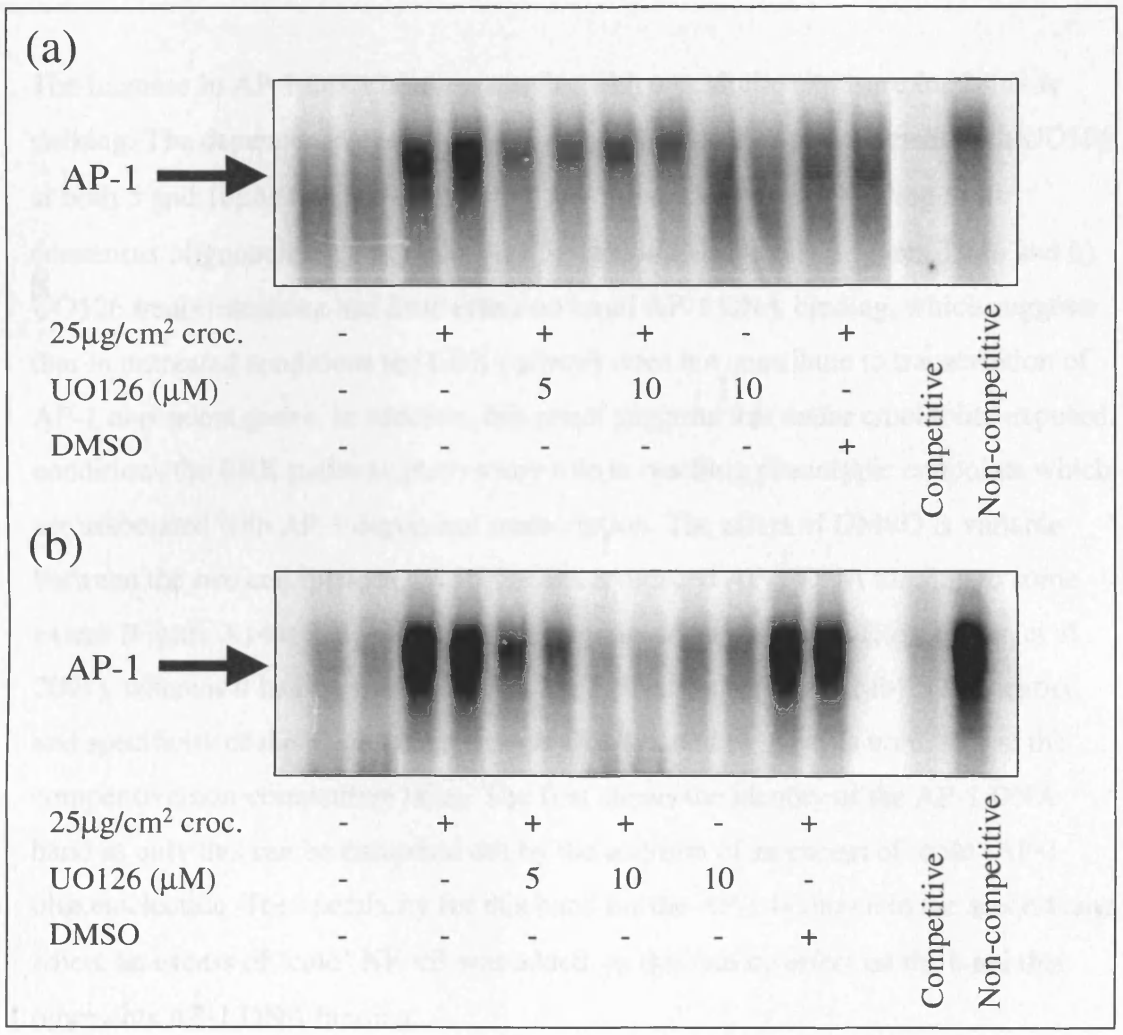


Figure 3.14 UO126 ameliorates the ability of UICC crocidolite to induce AP-1 DNA binding. 4/4 RM4 (a) or MET5A (b) cells were grown to 90% confluency before serum starvation for 24hrs. UO126 was added to cells for 2hrs before incubation with 25µg/cm^2 UICC crocidolite for 24hrs. Nuclear extracts were then isolated as described in materials and methods. 4µg of this extract was incubated with a ^{32}P-labelled oligonucleotide and the DNA AP-1 complex resolved by acrylamide gel electrophoresis. Refer to materials and methods for explanation of competitive/non-competitive lanes. Images shown are representative of at least 2 independent analyses.

Experiments to determine the downstream consequences of ERK1/2 activation focused on the ability of crocidolite to increase the DNA binding of the transcription factor containing AP-1 sequences. Electrophoretic mobility shift assays are a convenient way of investigating this event. Nuclear extracts were isolated from cells that had been treated with crocidolite in the presence or absence of a selective inhibitor of MEK1/2 (UO126), which would therefore prevent activation of ERK1/2

(Favata et al., 1998). This compound supersedes PD098059 because it binds to the active form of MEK1/2 and inhibits it non-competitively, whereas PD098059 binds the inactive form only and will therefore have no effect on MEK1/2 that is already active.

The increase in AP-1 DNA binding activity with crocidolite exposure for 24hrs is striking. The dependence upon the ERK pathway is clear as pretreatment with UO126 at both 5 and 10μM reduced the level of AP-1 that is capable of binding to its consensus oligonucleotide in both MET 5A and 4/4 RM4 cells (Figures 3.14a and b). UO126 treatment alone had little effect on basal AP-1 DNA binding, which suggests that in untreated conditions the ERK pathway does not contribute to transcription of AP-1 dependent genes. In addition, this result suggests that under crocidolite exposed conditions the ERK pathway plays a key role in reaching phenotypic endpoints which are associated with AP-1 dependent transcription. The effect of DMSO is variable between the two cell lines, in 4/4 RM4 cells it reduced AP-1 DNA binding to some extent (Figure 3.14a) which may be due its antioxidant properties (Rosenblum et al., 2001), whereas it had no such effects in MET 5A cells (Figure 3.14b). The identity and specificity of the band that represents DNA bound by AP-1 is borne out in the competitive/non-competitive lanes. The first shows the identity of the AP-1 DNA band as only this can be competed out by the addition of an excess of 'cold' AP-1 oligonucleotide. The specificity for this band for the AP-1 is shown in the second lane where an excess of 'cold' NF-κB was added, as this had no effect on the band that represents AP-1 DNA binding.

3.3 DISCUSSION

The main aim of the present studies were to investigate if effects previously noted in primary RPM cells were mirrored in human and rat mesothelial cell lines in terms of activation of the EGFR/ERK pathway. Initial experiments were directed toward investigating the modulation of EGFR protein expression by asbestos.
Both MET 5A and 4/4 RM4 cells were shown to increase expression of EGFR protein in a time dependent manner when treated with 25μg/cm^2 UICC crocidolite. This finding is supported by data in the literature derived from similar systems (Faux and

Houghton, 2000; Pache et al., 1998). Attempts to probe the phosphorylation status of the receptor proved unsuccessful, due to poor quality commercial antibodies. However, we may assume that this does occur as we could modulate the effect of asbestos on downstream signalling components using a selective inhibitor of EGFR tyrosine kinase activity, which in itself requires EGFR autophosphorylation. At this stage it is impossible to say whether increases in EGFR protein are due to *de novo* protein synthesis or decreased internalisation of the receptor. Studies that have probed the more long-term effects of asbestos on EGFR expression in mesothelial cells show that mRNA expression is not significantly altered immediately post exposure but is upregulated in pretumorous and asbestos-induced tumour tissue (Sandhu et al., 2000).

Basal levels of ERK1/2 were probed in both cell lines by western blotting and these experiments indicated that expression was very similar irrespective of cell type or passage number.

To determine the effect of crocidolite on the ERK pathway in these cells, phosphorylation of ERK1/2 on residues Thr^{202} and Tyr^{204} was probed by western blotting. These residues are recognised and phosphorylated specifically by MEK1/2, the upstream regulators of ERK1/2 activity (Butch and Guan, 1996). UICC crocidolite was capable of significantly increasing the proportion of ERK1/2 that was phosphorylated on these residues at doses of 10 and 25$\mu g/cm^2$, this largely agrees with the data in primary cultures of rat mesothelial cells (Zanella et al., 1996), although these studies noted larger increases with lower fibre dose. The reason for this discrepancy may be twofold: Firstly, established cell lines will have altered their phenotype in order to become immortalised and, therefore, may be more resistant to stressful stimuli. Secondly, the reference fibre preparation used in these studies contains fibres of shorter mean length, which are well characterised as being less pathogenic (Donaldson et al., 1993; Hart et al., 1994). However, there were a number of experiments that we could perform with the longer fibre preparations and the results of which are discussed below. Importantly, the expression of total ERK1/2 protein *i.e.* phosphorylated and non-phosphorylated, was unaltered in all treatments, this proves a true signalling event. Direct comparison of the dose-response curves in these two cell lines revealed that there were no significant differences between them.

Whilst ERK1/2 activation has been extremely well characterised and is known to be intrinsically linked to phosphorylation at the residues outlined above, *in vitro* kinase assays can be used to measure the actual activity of these kinases. In addition, we obtained sufficient quantities of NIEHS fibres to be able to gauge the effect of fibre length on ERK1/2 activity, an effect that had not been studied before. The dose-response curves plotted from this data show quite clearly how important fibre geometry is in activating the ERK pathway. In both cell lines the curve is steeper where longer fibres were used. This difference is most likely explained by the phagocytic properties of mesothelial cells. Although these cells are not professional phagocytes they do have phagocytic properties in order to remove foreign matter that invades the pleura (Kuwahara et al., 1994). In cases where foreign matter is too large to be enveloped, cells enter a state known as frustrated phagocytosis, this is characterised by the production of ROS (Weihong et al., 2000). We may therefore assume that cells attempting to phagocytose a population of longer fibres will be subjected to a higher level of oxidative stress, which as shown below, is vital for activation of the ERK signalling pathway. The time course of crocidolite-induced ERK1/2 phosphorylation gives insight into how these fibres are so pathogenic. After 24hrs of exposure, the ERK1/2 response was still increasing and this is indicative of how persistent the fibre stimulus is to this pathway. When one considers that these fibres stay in the lungs of exposed individuals for many years (Mossman and Sesko, 1990) it is not hard to speculate that prolonged activation of signalling pathways over this period will occur and that this may contribute to pathogenesis.

The role of oxidants in disease is well established (Halliwell, 1994) and this may arise from their ability to modulate signalling pathways (Guyton et al., 1996). The human body has a wide range of defenses aimed specifically to combat their deleterious effects, of particular importance are thiol containing compounds such as glutathione. NAC is a precursor of glutathione (Atkins et al., 2000) and is frequently used both *in vivo* and *in vitro* to protect against oxidative stress. Previous studies with asbestos have highlighted how many of its effects may be counteracted by preloading cells with NAC (Janssen et al., 1995; Faux and Houghton, 2000; Jimenez et al., 1997). Our studies show that the effects of crocidolite asbestos on the ERK pathway can be reversed by an 18hr pretreatment with either 1 or 5mM NAC in MET 5A and 4/4

RM4 cells. There is little difference between these cell lines, except to say that 4/4 RM4 cells are more sensitive to the effects of NAC and the ERK1/2 response is lowered to beyond untreated control levels. Also of note is the effect of NAC alone on these cell lines, this treatment in 4/4RM4 cells reduces basal levels suggesting they are in an inherent state of low oxidative stress. In MET 5A cells, however, there is no such effect.

A role for lipid peroxidation has never been explored in the activation of the ERK pathway by asbestos. Lipid peroxides are formed by the attack of ROS on lipids in the plasma membrane (Esterbauer et al., 1991), because they are not as highly reactive as ROS they are longer lived and free to diffuse from their site of origin and attack biomolecules elsewhere in the intracellular compartment (Esterbauer et al., 1991). Vitamin E is a lipophilic antioxidant and as such resides in the plasma membrane and specifically detoxifies lipid peroxides (Thomas and Stocker, 2000). Pretreatment of MET 5A or 4/4 RM4 cells with 0.5 or 1mM vitamin E was consistently able to reduce the effects of crocidolite on pERK1/2, suggesting that lipid peroxide species somehow contribute to the activation of the ERK pathway. The exact mechanism behind this phenomenon is unclear at this stage, but it does seem unlikely that these species would act directly on ERK1/2 as studies treating vascular smooth muscle cells with 4-HNE showed that the activation of ERK could be blocked by PD098059. Interestingly, vitamin E treatment alone was capable of increasing the level of ERK1/2 phosphorylation above untreated control levels, and this was significant in 4/4 RM4 cells. A possible explanation for this could be the pro-oxidant activity of vitamin E in the absence of co-antioxidants or in conditions where oxidative stress is relatively low *i.e.* no asbestos exposure (Thomas and Stocker, 2000). These results suggest that the use of chemoprevention agents that have antioxidant properties may prove useful in strategies designed to lower the risk of mesothelioma in individuals with known exposure to asbestos. This approach is the focus of much research in relation to a number of human cancers (Malone, 1991; Gescher et al., 1998; Dragsted, 1998) and if efficacious would mean the trauma and low success rate of radical surgery to resect mesothelioma tumours could be avoided.

The importance of EGFR tyrosine kinase activity in the activation of the ERK pathway by asbestos was previously reported in primary RPM cells (Zanella et al.,

1996) and using a clinically relevant inhibitor of this activity, the present studies show that this is also true in rat and human cell lines. PKI166 has been shown to have very favourable results in clinical trials, demonstrating *in vivo* antitumour activity in several EGFR overexpressing xenograft tumor models in nude mice (Traxler et al., 2001). It should be noted that the EGFR does not solely activate the ERK pathway, and so inhibition of associated tyrosine kinase activity will exert additional effects on other signalling pathways, which may in turn affect the phenotypic endpoint. This phenomenon is discussed in detail in later chapters.

The importance of the fibrous nature of crocidolite on activation of the ERK pathway was explored by using milled crocidolite at comparable mass per unit area doses. Studies by Zanella *et al.* (1996) showed that riebeckite, another non-fibrous analogue of crocidolite, did not activate the ERK pathway. However, we found quite the opposite effect to be true. Indeed, this response looked more potent than that seen with fibres. At first this was quite disappointing because it suggested that in fact the ERK response was non-specific and therefore not a true indicator of the carcinogenicity of crocidolite fibres. The reason for this discrepancy between our work and that described in the literature may be for a number of reasons e.g. milled crocidolite may be different from riebeckite in terms of its physical and chemical make up, thus instigating a different cellular response. Alternatively, it could be another indication of how cell lines react differently to primary cultures. However, this second explanation did seem a little unlikely as previous experiments had indicated the cell lines to be more robust than their primary culture counterparts.

Regardless, the findings of the present studies required further experimentation to explain it. As expected, the effects of milled crocidolite were almost entirely chemical in nature, more specifically, surface iron was shown to be crucial as by using agents that chelate surface $Fe^{2+/3+}$ we could almost abolish the ERK response. Whereas with fibres chelation had less effect. At first, this may seem contradictory to the effects noted when short and long fibres were compared, as longer fibres will have less total surface area than shorter fibres on a mass/mass basis. Therefore, these findings highlight the fact that fibres do not exert their pathogenicity by either chemical or physical properties, but a combination of both. When the situation *in vivo* is considered it becomes easier to rectify these somewhat conflicting findings. Whilst

inhaled milled crocidolite will clearly induce oxidative stress and resulting acute inflammatory responses through Fenton chemistry catalysed by $Fe^{2+/3+}$ on its surface (Kamp and Weitzman, 1999), its relatively small size means that it can be quickly cleared by phagocytic cells of the pulmonary immune system. In contrast, fibrous material that had penetrated the lower regions of the lung would be too big to phagocytose, thereby inducing frustrated phagocytosis as well as providing a catalyst for Fenton chemistry.

The importance of crocidolite-induced ERK1/2 activation in the upregulation of AP-1 DNA binding was demonstrated using electrophoretic mobility shift assays. Crocidolite alone is capable of increasing the level of protein in the nucleus that can bind to an AP-1 consensus oligonucleotide. Inhibiting the ERK pathway at the level of MEK1/2 by UO126 in both cell lines examined ameliorated this effect. This is most likely to be mediated by a reduction in *c-fos* expression, as this gene is known to be regulated by the ERK pathway in asbestos exposed mesothelial cells (Zanella et al., 1999). This effect is likely to be via phosphorylation of Elk-1, which is a TCF, by ERK1/2. Upon phosphorylation, Elk-1 has increased affinity for the SRF that binds to the SRE and the transcription of genes, including *c-fos,* are initiated (Treisman, 1994). However, attempts to show a reduction in Fos protein in the AP-1 complex under ERK pathway inhibited conditions by supershift assay proved unsuccessful due to poor antibody recognition. It would be interesting to further investigate the increase in AP-1 DNA binding activity by crocidolite with the use of PKI166.

Together these results suggest that rat and human mesothelial cells behave in a very similar fashion *in vitro* with respect to the ERK pathway. There remain some unanswered questions, however, the source of the increase in EGFR protein is as yet unknown. As the work of Sandhu *et al.* (2000) showed no increase in EGFR mRNA expression it seems likely that an inhibition of receptor internalisation could be responsible. The mechanism behind the activation of the ERK pathway is clearly an oxidative stress dependent process involving the EGFR. A summary of these findings is shown in Figure 3.15.

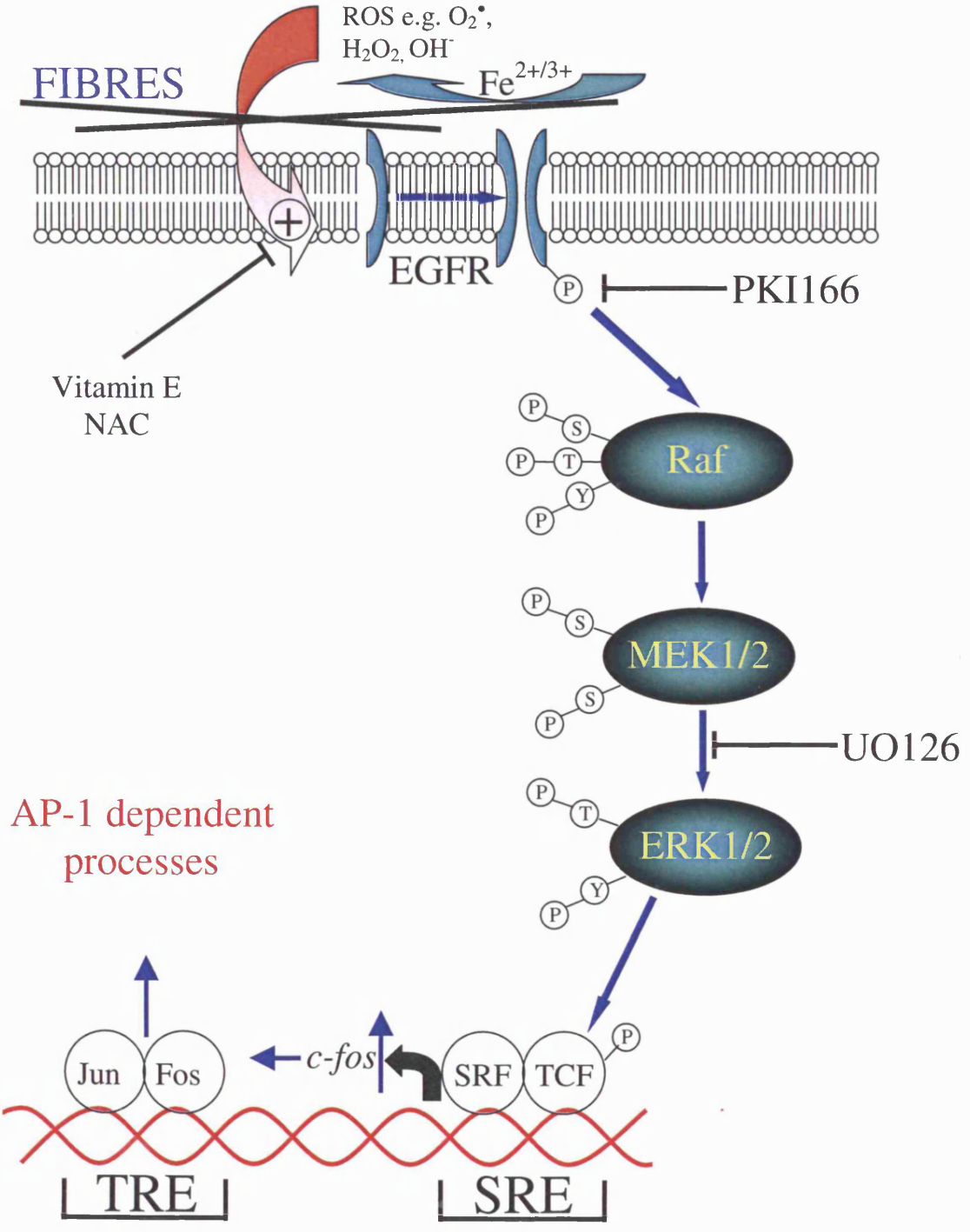


Figure 3.15 Summary of the effects of crocidolite on the ERK pathway in mesothelial cells.

CHAPTER 4

EFFECTS OF CROCIDOLITE ASBESTOS ON THE SAPK PATHWAYS IN MESOTHELIAL CELLS

CHAPTER 4

4.1 INTRODUCTION

Despite recent advances in the identification of upstream regulators of the SAPK pathways, the exact mechanism(s) responsible remain largely unclear due to the wide range of stimuli that can induce their activation. Downstream these pathways have many effectors, including transcription factors *e.g.* AP-1 (Derijard et al., 1994; van Dam et al., 1995) and more recently a role for p38 in activating Akt has been suggested (Rane et al., 2001). As crocidolite has previously been shown to upregulate AP-1 DNA binding (Heintz et al., 1993; Driscoll, 1996), it was hypothesised that one or more of the SAPK pathways were involved in this response.

As a result of the numerous functions of the SAPK pathways, the possible cellular outcomes are equally as diverse. SAPKs have been linked to apoptosis in a number of systems where cells that have accumulated genotoxic damage could otherwise survive and enter the first stage of carcinogenesis (Chen et al., 1996; Korsmeyer, 1995). However, early p38 and JNK activation have been linked to cell survival in response to TNFα (Roulston et al., 1998). The best-described roles for the SAPK pathways are their ability to modulate the expression of cytokines and therefore influence ensuing inflammatory responses. Cytokines Interleukin-1β (IL-1β) and TNF-α are produced early in the inflammatory process and rely on SAPK pathways to exert their effects (Krause et al., 1998; Yuasa et al., 1998) and also for their own regulation (Rutault et al., 2001; Hoffmeyer et al., 1999). In recent years researchers have postulated that an environment of chronic immune activation is well suited to induce malignancy and would involve the prolonged presence of a factor, either infectious or non-infectious, that could act as a persistent trigger for the immune system. It is thought that this scenario could eventually lead to imbalances between humoral immunity (HI) and cell mediated immunity (CMI), with the former becoming predominant, which may be sufficient to induce the carcinogenic process (O'Byrne and Dalgleish, 2001). Asbestos is a perfect example of the above: a non-infectious agent that is extremely bio-persistent and capable of inducing long-term inflammatory responses.

Previous studies have shown that pleural mesothelial cells secrete a variety of cytokines following asbestos exposure including monocyte chemoattractant protein-1

(Tanaka et al., 2000), fibronectin (Kuwahara et al., 1994) and IL-8 (Griffith et al., 1994) all of which have chemoattractant properties, which would recruit pleural macrophages to the site of injury. Upon arrival these macrophages propagate the immune response through production of pro-inflammatory cytokines such as IL-1β and TNFα. The maintenance of this response could rely upon SAPK activation both for IL-8 expression (Krause et al., 1998) and the response to IL-1β and TNFα (Yuasa et al., 1998).

The JNK pathway is not activated in primary RPM cells exposed to asbestos (Jimenez et al., 1997). However, no studies have investigated the activation of p38 under the same conditions. With the abundance of information in the current literature describing a pivotal role for p38 in diseases associated with chronic immune activation it was hypothesised that p38 may also play a role in asbestos-related disease. However, given previous discrepancies between rat and human mesothelial cells, the present studies also investigated the activation state of JNK in 4/4 RM4 and MET 5A cells exposed to crocidolite asbestos. The effect of asbestos on SAPK activity in 4/4 RM4 and MET 5A cells under the same experimental conditions previously shown to activate the ERK pathway was investigated. The mechanism behind the activation of p38 was then probed and was found to be oxidative stress dependent. Finally, the importance of p38 activity in the downstream formation of AP-1 DNA binding complexes was established.

4.2 RESULTS

4.2.1 Crocidolite does not activate JNK1/2 in 4/4 RM4 and MET 5A cell lines

The level of JNK1/2 activity was assessed in MET 5A and 4/4 RM4 cells, following 24 hrs of UICC and NIEHS crocidolite exposure, by *in vitro* kinase assay. In all cases anisomycin (a positive control for JNK1/2 activity) was capable of causing marked increases in JNK1/2 activity in both cell lines compared to untreated controls (Figure 4.1a-d). However, crocidolite at all doses examined had no noticeable effect on the level of JNK1/2 activity in either 4/4 RM4 cells (Figure 4.1a and b) or MET 5A (Figure 4.1c and d). These findings were reflected when the phosphorylation state of JNK1/2 was probed by western blotting. Anisomycin induced large increases in phosphorylation of JNK1/2 at residues Thr^{183}/Tyr^{185}, whereas UICC crocidolite had no such effects (Figure 4.2).

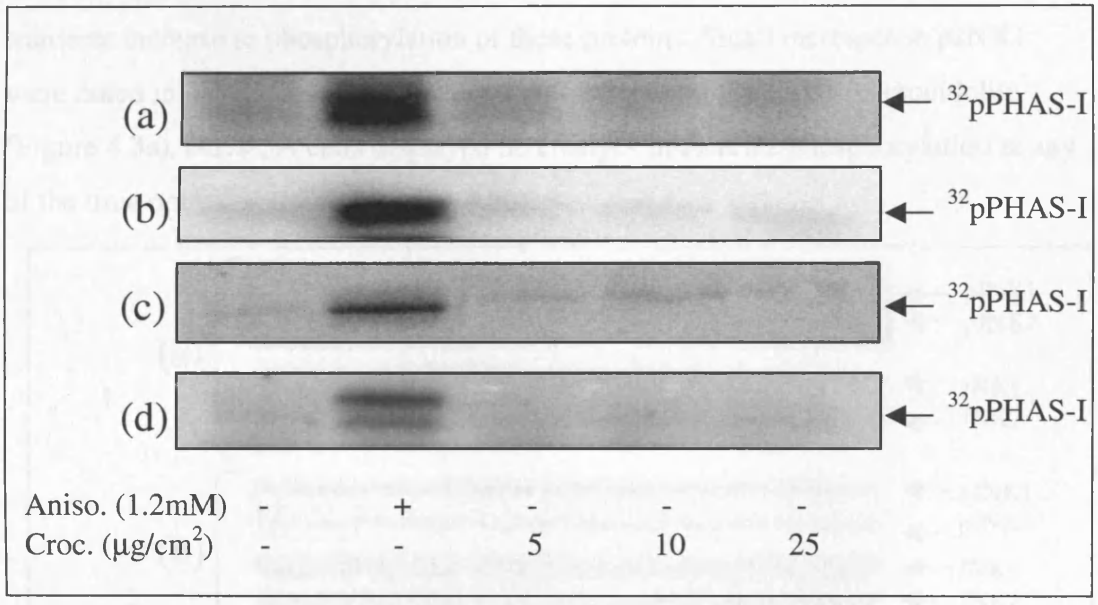


Figure 4.1 Effect of UICC or NIEHS crocidolite on JNK1/2 activity as determined by *in vitro* kinase assay in 4/4 RM-4 and MET 5A cells. 4/4 RM4 (a,b) and MET 5A (c,d) cells were serum starved for 24 hrs prior to treatment for 24 hrs with the indicated concentrations of (a,c) NIEHS crocidolite or (b,d) UICC crocidolite. In each case a 30 minute treatment with anisomycin (1.2mM) was included as a positive control. Cells were then lysed and JNK1/2 was immunoprecipitated from equal amounts of protein of each sample. JNK1/2 activity was measured by the ability of the purified kinase to phosphorylate PHAS-I substrate in an *in vitro* kinase assay. Blots shown are representative of three experiments.

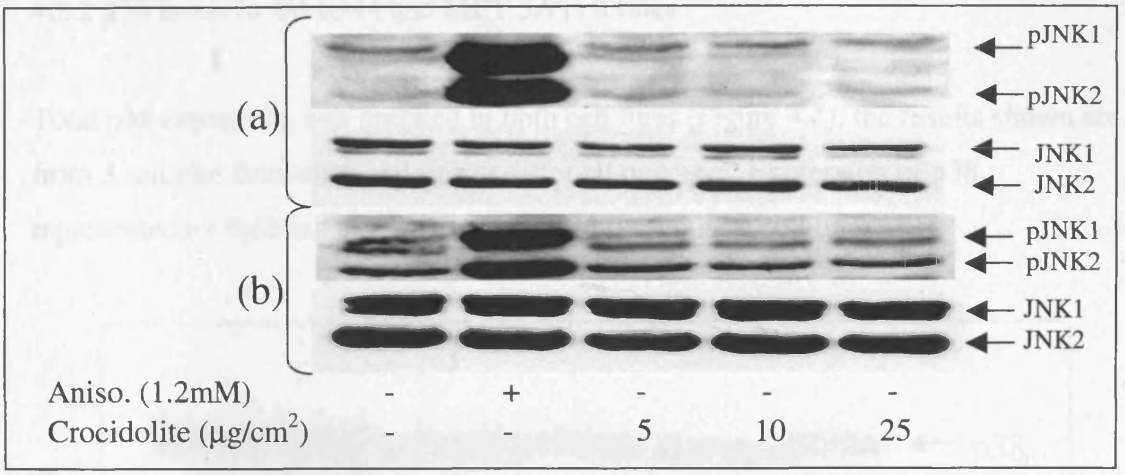


Figure 4.2 Effect of UICC crocidolite on JNK1/2 phosphorylation as determined by western blotting. 4/4 RM-4 (a) or MET5A cells (b) were serum starved for 24 hrs prior to treatment for 24 hrs with the indicated concentrations of UICC crocidolite. In each case a 30 minute treatment with anisomycin (1.2mM) was included as a positive control. Cells were then lysed and the proteins separated by SDS PAGE. The proteins were transferred onto a nitrocellulose membrane and the resultant filters immunoblotted with anti-pJNK1/2 antibody. Bands were visualised by chemiluminescence. Blots were then stripped and reprobed for native JNK1/2. Blots shown are representative of two experiments for each cell line.

Levels of pJNK1/2 were then assessed at 4, 8 and 24 hrs to ensure that there was no transient increase in phosphorylation of these proteins. Small increases in pJNK1 were noted in 4/4 RM4 cells following 4 and 8 hrs exposure to UICC crocidolite (Figure 4.3a). MET 5A cells displayed no changes in JNK1/2 phosphorylation at any of the time points examined (Figure 4.3b).

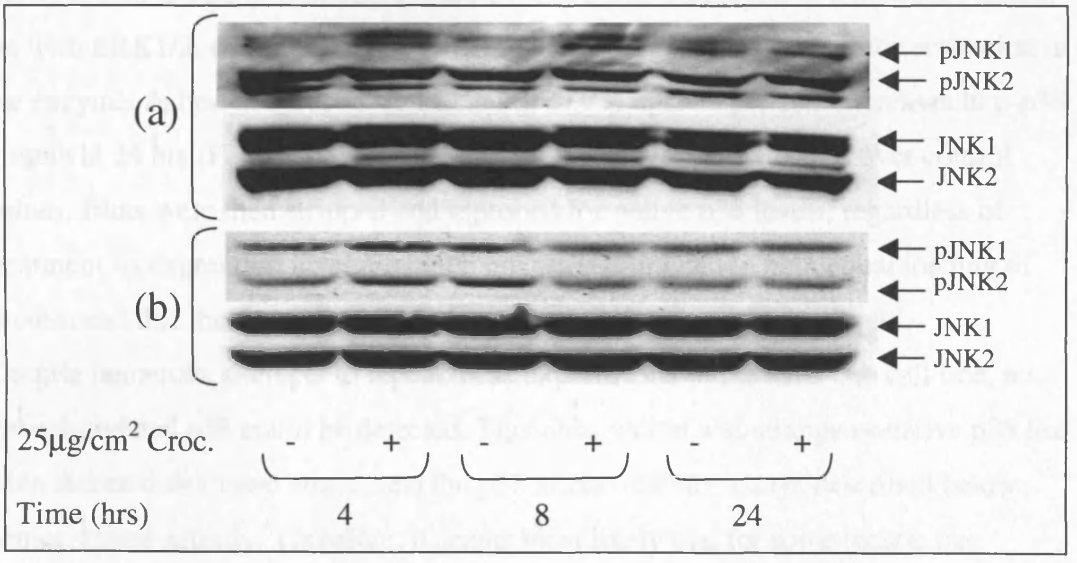


Figure 4.3 Time course of JNK phosphorylation by UICC crocidolite as determined by western blotting. 4/4 RM-4 (a) or MET 5A (b) cells were serum starved for 24 hrs prior to treatment with 25µg/cm^2 UICC crocidolite for times indicated, they were then lysed and the proteins separated by SDS PAGE. The proteins were transferred onto nitrocellulose membrane and the resultant filters immunoblotted with anti-pJNK1/2 antibody. Bands were visualised by chemiluminescence. Blots were then stripped and reprobed for JNK1/2. Blots shown are representative of two experiments.

4.2.2 p38 levels in 4/4 RM4 and MET 5A cell lines

Total p38 expression was checked in both cell lines (Figure 4.4), the results shown are from 3 samples from each cell line at different passages. Expression of p38, represented by the band at 38kDa, is very similar in these cell lines.

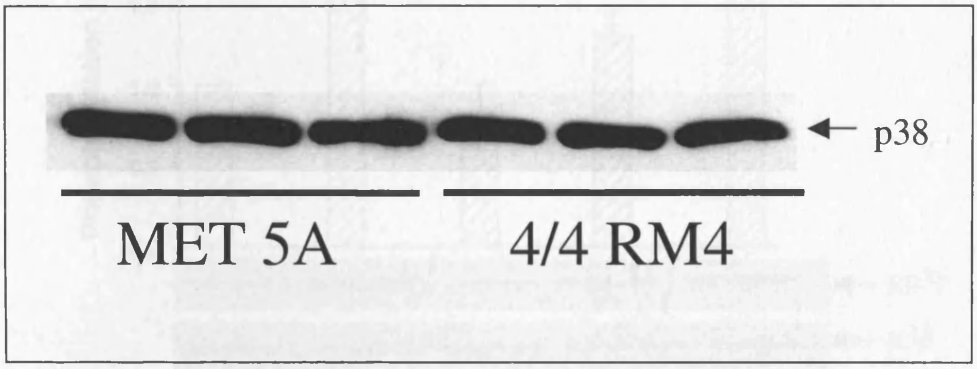


Figure 4.4 Levels of p38 in MET5A and 4/4 RM4 cells. Serum starved cells were lysed and proteins separated by SDS PAGE. . Following transfer onto nitrocellulose membrane the resultant filters were immunoblotted with anti-p38 antibody. Bands were visualised by chemiluminescence. For each cell line 3 different samples were tested.

4.2.3 Crocidolite activates p38 in MET 5A and 4/4 RM4 cells

Levels of phosphorylated p38 (Thr^{180}/Tyr^{182}) protein were examined after 24 hrs of fibre exposure, with anisomycin (1.2mM for 30mins) employed as a positive control. As with ERK1/2, the phosphorylation of these two residues is critical for activation of the enzyme. Asbestos induced significant ($p<0.05$) dose-dependent increases in p-p38 protein at 24 hrs (Figure 4.5) with maximum levels reaching 1.5 fold over control values. Blots were then stripped and reprobed for native p38 levels, regardless of treatment its expression level remained unchanged indicating both equal loading of protein and that the rises in p-p38 levels were independent of overall p38.
Despite numerous attempts to repeat these experiments in the MET 5A cell line, no phosphorylated p38 could be detected. This observation was strange as native p38 had been detected as shown above, and the p38 kinase activity assays described below demonstrated activity. Therefore, it seems most likely that for some reason the phospho-p38 antibody did not recognise this protein in MET 5A cells.

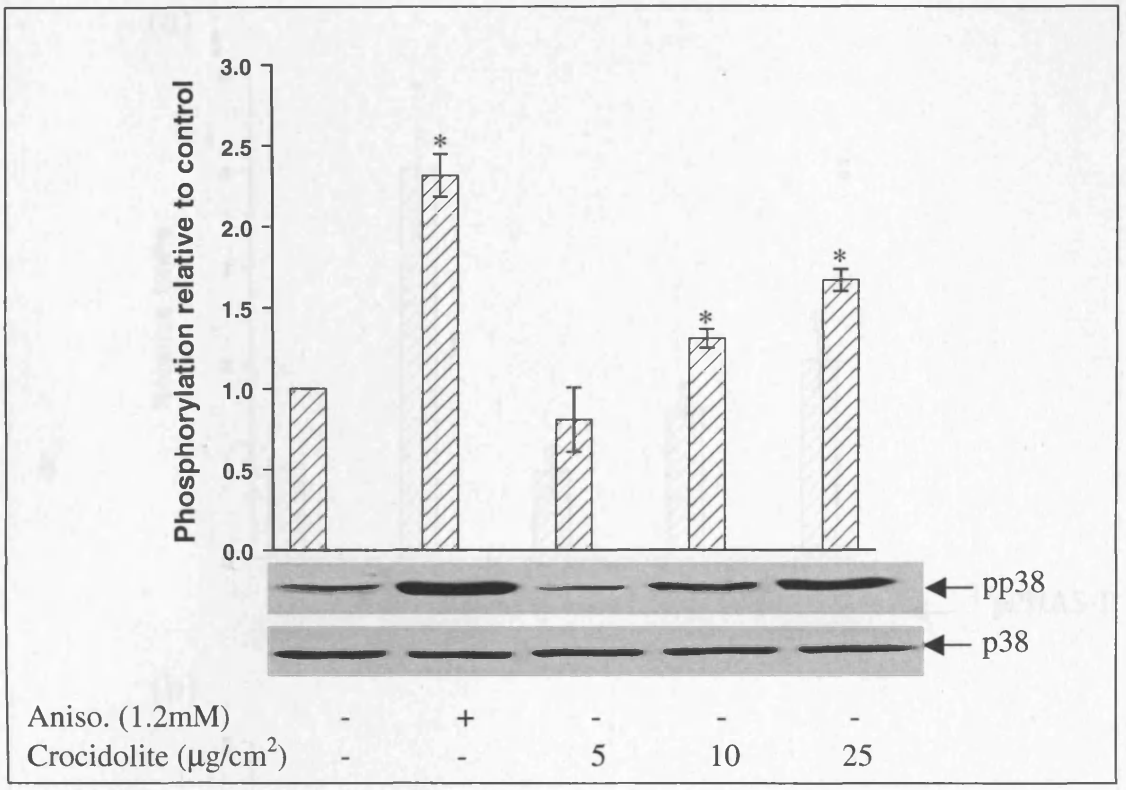


Figure 4.5 Effect of UICC crocidolite on p38 phosphorylation in 4/4 RM4 cells as determined by western blotting. 4/4 RM-4 cells were serum starved for 24 hrs prior to treatment for 24 hrs with the indicated concentrations of UICC crocidolite. In each case a 30 minute treatment with anisomycin (1.2mM) was included as a positive control. Cells were then lysed and the proteins separated by SDS PAGE. The proteins were transferred onto a nitrocellulose membrane and the resultant filters immunoblotted with anti-pp38 antibody. Bands were visualised by chemiluminescence. Blots were then stripped and reprobed for p38. Blots shown are representative of three experiments. The data from three separate experiments were quantified by densitometry and the means +/- S.E.M plotted. * $p<0.05$.

As with the studies investigating ERK1/2, *in vitro* kinase assays were used to investigate modulations in p38 activity by UICC and NIEHS crocidolite. Figure 4.6b shows how the activity of p38 is altered in 4/4 RM4 cells with increasing doses of UICC crocidolite and also with the known inducer of p38 activity, anisomycin. Levels of p38 activity with 5µg/cm^2 UICC crocidolite are essentially the same as the untreated control (Figure 4.6b). At higher doses (10 and 25µg/cm^2) however, p38 activity is increased to nearly double and was significantly different to untreated controls in both cases ($p<0.05$). Indeed, this level of induction is very similar to that observed in the analysis of pp38 by western blotting under identical conditions. The effect of NIEHS crocidolite was very similar in this cell line. Again 5µg/cm^2 had little or no effect and higher doses (10 and 25µg/cm^2) were capable of strongly inducing p38 activity (Figure 4.6a).

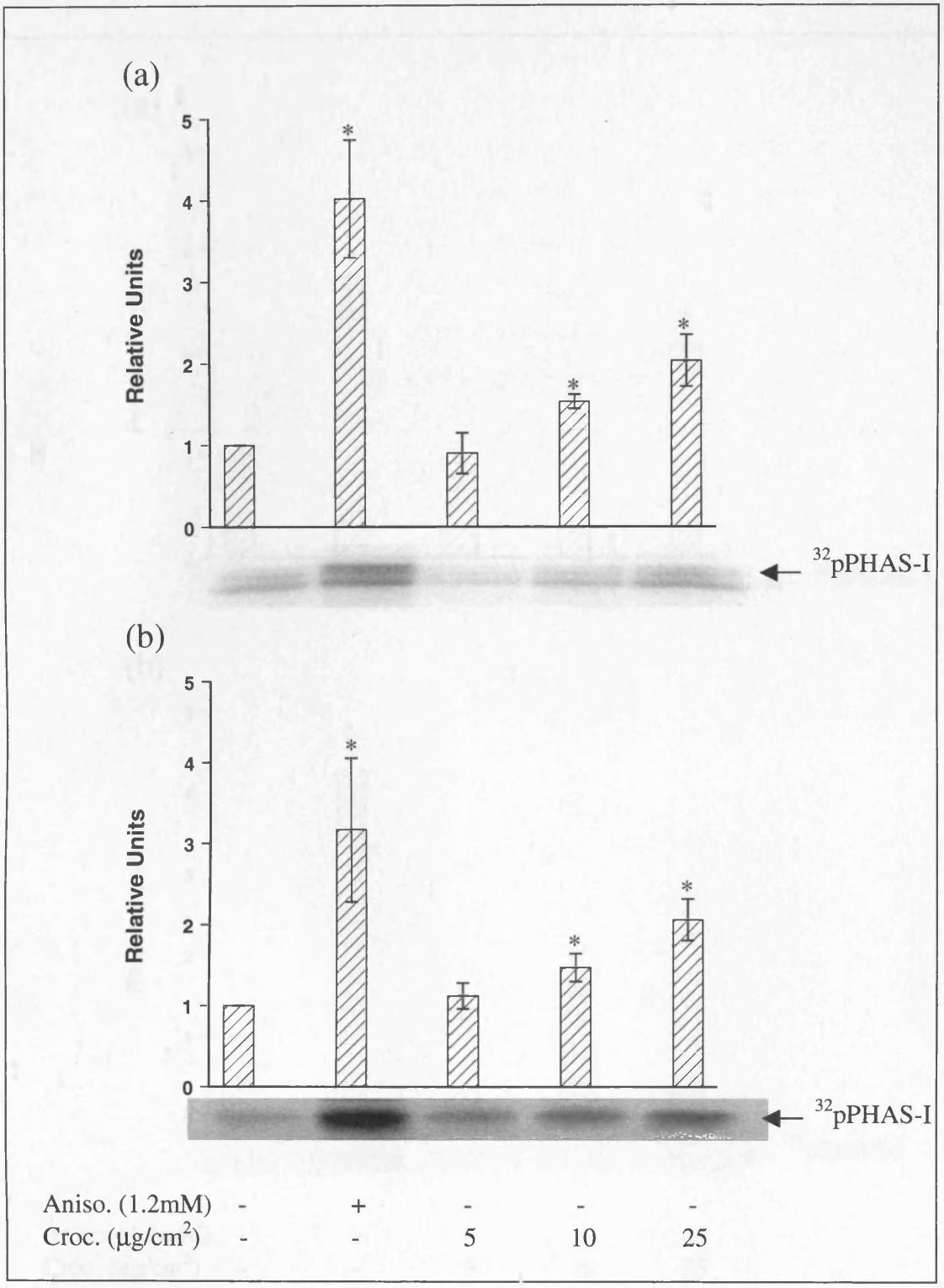


Figure 4.6 Effect of UICC or NIEHS crocidolite on p38 activity as determined by *in vitro* kinase assay in 4/4 RM-4 cells. Cells were serum starved for 24 hrs prior to treatment for 24 hrs with the indicated concentrations of (a) NIEHS crocidolite or (b) UICC crocidolite. In each case a 30 minute treatment with anisomycin (1.2mM) was included as a positive control. Cells were then lysed and p38 was immunoprecipitated from equal amounts of protein of each sample. p38 activity was measured by the ability of the purified kinase to phosphorylate PHAS-I substrate in an *in vitro* kinase assay. Blots shown are representative of three experiments and were quantified by densitometry and the means +/- S.E.M. plotted. * $p<0.05$.

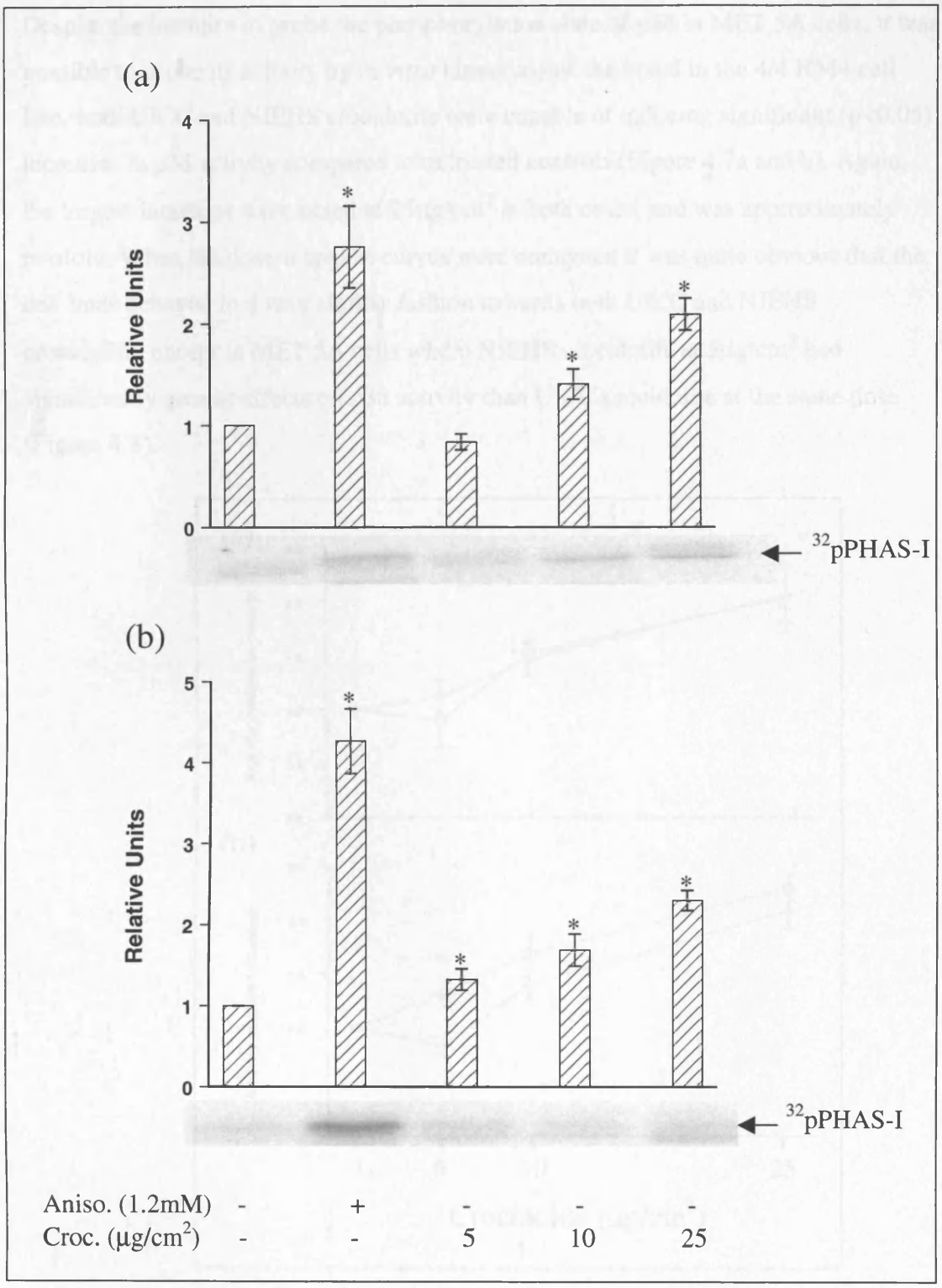


Figure 4.7 Effect of UICC or NIEHS crocidolite on p38 activity as determined by *in vitro* kinase assay in MET5A cells. Cells were serum starved for 24 hrs prior to treatment for 24 hrs with the indicated concentrations of (a) NIEHS crocidolite or (b) UICC crocidolite. In each case a 30 minute treatment with anisomycin (1.2mM) was included as a positive control. Cells were then lysed and p38 was immunoprecipitated from equal amounts of protein of each sample. p38 activity was measured by the ability of the purified kinase to phosphorylate PHAS-I substrate in an *in vitro* kinase assay. Blots shown are representative of three experiments and were quantified by densitometry and the means +/- S.E.M. plotted. * $p<0.05$.

Despite the inability to probe the phosphorylation state of p38 in MET 5A cells, it was possible to probe its activity by *in vitro* kinase assay. As noted in the 4/4 RM4 cell line, both UICC and NIEHS crocidolite were capable of inducing significant ($p<0.05$) increases in p38 activity compared to untreated controls (Figure 4.7a and b). Again, the largest increases were noted at 25μg/cm^2 in both cases, and was approximately twofold. When the dose-response curves were compared it was quite obvious that the cell lines behaved in a very similar fashion towards both UICC and NIEHS crocidolite, except in MET 5A cells where NIEHS crocidolite at 5μg/cm^2 had significantly greater effects on p38 activity than UICC crocidolite at the same dose (Figure 4.8).

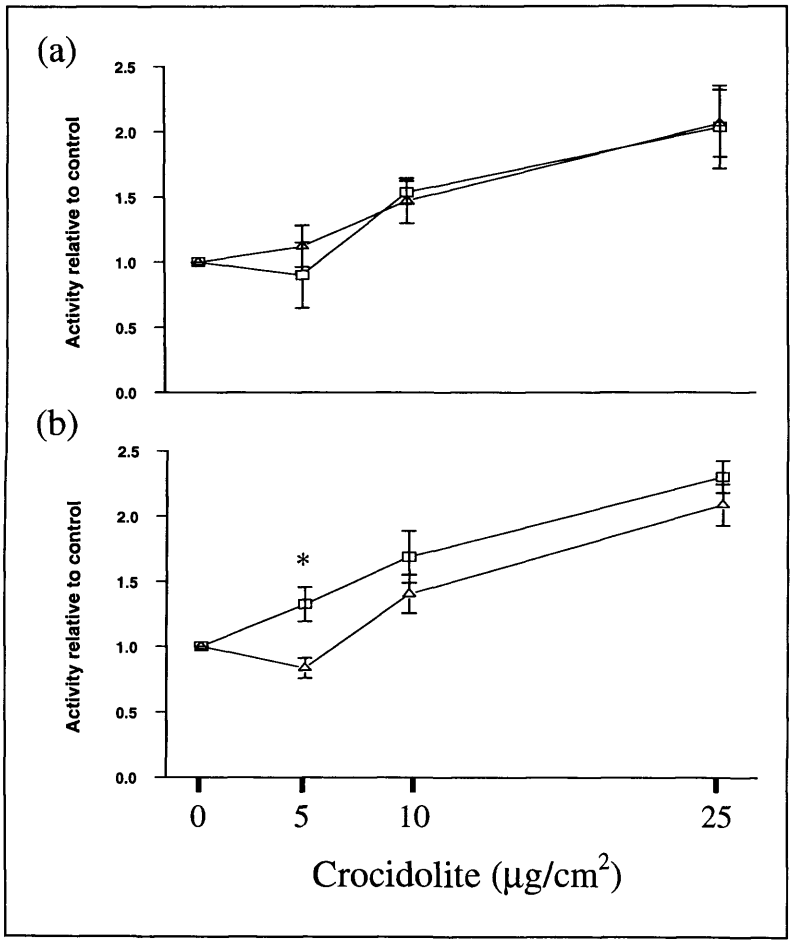


Figure 4.8 Dose-response curve of the effect of UICC (△) and NIEHS (□) crocidolite on p38 activity in (a) 4/4 RM4 and (b) MET 5A cells. Values shown are mean +/- S.E.M. * $p<0.05$.

It was decided that western blotting would be an adequate indicator of the level of activated p38 in 4/4 RM4 samples for further experimentation.

The time course of p38 phosphorylation was investigated by probing pp38 levels at 4, 8 and 24 hours after fibre treatments with untreated control samples taken at each time point to allow for fluctuations in background pp38. Figure 4.9 shows that crocidolite significantly ($p<0.05$) increases pp38 levels over the latter two time periods, however, at 4 hours these levels are essentially the same as control. This lag period may be explained by the time it takes fibres to settle onto the cell monolayer. Levels of pp38 in untreated controls also rose over the same period and is most likely explained by the extended presence of the cells in serum free media that is, in itself, a stressful stimuli (Kummer et al., 1997). Therefore, we could not extend the time course any further as the signal to noise ratio became too low (data not shown). These results were very similar to those obtained when studying the ERK pathway, both in terms of level of induction and time course.

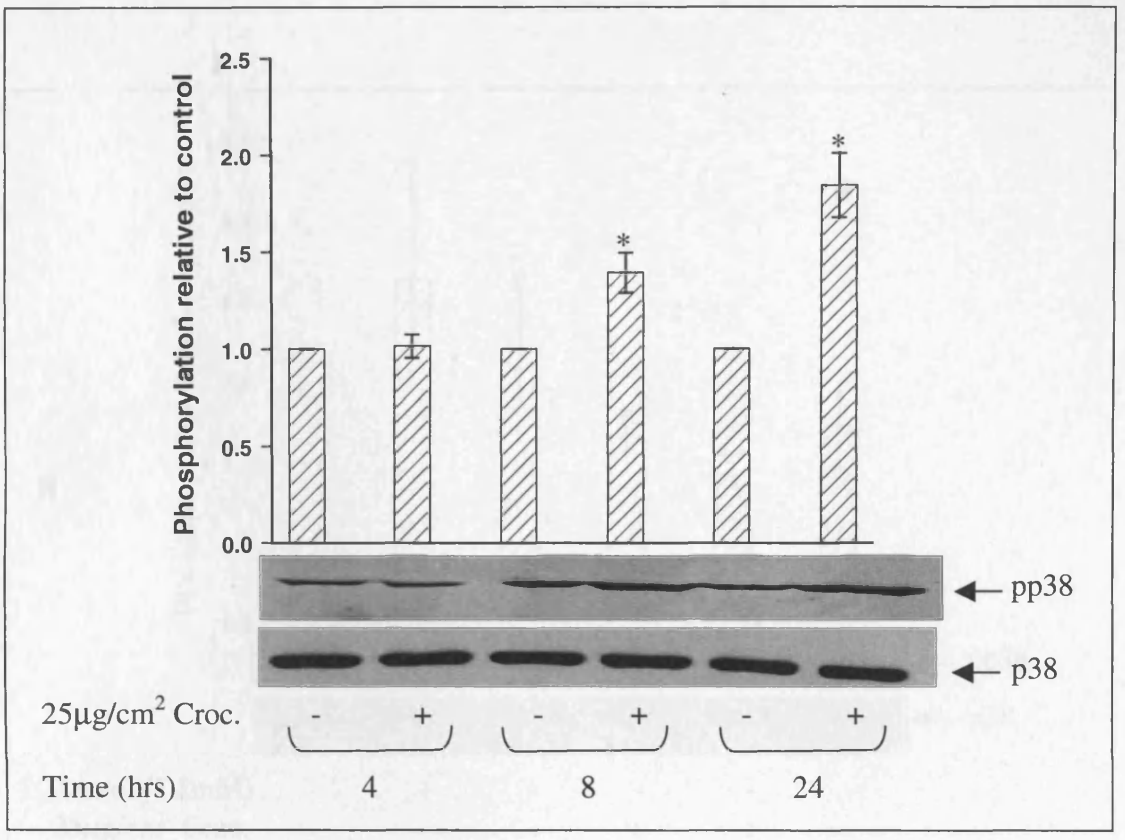


Figure 4.9 Time course of p38 phosphorylation by UICC crocidolite as determined by western blotting. 4/4 RM-4 cells were serum starved for 24 hrs prior to treatment with 25µg/cm^2 UICC crocidolite for times indicated, they were then lysed and the proteins separated by SDS PAGE. The proteins were transferred onto a nitrocellulose membrane and the resultant filters immunoblotted with anti-pp38 antibody. Bands were visualised by chemiluminescence. Blots were then stripped and reprobed for p38. Blots shown are representative of three experiments. The data from each experiment was quantified by densitometry and the means +/- S.E.M. plotted. * $p<0.05$.

4.2.4 Mechanism of p38 activation by crocidolite in 4/4 RM-4 cells

The importance of oxidants in the crocidolite-induced activation of p38 was investigated by pre-incubating the cells with 1 and 5mM NAC for 18hrs prior to exposure to 25µg/cm^2 UICC crocidolite for a further 24hrs. In common with crocidolite induced ERK activation, NAC abrogated the phosphorylation of p38 in a dose-dependent manner, where co-incubation with 5mM NAC returned pp38 to untreated control levels (Figure 4.10).

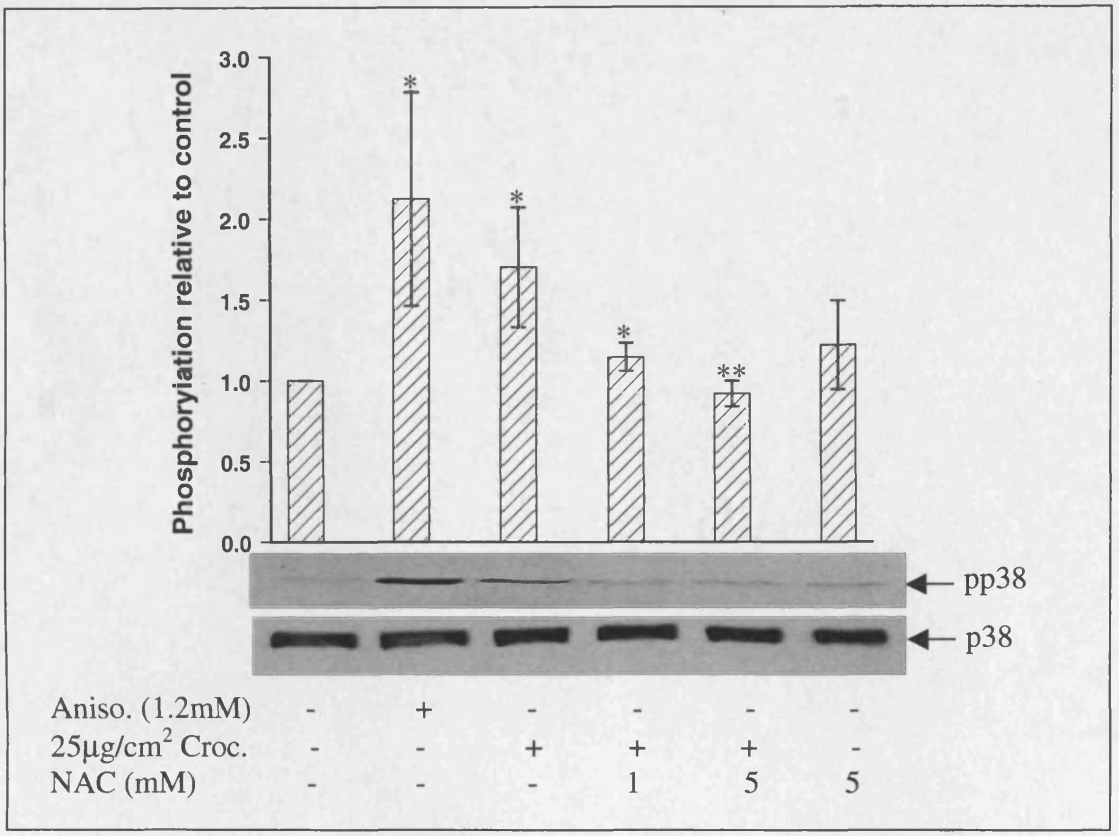


Figure 4.10 Effect of NAC pretreatment on the ability of UICC crocidolite to induce phosphorylation of p38 as determined by western blotting. 4/4 RM-4 cells were serum starved for 6 hrs before 18 hrs incubation with doses of NAC indicated, then 25µg/cm^2 UICC crocidolite for 24 hrs, plus anisomycin (1.2mM) for 30 mins as a positive control. Cells were then lysed and the proteins separated by SDS PAGE. Following transfer onto a nitrocellulose membrane the resultant filters were immunoblotted with anti-pp38 antibody. Bands were visualised by chemiluminescence. Blots were then stripped and reprobed for p38. Blots shown are representative of three experiments. The data from three separate experiments were quantified by densitometry and the means +/- S.E.M. plotted. * $p<0.05$ with respect to control. ** $p<0.05$ with respect to UICC crocidolite alone.

To further elucidate the oxidant-mechanism involved in p38 activation, we incubated cells for 2hrs with 0.5 and 1mM vitamin E, an inhibitor of lipid peroxidation which we had hypothesised may be important in the activation of p38 by asbestos. Figure 4.11 shows these doses decrease asbestos-induced p38 phosphorylation to near control levels in a dose-dependent manner. The data suggests that p38 activation by crocidolite is reliant upon the formation of lipid peroxides at the plasma membrane and this theory is strengthened by observations in other laboratories that crocidolite is capable of inducing lipid peroxidation in mesothelial cells under fibre stressed conditions (Weitzman and Weitberg, 1985).

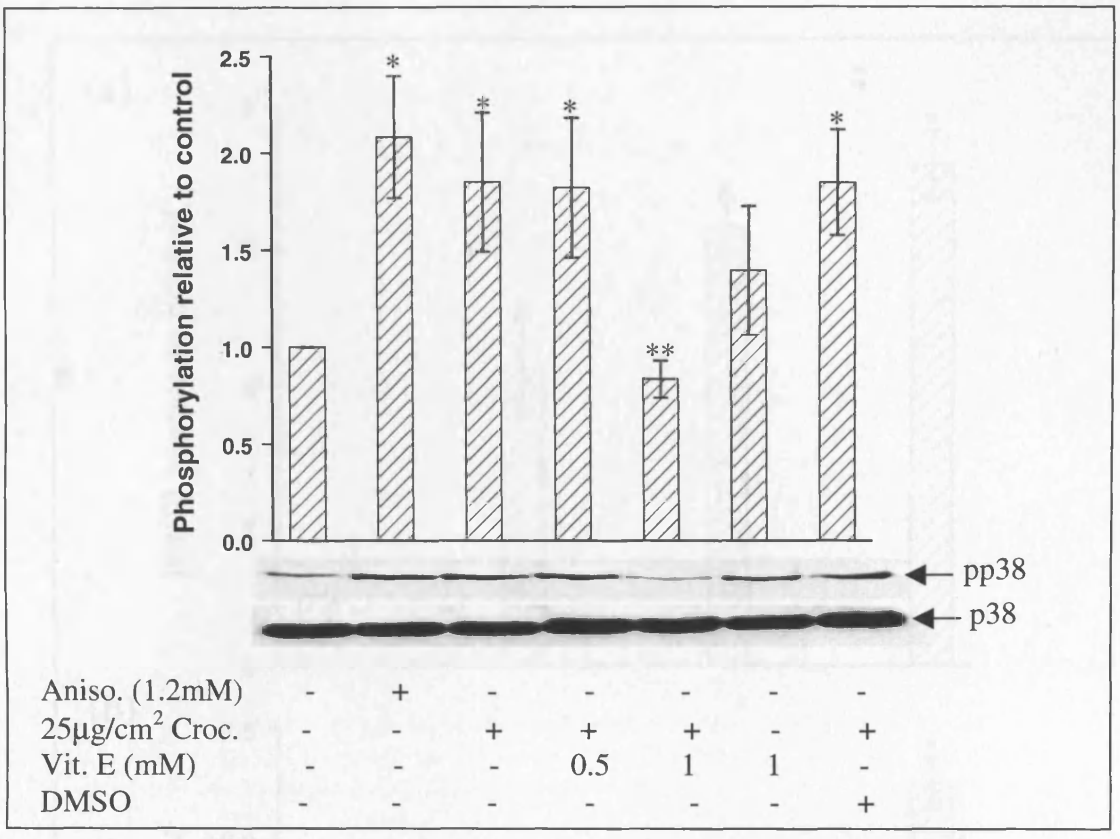


Figure 4.11 Effect of vitamin E pretreatment on the ability of UICC crocidolite to induce phosphorylation of p38 as determined by western blotting. 4/4 RM-4 cells were serum starved for 24 hrs before 2 hrs incubation with vit. E or DMSO vehicle control, then 25µg/cm^2 UICC crocidolite for 24 hrs, plus an anisomycin positive control. Cells were lysed and the proteins separated by SDS PAGE. Following transfer onto a nitrocellulose membrane the resultant filters were immunoblotted with anti-pp38 antibody. Bands were visualised by chemiluminescence. Blots were then stripped and reprobed for p38. Blots shown are representative of three experiments. The data from three separate experiments were quantified by densitometry and the means +/- S.E.M. plotted. * $p<0.05$ with respect to control. ** $p<0.05$ with respect to UICC crocidolite alone.

Previously, other laboratories have shown that one of the end-products of lipid peroxidation, 4-HNE, can increase p38 activity (Uchida et al., 1999) and this may also be true under crocidolite-exposed conditions as crocidolite can increase the level of 4-HNE in mesothelial cells as shown in Figure 4.12. The direct effect of 4-HNE on p38 activity was not determined as access to stable 4-HNE was not available for these experiments.

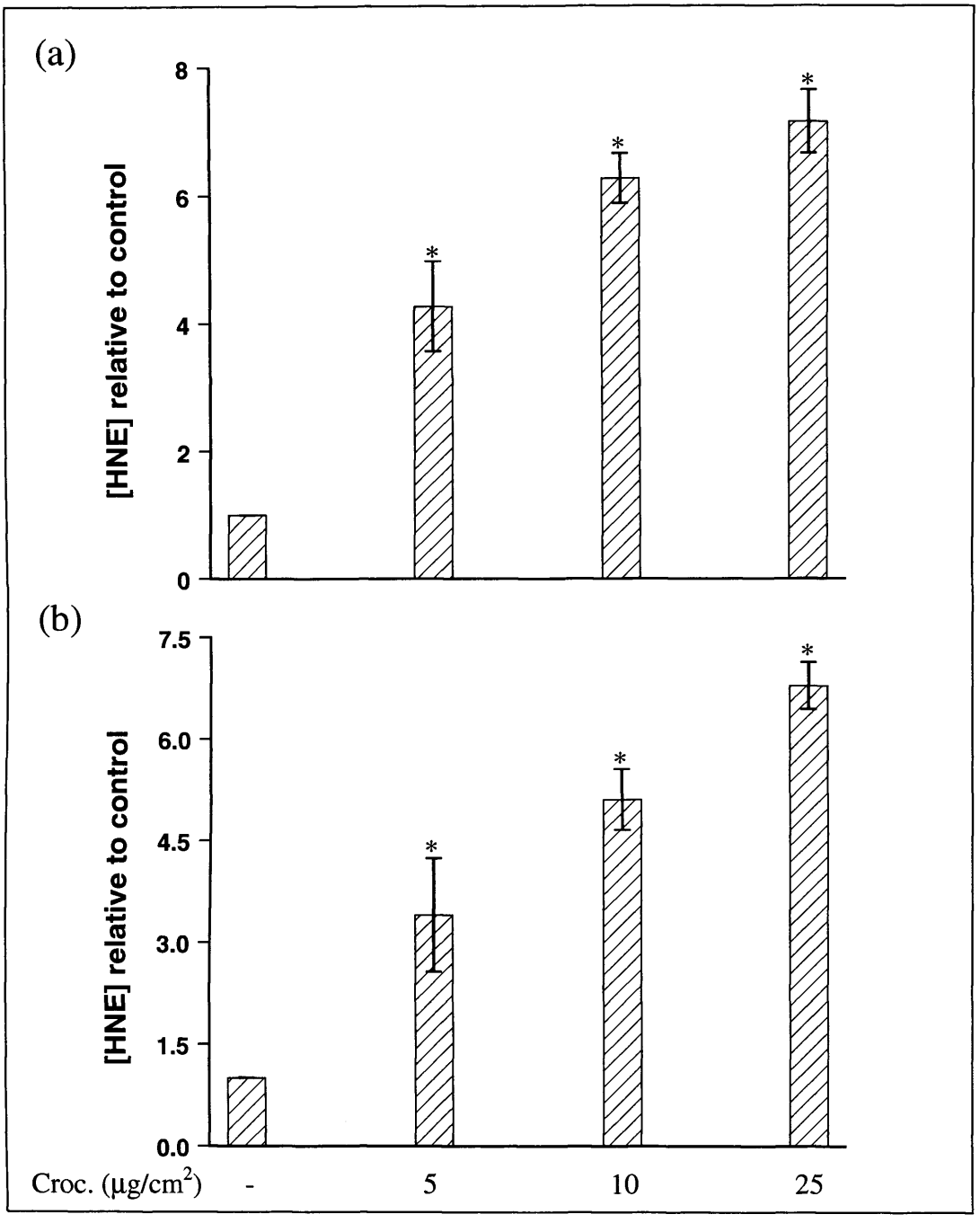


Figure 4.12 Effect of UICC crocidolite on 4-HNE production in 4/4 RM4 and MET 5A cells. 4/4 RM4 (a) or MET 5A (b) cells were grown to 90% confluency before serum starvation for 24 hrs then treated with indicated concentrations of UICC crocidolite. Cells were lysed and 4-HNE concentration was determined as described in materials and methods. Values shown are mean +/- S.E.M. * $p<0.05$ with respect to untreated control

A possible role for EGFR tyrosine kinase activity in crocidolite induced p38 activation was tested by incubating cells with PKI166 prior to asbestos exposure. These experiments showed that p38 did not lie downstream of EGFR activation, in fact, co-incubation with PKI166 appeared to slightly increase the level of p38 activation (Figure 4.13). However, insufficient repeats were performed of this experiment to statistically prove or disprove this observation.

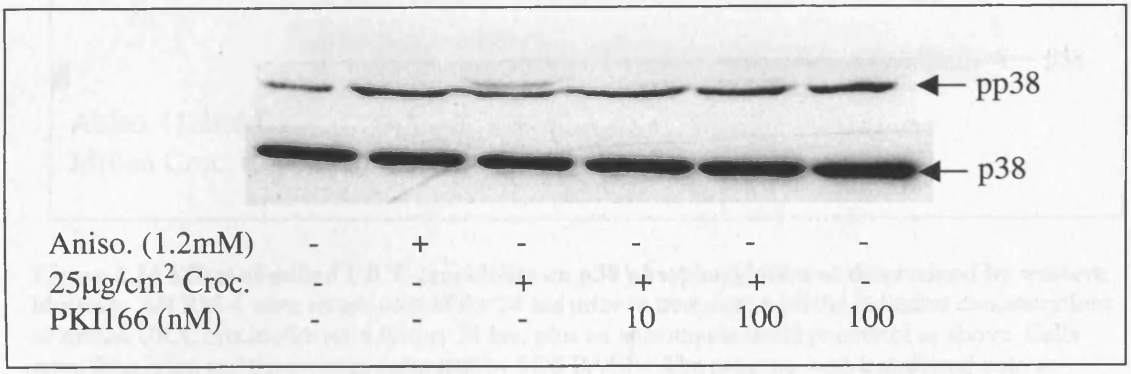


Figure 4.13 Effect of PKI166 pretreatment on the ability of UICC crocidolite to induce phosphorylation of p38 as determined by western blotting. 4/4 RM-4 cells were serum starved for 24 hrs before 2 hrs incubation with PKI166 or DMSO vehicle control, then 25µg/cm^2 UICC crocidolite for 24 hrs, plus an anisomycin positive control. Cells were then lysed and the proteins separated by SDS PAGE. Following transfer onto a nitrocellulose membrane the resultant filters were immunoblotted with anti-pp38 antibody. Bands were visualised by chemiluminescence. Blots were then stripped and reprobed for p38. Blots shown are representative of two experiments.

4.2.5 Effect of non-fibrous analogues on crocidolite-induced p38 activation

To examine if the p38 response is fibre-specific mesothelial cells were exposed to comparable concentrations (mass/unit area) of milled crocidolite, which enables crude dissection of the chemical and physical properties of these fibres. In Figure 4.14, the milled sample does, indeed, cause large increases in pp38 over sham treated controls. Additionally, these increases appeared larger than was seen with comparable mass/unit area doses of fibrous crocidolite and we concluded that, as was the case with ERK pathway activation, this response was due to the increased surface area of the milled sample compared to the fibrous on a mass/mass basis and could therefore be explained by non-specific effects of bioavailable iron on the particle surface. To test this hypothesis the iron from both milled and fibrous crocidolite samples was chelated by deferoxamine and ferrozine. When cells were exposed to the chelated

fibre sample the level of pp38 was reduced compared to sham chelated fibres although it was still higher than in untreated controls. However, chelation of the milled sample significantly reduced the ability of the preparation to induce p38 phosphorylation above untreated control levels (Figure 4.15).

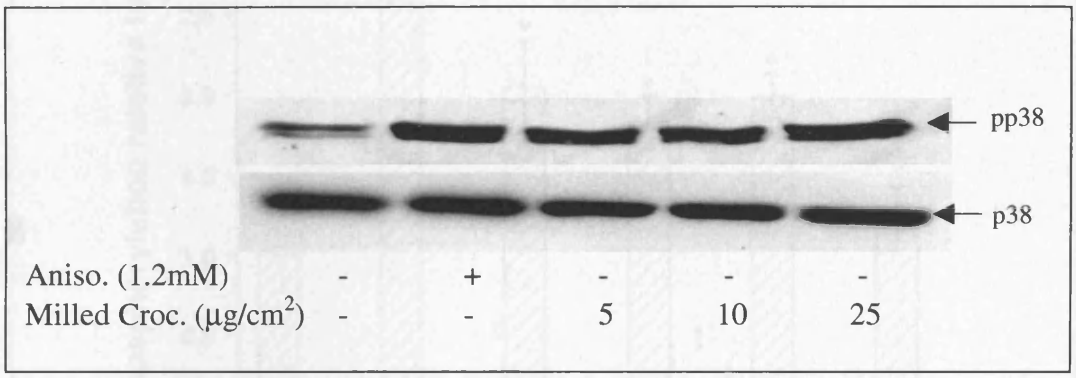


Figure 4.14 Effect of milled UICC crocidolite on p38 phosphorylation as determined by western blotting. 4/4 RM-4 were serum starved for 24 hrs prior to treatment with the indicated concentrations of milled UICC crocidolite for a further 24 hrs, plus an anisomycin positive control as above. Cells were then lysed and the proteins separated by SDS PAGE. The proteins were transferred onto a nitrocellulose membrane and the resultant filters immunoblotted with anti-p38 antibody. Bands were visualised by chemiluminescence. Blots were then stripped and reprobed for p38. Blot shown is from one experiment.

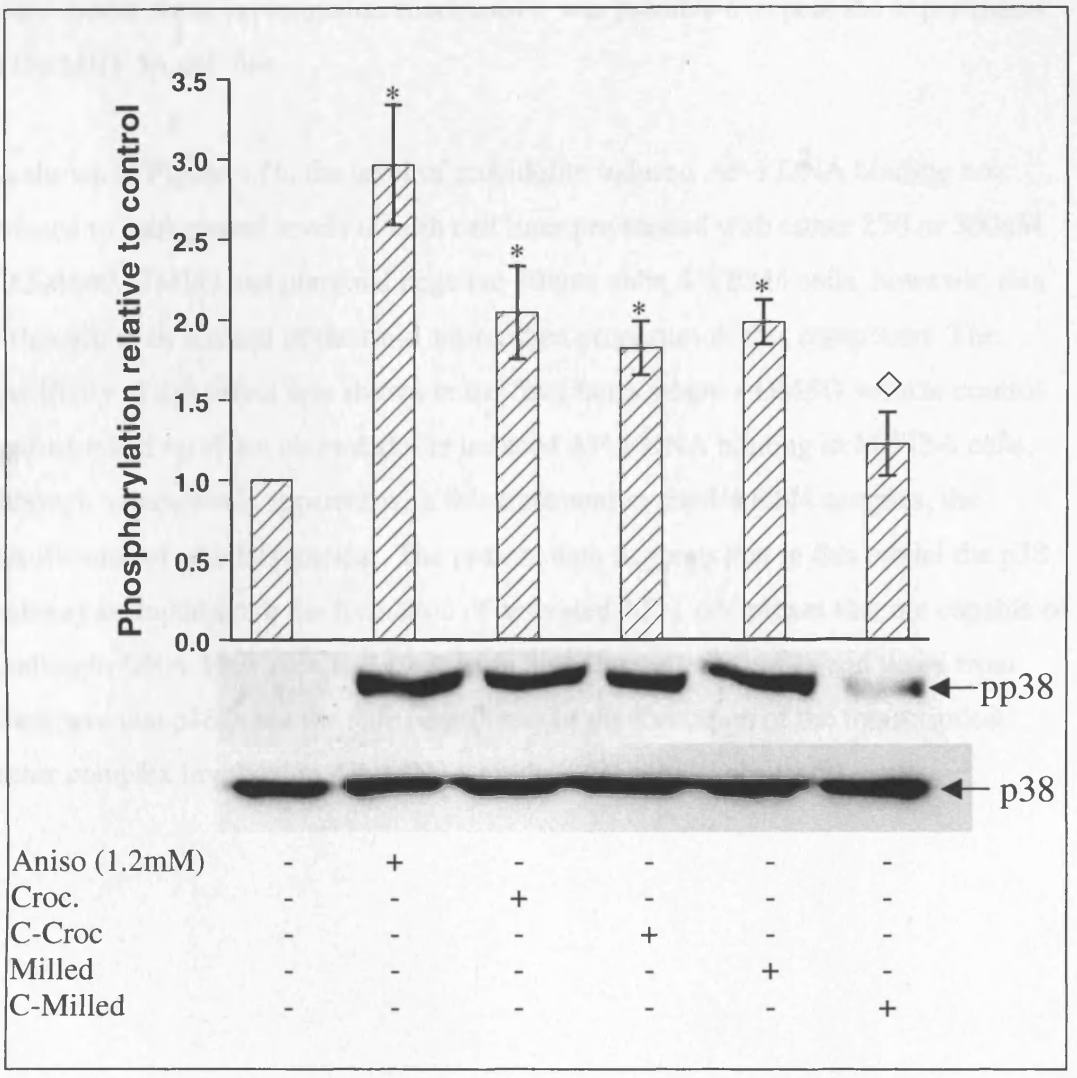


Figure 4.15 Effect of iron chelation (C) on the ability of fibrous and milled UICC crocidolite to induce phosphorylation of p38 as determined by western blotting. 4/4 RM-4 cells were serum starved for 24 hrs prior to treatment with 25μg/cm^2 of chelated/sham-chelated particle for 24 hrs, plus an anisomycin positive control as above. Cells were then lysed and the proteins separated by SDS PAGE. Following transfer onto a nitrocellulose membrane the resultant filters were immunoblotted with anti-pp38 antibody. Bands were visualised by chemiluminescence. Blots were then stripped and reprobed for p38. Blots shown are representative of three experiments for each cell line. The data from three separate experiments were quantified by densitometry and the mean +/- S.E.M. plotted. * $p<0.05$ with respect to untreated control. ◇ $p<0.05$ with respect to sham-chelated milled crocidolite.

4.2.6 Role of the p38 pathway in crocidolite-induced AP-1 DNA binding

The importance of p38 activation on downstream effectors has been discussed above and the hypothesis that p38 was involved in crocidolite-induced AP-1 DNA binding was tested. Prior to exposure to doses of crocidolite known to activate AP-1, cells were incubated for 2hrs with SB203580 a well-documented inhibitor of p38 to see if this effect could be ameliorated, before analysis of samples by gel mobility shift

assay. Under these experimental conditions it was possible to repeat the experiments in the MET 5A cell line.

As shown in Figure 4.16, the level of crocidolite induced AP-1 DNA binding was reduced to background levels in both cell lines pre-treated with either 250 or 500nM SB203580. DMSO had marginal negative effects on in 4/4 RM4 cells, however, this is thought to be a result of the mild antioxidant properties of this compound. The specificity of this effect was shown in the final lanes where a DMSO vehicle control treatment had no effect on crocidolite induced AP-1 DNA binding in MET5A cells, although a decrease is apparent with this treatment in the 4/4 RM4 samples, the significance of which is unclear. The present data suggests that in this model the p38 pathway is important in the formation of activated AP-1 complexes that are capable of binding to DNA. However, it is clear from both the present studies and those from elsewhere that p38 is not the sole contributor to the formation of the transcription factor complex involved in AP-1 DNA binding (Zanella et al., 1999).

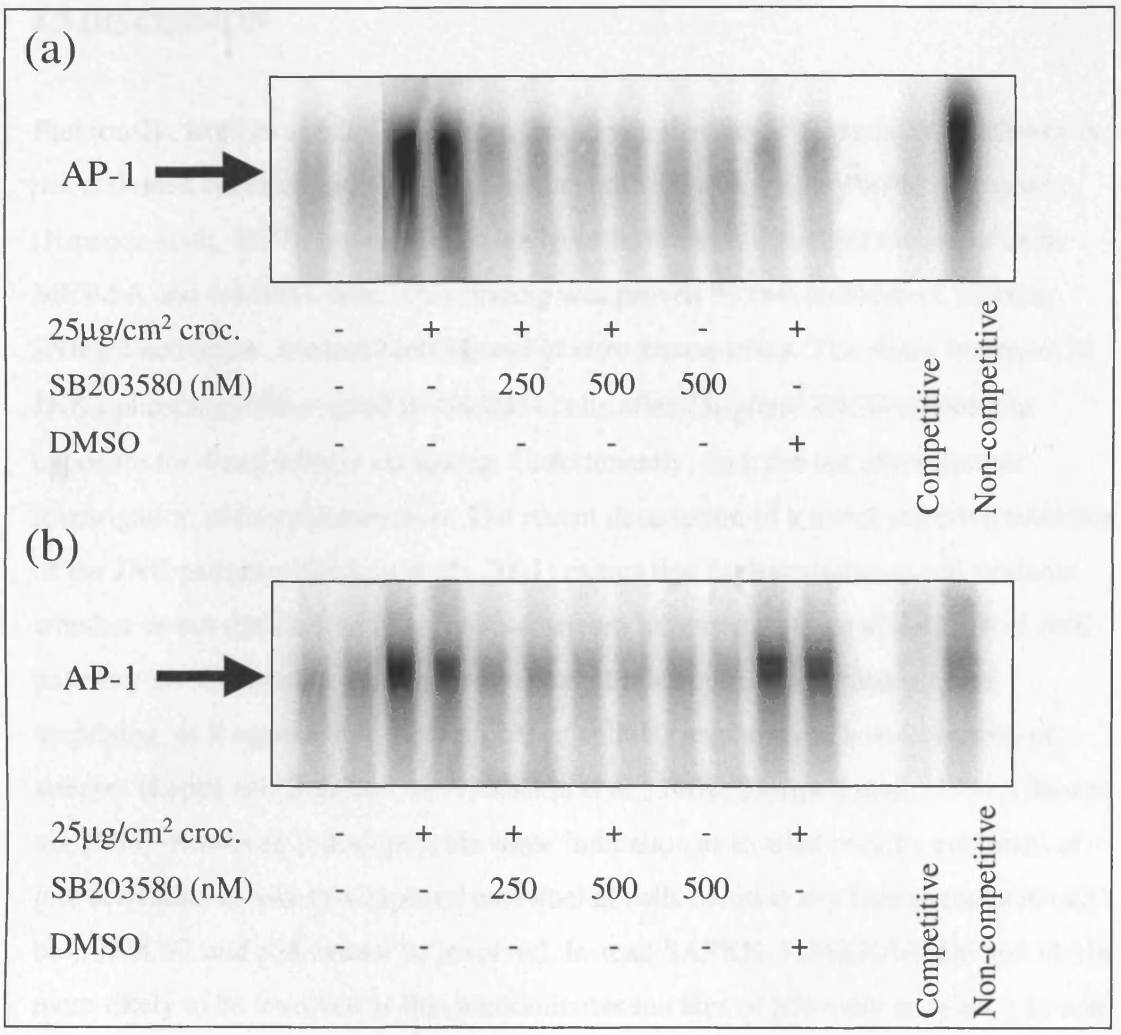


Figure 4.16 SB203580 ameliorates the ability of UICC crocidolite to induce AP-1 DNA binding. 4/4 RM4 (a) or MET5A (b) cells were grown to 90% confluency before serum starvation for 24hrs. SB203580 was added to cells for 2hrs before incubation with 25µg/cm^2 UICC crocidolite for 24hrs. Nuclear extracts were then isolated as described in materials and methods. 4µg of this extract was incubated with a ^{32}P-labelled AP-1 consensus oligonucleotide and the DNA AP-1 complex resolved by acrylamide gel electrophoresis. Refer to materials and methods for explanation of competitive/non-competitive lanes. Images shown are representative of at least 2 independent analyses.

4.3 DISCUSSION

Previously, work in another laboratory has shown that the JNK signalling pathway is not activated under crocidolite stimulated conditions in a primary RPM cell system (Jimenez et al., 1997). In our studies we have shown that this observation holds in MET 5A and 4/4 RM4 cells. This finding was proven by two methods of probing JNK1/2 activation, western blotting and *in vitro* kinase assay. The slight increases in JNK1 phosphorylation noted in 4/4 RM4 cells after 25μg/cm^2 UICC crocidolite exposure for 4 and 8 hrs is intriguing. Unfortunately, time did not allow further investigation of this phenomenon. The recent description of a novel selective inhibitor of the JNK pathway (Bennett et al., 2001) means that further studies could evaluate whether or not this finding has any downstream importance. Overall the lack of JNK pathway involvement in the response of mesothelial cells to asbestos is quite surprising, as it appears key to a number of cellular responses to a wide variety of stresses (Leppa and Bohman, 1999; Shukla et al., 2001; Derijard et al., 1994; Chen et al., 1996). However, it does provide some indication as to what may lie upstream of p38 activation in asbestos exposed mesothelial cells because any factors that activate both JNK1/2 *and* p38 cannot be involved. Instead SAPKK-3 (MKK/MEK6 and 6b) is more likely to be involved as this predominates in cases of p38-only activation in non-neuronal cell types (Meier et al., 1996).

The present studies have shown that the second arm of the SAPK cascade, p38 MAP kinase, is in fact, activated in mesothelial cells in a dose-dependent manner by use of western blots with antibodies against dually phosphorylated active protein and also *via* immune complex kinase assays. The latter technique is a direct measure of the actual activity of total cellular p38, and gave confirmation of the immunoblot data. The effect of fibre length is negligible in these studies. In both cell lines, these curves deviate only slightly from each other, most notably in MET 5A cells at a dose of 5μg/cm^2 where NIEHS crocidolite induced a significantly higher increase in p38 activity than its UICC counterpart.

The reason behind lack of epitope recognition by the pp38 antibody for pp38 in MET 5A cells is uncertain. The amino acid sequence of p38 around the activation motif,

which is recognised by the antibody, is highly conserved throughout different mammalian species. A number of alterations to the western blot protocol were attempted and several different batches of the antibody were tested to no avail. The idea that this pathway may be redundant in MET 5A cells was rejected following results from the *in vitro* kinase assay, which demonstrated that the activity of p38 in this cell line was very similar to that in 4/4 RM4 cells. The findings employing the selective inhibitor SB203580 further support the idea of non-recognition by the antibody, as the inhibitory effects of the compound would not be seen if the pathway was not active. The time course data indicates that the activation of p38 by asbestos is a persistent event, over a 24 hr period at least. It was unfortunate that the serum starved experimental conditions did not allow investigations beyond this time point. This procedure was used to keep the background levels of pERK1/2 low for the experiments described in the previous chapter and it was felt necessary to keep the same conditions in these studies so that these findings could all be considered on an equal basis. The parameters of p38 activation described above appear to correlate well with the activation of the ERK pathway under similar conditions in this cellular model (Zanella et al., 1996).

The mechanisms of p38 activation we have investigated reflect observations seen throughout. The *in vitro* effects of asbestos fibres appear to rely upon the generation of ROS either directly from the fibre surface (Fubini and Mollo, 1995) or indirectly through a process of frustrated phagocytosis (Kamp et al., 1992). In the *in vitro* system employed here it appears that both could be relevant as chelation of iron from the fibre surface abrogated but did not abolish p38 activation. The residual levels of p38 activation could be accounted for by the phagocytic response, which would still ensue assuming the physical properties of the fibres were unaltered by the chelation process. However, it is quite possible that not all the iron was chelated and that residual levels may be responsible for this observation. Whilst the exact source of ROS may be slightly unclear, the importance of ROS in the activation of p38 is not as both NAC and α-tocopherol could completely abrogate its activation. NAC is a cell membrane permeable precursor of glutathione, which is a major substrate for antioxidant enzymes in eukaryotic cells (Kamata and Hirata, 1999), allowing detoxification of peroxide species such as H_2O_2. Indeed, a number of these enzymes,

for example, catalase and superoxide dismutase, have previously been shown to be upregulated by asbestos (Janssen et al., 1992; Janssen et al., 1995). α-Tocopherol is a more specialised antioxidant due to its lipophilic nature. It is found in cell membranes and specifically acts to neutralise lipid peroxides that may trigger oxidant-producing chain reactions (Halliwell, 1994). These studies have shown increases in one of these lipid peroxides, 4-HNE, in asbestos exposed mesothelial cells. This finding may represent a possible mechanism whereby the stimulus from extracellular fibres may be transmitted to intracellular responses as 4-HNE can act as a signalling intermediary capable of activating stress signalling pathways such as p38 (Uchida et al., 1999). Additionally, 4-HNE has been shown to be important in vascular smooth muscle cell proliferation in atherosclerosis (Kakishita and Hattori, 2001), and it may have a similar proliferative effect following asbestos exposure. The ability of exogenous 4-HNE to modulate these signalling events is not certain from these studies and could be investigated in future work. It cannot be discounted that receptor mediated events are also upstream of p38 activation as this is a common feature in other cellular systems where stress-induced p38 activation occurs (Yuasa et al., 1998; Ichijo, 1999). Investigations into this aspect of p38 signalling would be interesting for future work, as both provide possible points for clinical intervention.

These studies have also examined the downstream effect of p38 activation on crocidolite-induced AP-1 DNA binding, a phenomenon which has previously been reported (Heintz et al., 1993; Ding et al., 1999). The data presented strongly suggest that the activation of p38 by crocidolite contributes to the formation of AP-1 complexes that can bind to consensus oligonucleotides containing the AP-1 site, as pharmacological inhibition of this pathway by SB203580 blocked the effect of asbestos on this transcription factor. Therefore, the prolonged activation of p38 may contribute to AP-1 dependent phenotypic endpoints reached in mesothelial cells exposed to asbestos. The mechanism is likely to be mediated by phosphorylation of ATF-2 enhancing its transcriptional activity and upregulating expression of the *c-jun* protooncogene. With previous studies having described increases in *c-jun* expression by asbestos (Heintz et al., 1993), it would be interesting to see if inhibition of p38 could modulate this effect. If these findings are considered alongside the effects of MEK1/2 inhibition on AP-1 DNA binding, it becomes apparent that both pathways

are required for AP-1 dependent gene transcription because inhibition of either of these pathways is sufficient to return AP-1 DNA binding to untreated control levels.

In conclusion, we have demonstrated for the first time that crocidolite can activate the p38 arm of the stress-activated protein kinase pathway and that it lies upstream of crocidolite mediated AP-1 DNA binding. The effect of crocidolite-induced p38 activation in mediating cell death is discussed in chapter 6, however, further studies are required to fully elucidate additional outcomes which may be important in the pathogenic process. The importance of the p38 pathway in controlling cytokine gene expression during inflammatory responses is discussed above and it would therefore be interesting to investigate if cytokine synthesis by mesothelial cells exposed to asbestos could be modulated by inhibition of p38. These novel findings represent a mechanism whereby mesothelial cells *in vivo* may enter a chronic inflammatory state induced by the persistent presence of asbestos fibres in the lung. In recent years chronic inflammation has emerged as a common feature in a wide array of human cancers (O'Byrne and Dalgleish, 2001) and much work has highlighted the variety of immune responses that may be elicited by asbestos (Li et al., 1993; Luster and Simeonova, 1998; Mongan et al., 2000; Simeonova and Luster, 1996).

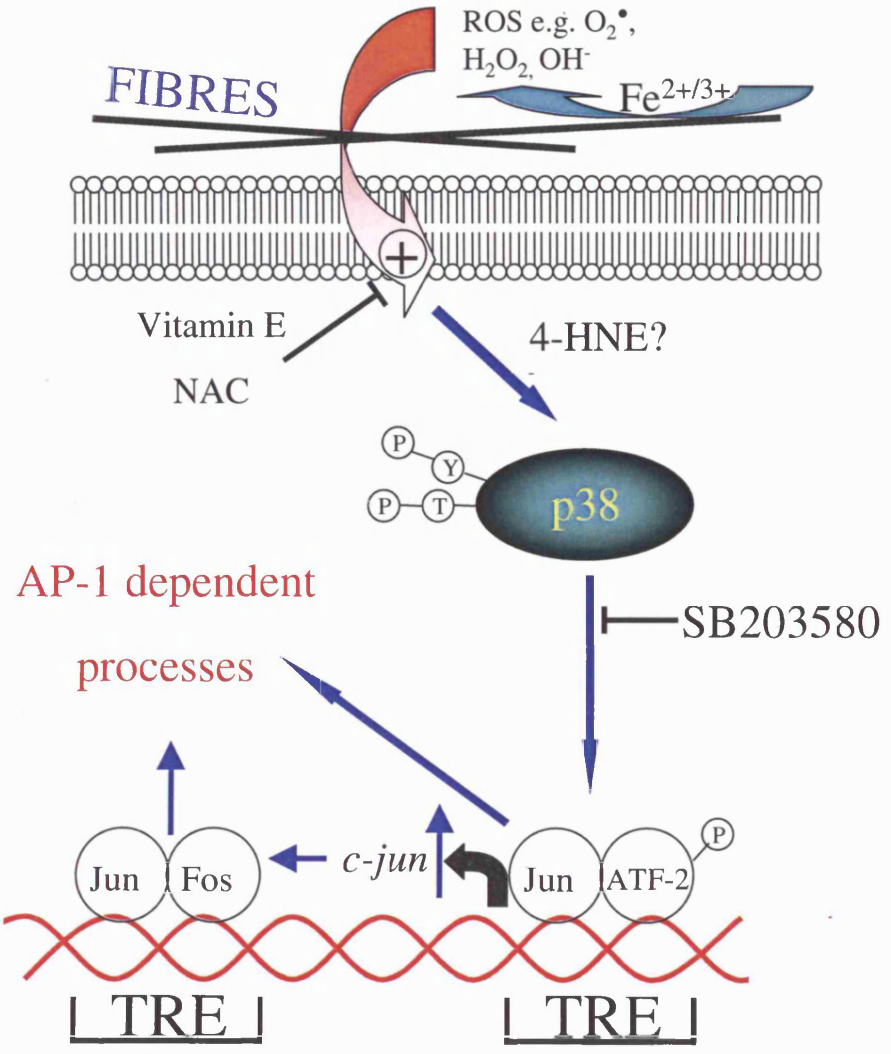


Figure 4.17 Proposed scheme of p38 pathway and AP-1 activation by crocidolite.

CHAPTER 5

EFFECTS OF CROCIDOLITE ASBESTOS ON THE Akt PATHWAY IN MESOTHELIAL CELLS

CHAPTER 5

5.1 INTRODUCTION

As discussed in chapter 1, Akt lies downstream of EGFR, in addition to the ERK pathway. Overall, Akt signalling appears to have a role in the cytoprotection of cells exposed to stressful conditions which would otherwise lead to apoptosis (Downward, 1998a). Therefore, in cases of aberrant control of Akt signalling, cells that have accumulated oncogenic mutations may survive and enter the multistage carcinogenesis paradigm (Maehama and Dixon, 1999).

Downstream effectors of Akt are very diverse and reflect its numerous functions. Of particular importance to cell survival is the effect of Akt activation on NF-κB signalling. The transcriptional activity of NF-κB can be controlled in two ways. The first of which classically involves phosphorylation of IκB on critical serine residues by the IKK signalsome complex (DiDonato et al., 1996). Phosphorylation of IκB causes it to disassociate from NF-κB and enter the ubiquitin-dependent degradation process thus allowing NF-κB to translocate to the nucleus and upregulate the transcription of NF-κB-responsive genes (Ghosh et al., 1998; Baldwin, Jr., 1996). Secondly, NF-κB dependent gene expression can be modulated by stimulation of the p65/RelA subunit without altering translocation of NF-κB, a process termed *trans*activation (Madrid et al., 2000). This function is performed by a distinct group of kinases, one of which is thought to be p38 (Vanden Berghe et al., 1998) that is, itself, regulated by Akt and IKKα/β (Madrid et al., 2001). In common with AP-1, NF-κB is a transcription factor whose activity is increased in times of cellular stress to upregulate the expression of genes important in enabling the cell to respond to these conditions (Mercurio and Manning, 1999). There are, therefore, numerous outcomes following transient or persistent NF-κB activation. A common feature however, is the contribution to inflammatory processes through enhanced expression of key cytokines (Makarov, 2000). As discussed previously, chronic immune activation may be intrinsic to a number of cancers and several studies have demonstrated that dysregulation of NF-κB activity in these situations may be important in carcinogenesis and resistance to chemotherapy (Wang et al., 1996).

CHAPTER 5

Previous studies have shown that asbestos induces nuclear accumulation of NF-κB, a phenomenon first noted in hamster tracheal epithelial cells that was shown to be dependent on oxidative stress (Janssen et al., 1995). Additionally, it was shown that these conditions could increase the expression of an NF-κB dependent luciferase reporter construct (Janssen et al., 1995). Further studies from the same laboratory showed that the same was also true in rat pleural mesothelial cells both *in vitro* and *in vivo* (Janssen et al., 1997). To date, no studies have addressed the exact mechanism responsible for this other than its dependence on oxidative stress and lipid peroxidation (Faux and Howden, 1997). The present studies sought to investigate the series of cellular signalling events that are responsible for NF-κB translocation following asbestos exposure. In particular, the relative importance of the EGFR/PI3K/Akt signalling module in reaching this endpoint was ascertained.

5.2 RESULTS

5.2.1 H_2O_2 stimulates Akt activity in MET 5A cells

Following research from other laboratories, H_2O_2 was chosen as a positive control for Akt activity. Due to the novelty of these studies, initial experiments were used to find optimal dose and times for H_2O_2-induced Akt activity in mesothelial cells. Initially, 300 or 500μM H_2O_2 was added to subconfluent cultures of MET 5A cells for 5, 15, 30 or 60 mins. Figure 5.1 illustrates the transient nature of Akt activation by H_2O_2 and following these results a dose of 500μM H_2O_2 for 15 mins was chosen as a positive control.

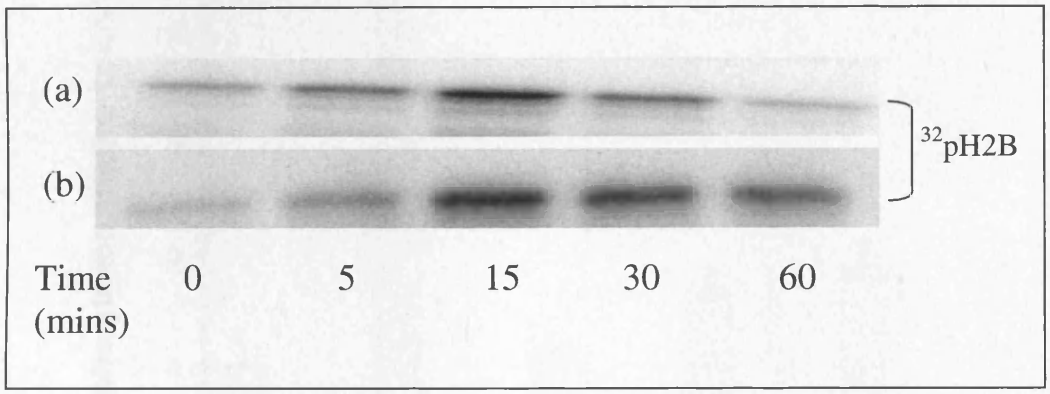


Figure 5.1 Effect of H_2O_2 on Akt activity as determined by *in vitro* kinase assay. MET5A cells were serum starved for 24hrs before incubation with (a) 300μM or (b) 500μM H_2O_2 for times indicated. Cells were then lysed and Akt was immunoprecipitated from equal amounts of protein of each sample. Akt activity was measured by the ability of the purified kinase to phosphorylate histone H2B substrate in an *in vitro* kinase assay. Blots shown are representative of two experiments.

5.2.2 Crocidolite activates Akt in MET 5A cells

Although Akt requires phosphorylation on a number of sites for activation (Huang et al., 2001) phosphorylation at ser^{473} was probed in early experiments. Initial experiments showed no effect of crocidolite on pAkt levels after 24 hrs, however, previous studies had demonstrated maximal NF-κB translocation at 8 hrs (Janssen et al., 1997). It was therefore hypothesised that increases in Akt phosphorylation by crocidolite could be a transient effect and because this event was thought to be

upstream of NF-κB activation experiments were repeated with just 6 hrs of crocidolite exposure. Figure 5.2 shows that crocidolite causes dose-dependent increases in phosphorylation at ser^{473} at this time point and that this increase is significant at 10 and 25μg/cm^2 ($p<0.05$). To ensure this finding was not due to upregulation of Akt expression or errors in protein loading, blots were reprobed for native Akt, which showed that levels remained unchanged throughout. In each experiment H_2O_2 elicited a more potent response than crocidolite.

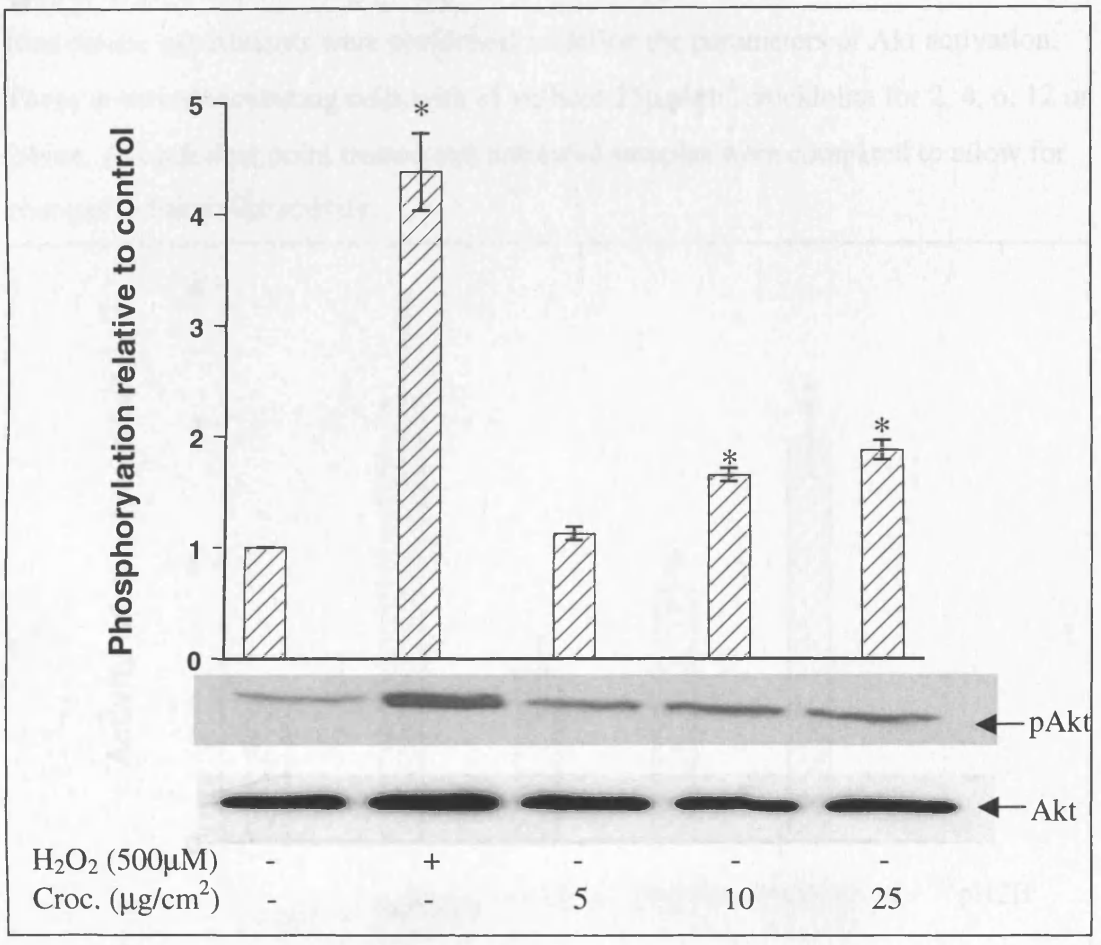


Figure 5.2 Effect of UICC crocidolite on Akt phosphorylation as determined by western blotting. MET5A cells were serum starved for 24hrs prior to treatment for 6hrs with the indicated concentrations of UICC crocidolite. In each case a 15 min treatment with H_2O_2 (500μM) was included as a positive control. Cells were then lysed and the proteins separated by SDS PAGE. The proteins were transferred onto nitrocellulose membrane and the resultant filters immunoblotted with anti-pAkt antibody. Bands were visualised by chemiluminescence. Blots were then stripped and reprobed for Akt. Blots shown are representative of three experiments. The data from three separate experiments were quantified by densitometry and the means +/- S.E.M plotted. * $p<0.05$

Having established that there was a change in phosphorylation status of Akt following crocidolite exposure, *in vitro* kinase assays were performed to examine whether or not these increases were reflected in the activity of Akt. Figure 5.3 shows a dose-dependent increase in actual Akt activity compared to untreated samples at this time point (6hrs), which was significant at 10 and 25μg/cm^2 crocidolite ($p<0.05$). In these studies it was not possible to probe the residues involved in Akt activation by western blotting simultaneously due to their distance from each other (165 amino acids apart). Therefore *in vitro* kinase assays were used henceforth to assess Akt activity. Taken together, these results strongly suggest the above hypothesis to be correct, however, time course experiments were performed to define the parameters of Akt activation. These involved incubating cells with or without 25μg/cm^2 crocidolite for 2, 4, 6, 12 or 24 hrs. At each time point treated and untreated samples were compared to allow for changes in basal Akt activity.

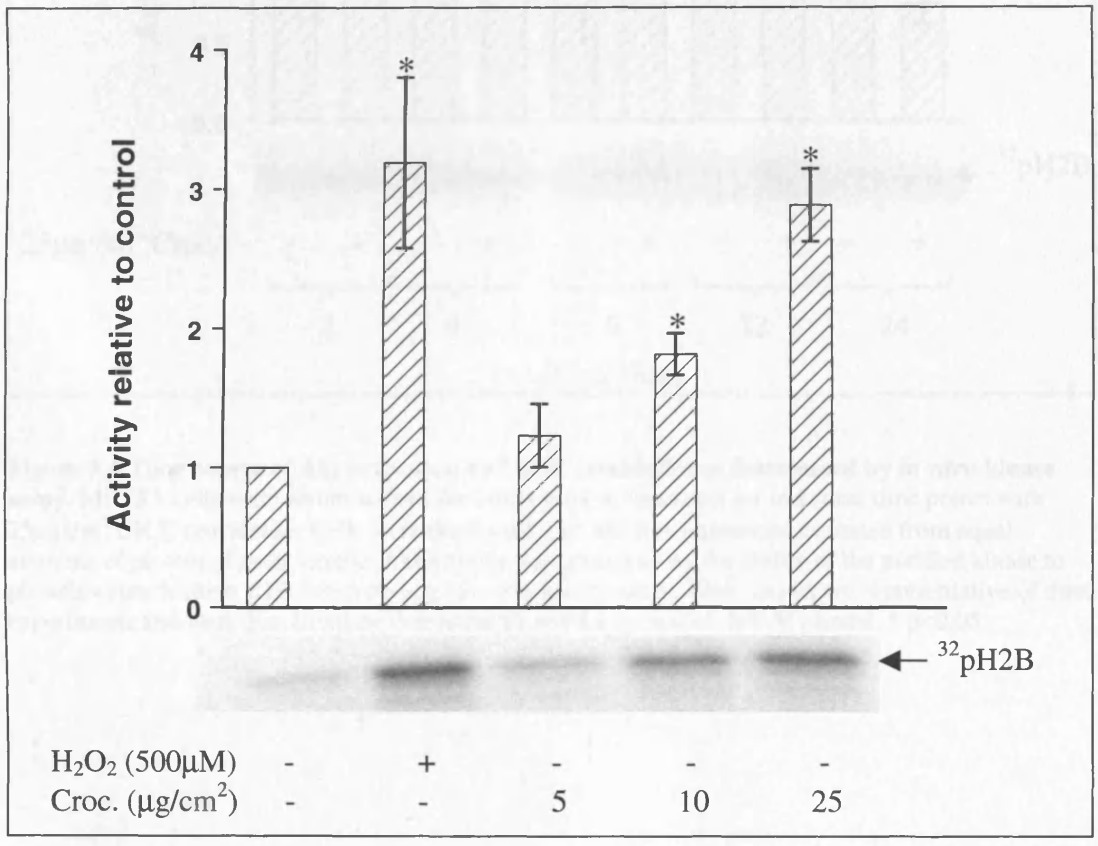


Figure 5.3 Effect of UICC crocidolite on Akt activity as determined by *in vitro* kinase assay. MET 5A cells were serum starved for 24hrs prior to treatment for 6hrs with the indicated concentrations of UICC crocidolite. In each case a 15 min treatment with H_2O_2 (500μM) was included as a positive control. Cells were then lysed and Akt was immunoprecipitated from equal amounts of protein of each sample. Akt activity was measured by the ability of the purified kinase to phosphorylate histone H2B substrate in an *in vitro* kinase assay. Image shown is representative of three experiments and these were quantified by densitometry and the means +/- S.E.M plotted. * $p<0.05$

The activation of Akt was shown to be a transient event with the peak at 6hrs (Figure 5.4), which proved the above hypothesis to be correct.

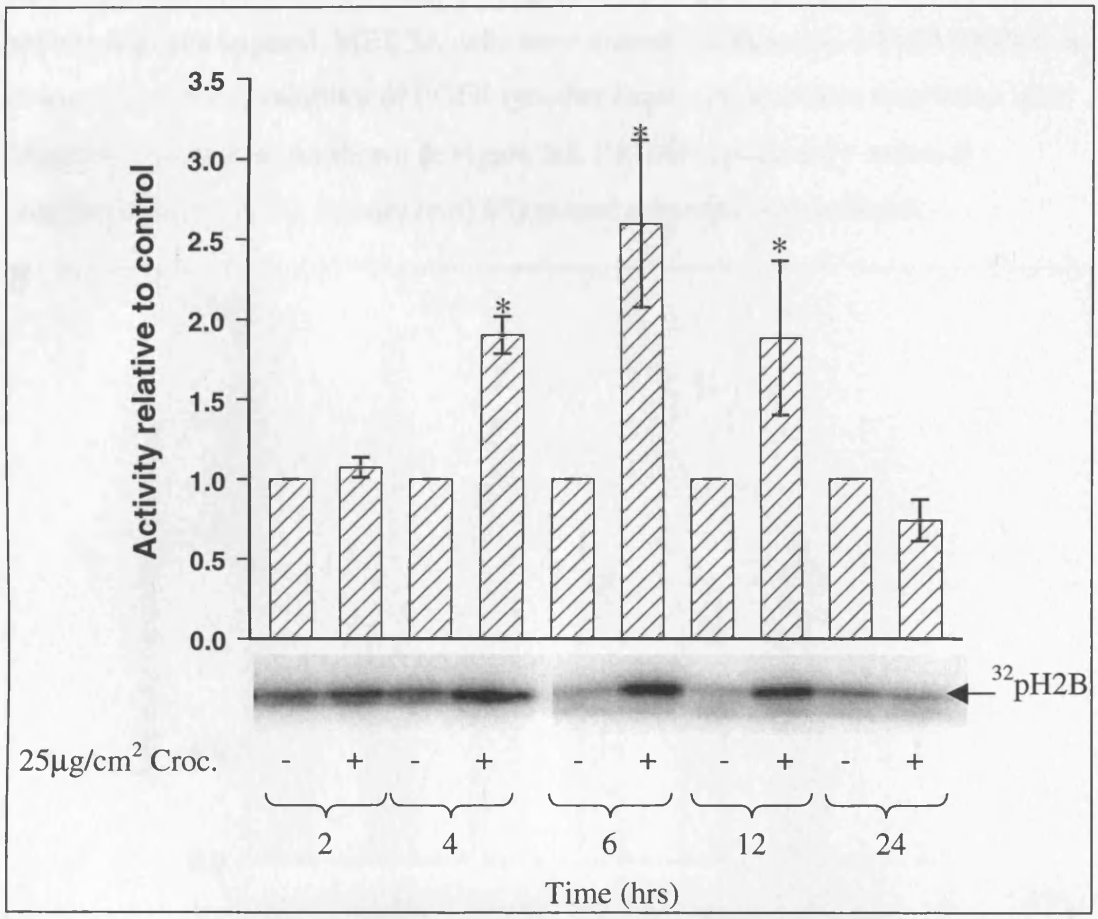


Figure 5.4 Time course of Akt activation by UICC crocidolite as determined by *in vitro* kinase assay. MET5A cells were serum starved for 24hrs prior to treatment for indicated time points with 25µg/cm^2 UICC crocidolite. Cells were then lysed and Akt was immunoprecipitated from equal amounts of protein of each sample. Akt activity was measured by the ability of the purified kinase to phosphorylate histone H2B substrate in an *in vitro* kinase assay. Blots shown are representative of three experiments and were quantified by densitometry and the means +/- S.E.M plotted. * $p<0.05$

5.2.3 Mechanism of Akt activation by crocidolite

Having strong evidence for activation of Akt in this system the mechanism behind its regulation was examined. Initially, the importance of upstream EGFR tyrosine kinase activity was investigated. MET 5A cells were treated for 2hrs with 100nM PKI166, a potent and selective inhibitor of EGFR tyrosine kinase, prior to 6hrs incubation with 25μg/cm^2 crocidolite. As shown in Figure 5.5, PKI166 significantly reduced crocidolite-induced Akt activity ($p<0.05$) to near untreated control levels.

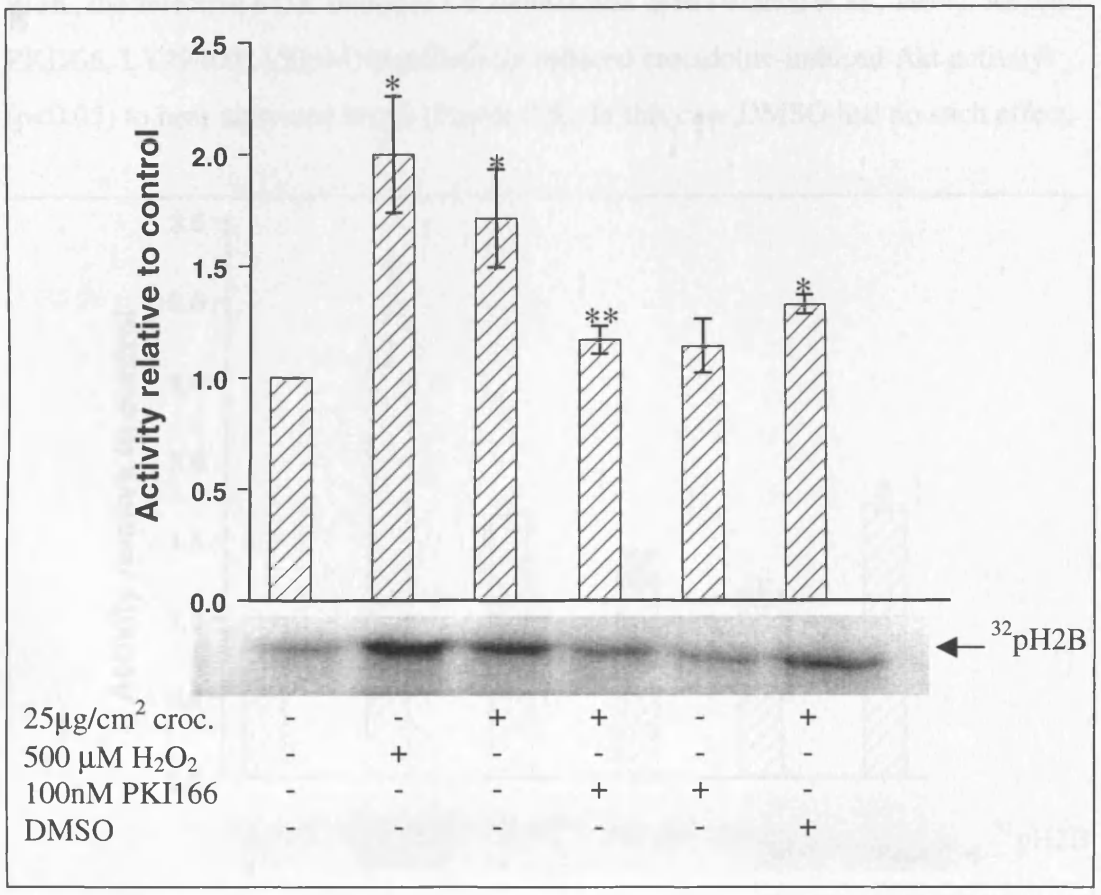


Figure 5.5 Effect of PKI166 pretreatment on the ability of UICC crocidolite to induce Akt activity as determined by *in vitro* kinase assay. MET5A cells were serum starved for 24hrs before 2hrs incubation with 100nM PKI166, then 25μg/cm^2 UICC crocidolite for 6hrs. H_2O_2 (500 μM) was used as a positive control. Cells were then lysed and Akt was immunoprecipitated from equal amounts of protein of each sample. Akt activity was measured by the ability of the purified kinase to phosphorylate histone H2B substrate in an *in vitro* kinase assay. Blots shown are representative of three experiments and were quantified by densitometry and the means +/- S.E.M plotted. * $p<0.05$ with respect to untreated control. ** $p<0.05$ with respect to UICC crocidolite alone.

This data also suggests an inhibitory effect of DMSO on Akt activity, although this was not significant and may occur through its antioxidant properties. The level of Akt

activity with crocidolite and DMSO was significantly higher ($p<0.05$) than observed with crocidolite and PKI166. To further support this hypothesis the effect of DMSO in the experiment below, where the volume of DMSO added was actually higher, is negligible. The apparent lack of effect of PKI166 in the absence of crocidolite suggests EGFR tyrosine kinase activity does not play a significant role in maintaining basal Akt activity.

To discover whether or not crocidolite-induced Akt activity was dependent upon PI3K, the selective PI3K inhibitor LY294002 was used (Vlahos et al., 1994). As with PKI166, LY294002 (50μM) significantly reduced crocidolite-induced Akt activity ($p<0.05$) to near untreated levels (Figure 5.6). In this case DMSO had no such effect.

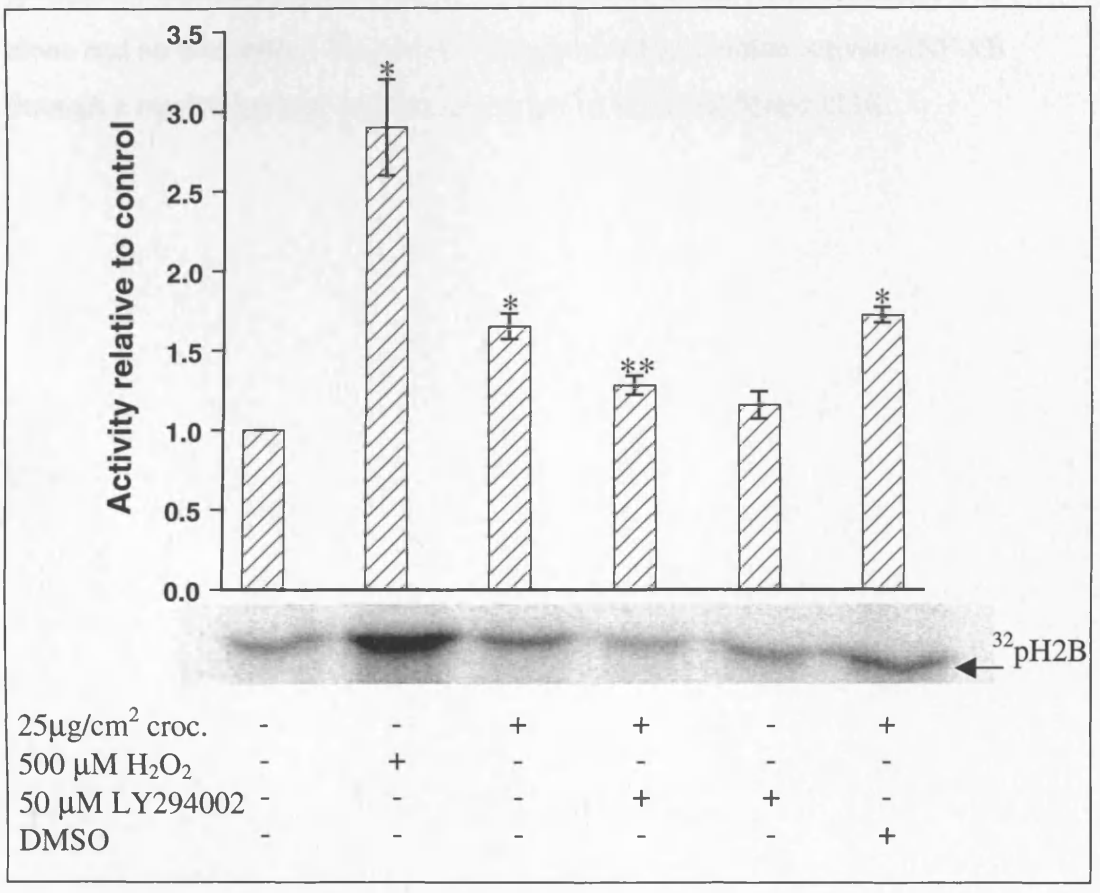


Figure 5.6 Effect of LY294002 pretreatment on the ability of UICC crocidolite to induce Akt activity as determined by *in vitro* kinase assay. MET5A cells were serum starved for 24hrs before 2hrs incubation with 50μM LY294002, then 25μg/cm^2 UICC crocidolite for 6hrs. H_2O_2 (500 μM) was used as a positive control. Cells were then lysed and Akt was immunoprecipitated from equal amounts of protein of each sample. Akt activity was measured by the ability of the purified kinase to phosphorylate histone H2B substrate in an *in vitro* kinase assay. Blots shown are representative of three experiments and were quantified by densitometry and the means +/- S.E.M plotted. * $p<0.05$ with respect to untreated control. ** $p<0.05$ with respect to crocidolite.

5.2.4 Effect of the EGFR/PI3K pathway on crocidolite-induced NF-κB DNA binding

Having established that activation of Akt by crocidolite was dependent on EGFR tyrosine kinase activity and PI3K, subsequent studies were designed to elucidate their role in NF-κB DNA binding following crocidolite exposure. Nuclear protein extracts were isolated from MET 5A cells treated with crocidolite in the presence and absence of the selective EGFR tyrosine kinase inhibitor PKI166, the PI3K inhibitor LY294002 or DMSO as a vehicle control. Using gel mobility shift assays crocidolite was shown to increase the DNA binding of NF-κB to a consensus oligonucleotide above control incubations after 8hrs of exposure (Figure 5.7). Inhibition of PI3K or EGFR tyrosine kinase activity by LY294002 (Figure 5.7a) or PKI166 (Figure 5.8b) respectively, prior to asbestos exposure reduced its ability to increase NF-κB DNA binding. DMSO alone had no such effect. These results suggest that crocidolite activates NF-κB through a mechanism that requires activation of both EGFR and PI3K.

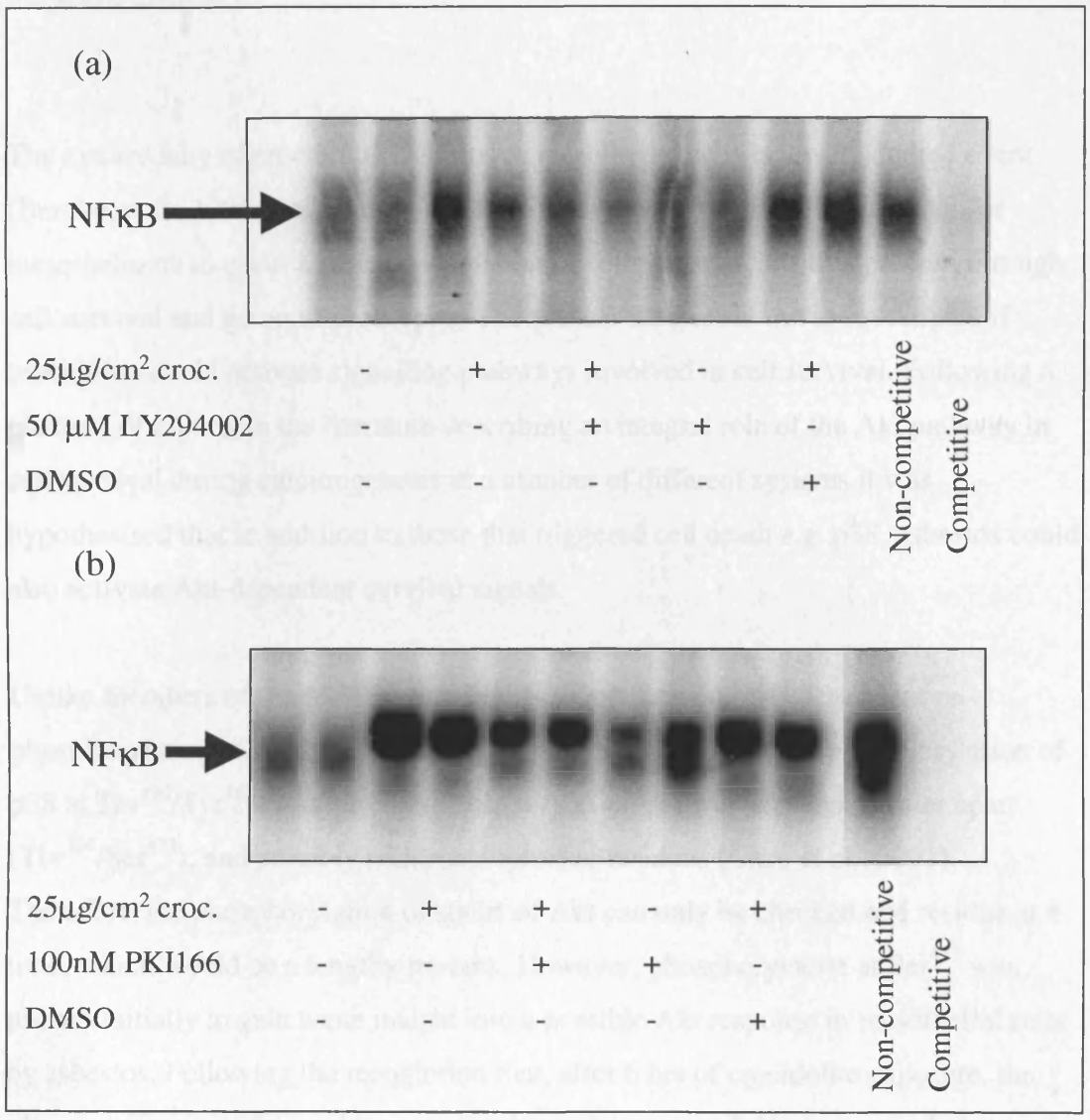


Figure 5.7 PKI166 and LY294002 ameliorate the ability of UICC crocidolite to induce NFκB translocation and DNA binding. MET5A cells were grown to 90% confluency before serum starvation for 24hrs. PKI166/LY294002 was added to cells for 2hrs before incubation with 25μg/cm^2 UICC crocidolite for 8hrs. Nuclear extracts were then isolated as described in materials and methods. 4μg of this extract was incubated with a 32P-labeled NF-κB consensus oligonucleotide and the DNA NF-κB complex resolved by acrylamide gel electrophoresis. 'Cold' NF-κB was used to compete out the band of interest and 'cold' AP-1 shows the interaction is specific for the NF-κB consensus sequence. Images shown are representative of at least 2 independent repeats.

5.3 DISCUSSION

The cytotoxicity of crocidolite asbestos to mesothelial cells is a well studied event (Berube et al., 1996; Broaddus et al., 1996), however, it is clear that in order for mesothelioma to occur a certain population of cells must escape this process through cell survival and go on to proliferate. The present studies set out to investigate if crocidolite could activate signalling pathways involved in cell survival. Following a plethora of reports in the literature describing an integral role of the Akt pathway in cell survival during carcinogenesis in a number of different systems it was hypothesised that in addition to those that triggered cell death *e.g.* p38, asbestos could also activate Akt-dependent survival signals.

Unlike members of the MAP kinase family whose activation is dependent on phosphorylation of residues in close proximity to each other (*e.g.* phosphorylation of p38 at Thr^{180}/Tyr^{182}) Akt requires phosphorylation at residues much further apart (Thr^{308}/Ser^{473}), and possibly additional tyrosine residues (Chen et al., 2001). Therefore, the phosphorylation of status of Akt can only be checked one residue at a time, which would be a lengthy process. However, phosphorylation at Ser^{473} was probed initially to gain some insight into a possible Akt response in mesothelial cells by asbestos. Following the recognition that, after 6 hrs of crocidolite exposure, the phosphorylation of this residue was significantly increased, kinase assays were used to obtain a more complete picture of the activation status of Akt under these conditions. These assays yielded similar results, although they did show a higher level of Akt activity (approximately three fold) than suggested by western blots (approximately two fold), which shows the importance of factors other than phosphorylation at Ser^{473}. In addition, the *in vitro* kinase assays avoided the need to probe multiple residues for each sample and were chosen for the remaining investigations.

The initial failure to note any increases in pAkt after 24 hrs of asbestos exposure had indicated that any modulations in Akt activity would be transient and this was proven in a series of experiments designed to measure crocidolite-induced Akt activity over a 24 hr time period. This observation raises some important questions because increases

in downstream NF-κB DNA binding by crocidolite was shown still to be rising after 24 hrs of exposure in previous studies (Janssen et al., 1997; Faux and Howden, 1997). This finding could mean that Akt activation is a trigger in terms of NF-κB activation and additional factors are necessary to maintain the response but it also suggests the activation of factors that negatively regulate Akt activity. A possible candidate is the tumour suppressor, PTEN, which is deleted in a number of tumours (Li et al., 1997; Steck et al., 1997). This phosphatase enzyme essentially antagonises the role of PI3K by dephosphorylating phosphatidylinositol species at the 3 position of the inositol ring (Maehama and Dixon, 1998). Therefore, PTEN should be a target in future studies both in asbestos stimulated mesothelial cells and mesothelioma tumour samples.

The importance of EGFR tyrosine kinase and PI3K activity in crocidolite-induced Akt activation was deduced by use of selective inhibitors of these kinases. The ability of PKI166 to abrogate ERK1/2 activation by crocidolite has already been discussed. The studies presented here show that this is not the sole function of EGFR activation as Akt activity was affected in a similar way. This finding puts EGFR in a key position for the propagation of two divergent signalling pathways leading to the activation of two separate transcription factors, AP-1 and NF-κB, and as shown in chapter 6 the promotion of cell survival. The results of the present studies suggest that upregulation of EGFR and concomitant increases in EGFR-dependent signalling may be key to the development of mesothelioma and as such would be a useful target in future therapies. Previously, levels of EGFR expression have been shown to be strong in both malignant mesothelioma and non-neoplastic human tissue, although the three subtypes of mesothelioma (sarcomatoid, epithelial and mixed cell) did display significantly different levels of expression (Ramael et al., 1991).

Downstream of EGFR, Akt activity is mediated through PI3K and PDKs (Downward, 1998a). Using the selective inhibitor of PI3K, LY294002, alongside crocidolite exposure the results of the present studies suggest that PI3K is activated by asbestos, a novel finding in itself. Akt activation is not the only role for PI3K. A number of additional cellular processes are regulated by PI3K. One of these is PKC, certain isoforms of which have been shown to be activated in cells transfected with constitutively active PI3K (Moriya et al., 1996; Akimoto et al., 1996). Due to the wide

range of intracellular processes influenced by PKC (Yatomi et al., 1992) the importance of PI3K activation is further underlined. Previously, inhibition of PKC has been shown to significantly reduce asbestos-induced expression of *c-jun* and *c-fos* mRNA (Fung et al., 1997b). Therefore, it would be interesting to see if PI3K played a part upstream in this system. All kinase-dependent signalling pathways require antagonistic phosphatases for proper regulation. PI3K has been shown to regulate the activity of two of these: protein phosphatases-1 (PP1) and protein phosphatases-2A (PP2A). The findings that asbestos is capable of modulating PI3K function may have implications on global cellular signalling and as such is a worthy target for future research (Ragolia et al., 1997; Begum and Ragolia, 1996).

Due to time constraints it was not possible to investigate the effect of NAC on crocidolite-induced Akt activity and this could be examined in future studies. It seems highly likely that these investigations would show an oxidative stress dependency of Akt activation due to the hypothesised redox regulation of upstream EGFR activation. Additionally, experiments involving non-fibrous analogues were omitted but again it is expected that a similar effect would be noted as described for the p38 and ERK1/2 pathways *i.e.* non-specific effects of bio-available iron due to increased surface area.

The downstream effects of the EGFR/PI3K/Akt signalling module on the transcription factor NF-κB were investigated using selective inhibitors. Both LY294002 and PKI166 were capable of reducing crocidolite-induced NF-κB DNA binding to near untreated control levels, which shows for the first time a link between EGFR autophosphorylation, PI3K and NF-κB in mesothelial cells when stimulated with asbestos. The transcription factor, NF-κB, has been implicated in the regulation of gene expression related to the inhibition of apoptosis by the up-regulation of anti-apoptotic genes (Van Antwerp et al., 1996; Wang et al., 1996). Recently, the NF-κB cascade has been shown to be important in Bcl-x expression and for the anti-apoptotic effects in primary human lymphocytes (Khoshnan et al., 2000). Furthermore Bcl-x is down-regulated in cells undergoing apoptosis (Arriola et al., 1999). The cell signalling pathways following autophosphorylation of EGFR leading to the activation of NF-κB are complex and may involve crosstalk between a number of different mediators. Recently in the lung epithelium the MAP kinase cascade has been shown

to regulate NF-κB through a Ras-dependent pathway involving ROS, reactive nitrogen species and a Ras-independent pathway involving TNF-α (Janssen-Heininger et al., 1999). Specifically, inhibition of the MEK-ERK pathway activates NF-κB (Janssen-Heininger et al., 1999) whereas the MEKK-JNK/p38 pathways (Janssen-Heininger et al., 1999; Carter et al., 1999; Craig et al., 2000) enhance the activation of NF-κB. However, differences have been observed in different cell types with respect to the specific MAP kinase pathways that are involved. It is therefore important to further investigate the signalling pathways that are involved in the activation of NF-κB. In particular the contribution of different MAP kinase pathways in the activation of NF-κB in mesothelial cells treated with asbestos should be studied to obtain a clearer understanding of the involvement of these signalling cascades in the development and pathogenesis mesothelioma. The direct involvement of Akt in NF-κB regulation in this system can only be inferred at this stage due to the current lack of selective inhibitors for this activity and the inability to transfect MET 5A cells.

In summary, these results show that the induction of NF-κB by crocidolite asbestos in mesothelial cells is initiated through a signalling pathway linked to autophosphorylation of the EGFR and PI3K activity. Additionally, the activation of Akt by asbestos is described and is also reliant upon EGFR and PI3K. Akt may be a key player in linking the transduction of the survival signal from the cell membrane to the cytoplasm, before the activation of NF-κB. The effect of this signalling process on phenotypic endpoint responses is discussed at length in chapter 6.

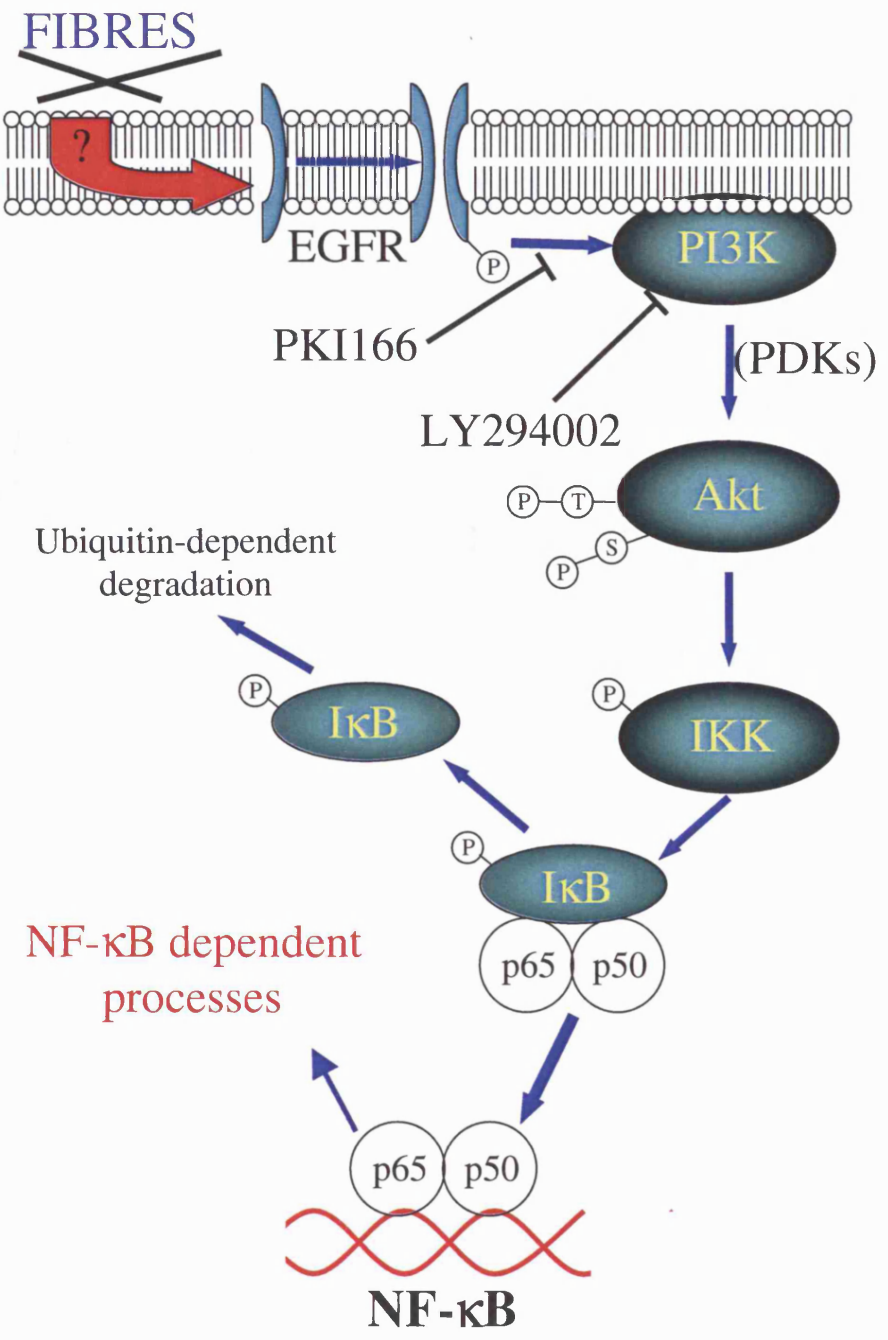


Figure 5.8 Proposed scheme of crocidolite-induced NF-κB activation via the EGFR/PI3K/Akt pathway.

CHAPTER 6

EFFECTS OF CROCIDOLITE-INDUCED CELL SIGNALLING ON CYTOTOXICITY AND APOPTOSIS IN MESOTHELIAL CELLS

CHAPTER 6

6.1 INTRODUCTION

The activation of cell signalling pathways described in the preceding chapters by asbestos may lead to an array of endpoints, ranging from the modulation of gene expression to the commitment of the cell to apoptosis and these may be intrinsically linked. In cancer research the study of the balance between cell death and proliferation is vital, as dysregulation in either of these processes may be a vital step in carcinogenesis (Kaufmann and Gores, 2000; Kerr et al., 1994). Apoptosis is a mechanism that enables cells to commit suicide either as part of tissue remodelling processes or in conditions where they may have been damaged in such a way as to have gained mutagenic potential (Kerr et al., 1972; Alison and Sarraf, 1995). The involvement of signalling pathways in both the initiation and inhibition of apoptosis is well reported (Xia et al., 1995; Roberts et al., 2000; Saldeen et al., 2001). The exact sequence of events will depend upon the context of the signal and also the cell type involved, but investigating the role signalling pathways play in defined pathogenic systems may aid our understanding of early stages in the disease process and highlight possible targets for pharmacological intervention (Makin and Dive, 2001).

Mesothelial cells *in vitro* demonstrate a characteristic response to crocidolite insult. Initially, fibres are cytotoxic by their ability to induce the apoptotic machinery (Berube et al., 1996), although it is likely that some necrosis will also occur. The apoptotic process in this system has since been shown to be dependent on oxidative stress (Broaddus et al., 1996) and as a consequence of ERK pathway activation (Jimenez et al., 1997). More recently it has been suggested that induction of *c-fos* expression by activation of the ERK cascade is a key factor, most probably through modulation of a subset of AP-1 dependent genes (Zanella et al., 1999) because the particular composition of the AP-1 complex, in terms of Jun:Jun/Fos:Jun makeup, may influence its affinity for certain promoters (Angel and Karin, 1991). These results are interesting because they are in contrast to widely reported observations in the literature that the ERK pathway is involved in growth factor-dependent survival and/or proliferation in conditions that may otherwise induce apoptosis (Berra et al., 1998; Stadheim and Kucera, 1998).

In contrast to the role of ERK, the SAPK arms of the MAPK family are mostly linked to signalling events that favour the entry of the cell into the apoptotic process (Xia et al., 1995), although there are also exceptions to this rule under certain conditions (Roulston et al., 1998). The predominance of the SAPK pathways in cell death-inducing conditions arises from the range of factors that stimulate their activation *e.g.* UV irradiation, osmotic shock and proinflammatory cytokines (Chen et al., 1996). In these studies the JNK pathway was shown not to be active under asbestos stimulated conditions, whereas the opposite was true with p38. The exact mechanism whereby p38 promotes apoptosis is largely unclear, although associated downstream activation of caspases is likely to be involved (Cheng et al., 2001a; Lee et al., 2002; Zou et al., 2002). Additionally, the activation of the p38 pathway may affect apoptosis by a more indirect mechanism involving the alteration of gene expression through modulation of the transcriptional activity of key factors such as AP-1 (Guo et al., 2001).

The Akt pathway is also crucial in the life or death decision-making process in cellular systems. Akt signalling appears to have a role in the cytoprotection of cells exposed to stressful conditions, which would otherwise lead to apoptosis (Downward, 1998a). In cases of dysregulation of Akt signalling cells that have accumulated oncogenic mutations will survive and enter the multistage carcinogenesis paradigm (Maehama and Dixon, 1999). This phenomenon may occur through a number of different mechanisms including phosphorylation and inactivation of proapoptotic bcl-2 family members such as Bad (Peruzzi et al., 1999). Alternatively, Akt may phosphorylate caspase 9, which leads to a reduction in its protease activity (Cardone et al., 1998). Akt can also influence the activation and nuclear translocation of NF-κB, which in turn upregulates the transcription of many survival factors and this is discussed more fully in chapter 5.

Caspases are intimately involved in reaching apoptotic endpoints and may be activated through intrinsic or extrinsic pathways as discussed in chapter 1. Both of these pathways converge downstream on executioner caspases, such as caspase 3, which modulate their downstream targets by proteolysis *e.g.* caspase 3 can inactivate the antiapoptotic protein Akt (Widmann et al., 1998).

CHAPTER 6

In addition to the characterisation of the apoptotic responses of rat mesothelial cells to asbestos, investigators have previously examined apoptosis in mesothelioma cell lines. These studies have demonstrated that mesothelioma cell lines were more resistant to apoptosis induced by both oxidant (asbestos, H_2O_2) and nonoxidant stimuli (calcium ionophore) when compared to rabbit primary mesothelial cells. The resistance to apoptosis, however, was shown not to be associated with overexpression of Bcl 2, and they conclude that other factors must be involved (Narasimhan et al., 1998).

The importance of these signalling pathways in the attainment of cytotoxic endpoints was the focus of this section of work. Initially, effects on overall cell viability were tested using MTT assays in asbestos stimulated conditions alone and then in the presence of selective pharmacological inhibitors of the pathways outlined above. Due to the difficulty in employing FACS techniques for analysing fibre-exposed cells, the activity of caspase 3 under similar conditions as above was used as a marker of apoptosis.

6.2 RESULTS

6.2.1 Effect of fibre length on crocidolite-induced cytotoxicity

The importance of fibre geometry in the ability of crocidolite asbestos to exert its pathological effects has been described previously (Donaldson et al., 1993). In the initial experiments in the present studies, 4/4 RM4 and MET 5A cells were exposed to comparable doses (mass/mass) of UICC, NIEHS and milled crocidolite. To gauge the level of cytotoxicity/cell viability, MTT assays were performed. In all cases the viability of untreated cells was assumed as 100%.

For these initial experiments, comparable doses and times to cell signalling investigations were used *i.e.* 5, 10 and 25μg/cm^2 for 24hrs. UICC, NIEHS and milled preparations were used to enable crude comparisons between particles having different aspect ratios. As shown in Figure 6.1, all particles were capable of reducing cell viability in a dose-dependent manner in both 4/4 RM4 and MET 5A cells. In rat cells these decreases were significant at all doses tested irrespective of particle used (Figure 6.1a). However, this data does suggest that NIEHS is more cytotoxic than either UICC or milled crocidolite, which have similar cytotoxic effects. This observation is reflected in the mean cell viability values at the highest dose tested (25μg/cm^2), with NIEHS causing a 40% reduction, UICC 20% and milled 18%. Whilst MET 5A cells display a similar overall dose response pattern to the effects of these particles, the difference between each of them is much lower (Figure 6.1b). The difference in dose-response curves between the cell lines is rather intriguing, whereas there is an initially steep drop followed by a gradual decrease in cell viability in 4/4 RM4 cells, MET 5A cells display resistance to the asbestos insult at 5μg/cm^2, but at higher doses the curve drops more steeply.

Having established the cytotoxic effects of crocidolite in these cell lines, the importance of the p38, ERK and PI3K/Akt pathways in reaching this endpoint was investigated. For these experiments a similar approach to that used in examining the effect of these pathways on transcription factors was adopted. In these studies, selective inhibitors of the aforementioned signalling pathways were added 2 hrs before the addition of crocidolite, or alone to gauge their effects under non-stimulated conditions.

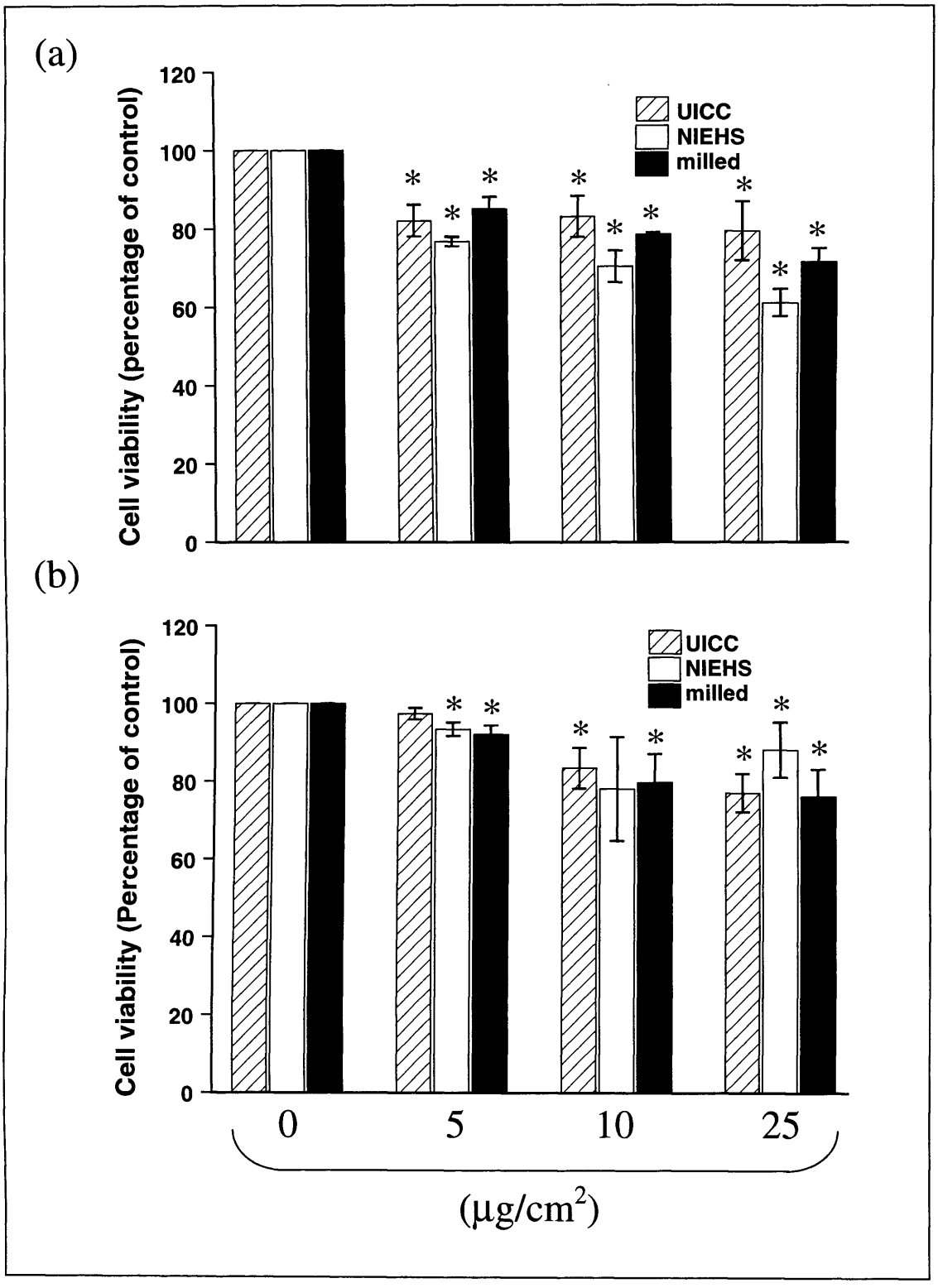


Figure 6.1 Effect of UICC/NIEHS/milled crocidolite on cell viability as determined by MTT assay. $3x10^4$ 4/4 RM4 (a) or MET5A cells (b) in 96 well plates were serum starved for 24 hrs prior to treatment for 24 hrs with the indicated concentrations of UICC (hatched bars), NIEHS (open bars) or milled (filled bars) crocidolite. 100μg MTT was then added to each well for 1 hr at 37°C. Following media removal, the formazan dye product was dissolved in DMSO and the absorbance of each well was read at 540nm. Means +/- S.E.M are plotted, n=3. *=$p<0.05$ when compared to untreated control.

6.2.2 Role of ERK in crocidolite-induced cytotoxicity

Initially, the role of the ERK pathway was investigated as this pathway has been shown previously to be involved in crocidolite-induced apoptosis in primary cultures of rat mesothelial cells. In previous experiments outlined in the thesis MET 5A cells had broadly followed the same kind of response patterns as 4/4 RM4 cells, this was not the case these cytotoxicity studies. Figure 6.2a shows the response of both cell lines to increasing doses of the MEK inhibitor, UO126. It is clear that the response of these cell lines is quite different; in fact they are quite opposite. In 4/4 RM4 cells, inhibition of the ERK pathway by UO126 seems to disengage whatever is preventing them from dividing, as the increase in absorbance shown (~45%) implies an increase in cell number (Figure 6.2a). Under asbestos exposed conditions this compound returns cell viability to near untreated control levels at the lowest dose tested, higher doses increased this still further, to a maximum of ~150% (Figure 6.2b). This finding was not too surprising as it agrees with published studies (Jimenez et al., 1997). The real surprise was the response in the MET 5A cell line. UO126 alone decreased the cell viability in a dose-dependent manner, to about 75% at 10μM UO126 (Figure 6.2a). Under crocidolite-exposed conditions, the effect of UO126 followed a similar pattern, whilst doses of 1 and 5μM had little effect, 10μM was capable of significantly ($p<0.05$) reducing cell viability compared to crocidolite alone (Figure 6.2b). In all cases DMSO alone had no significant effect.

These results are fascinating because they highlight perfectly how important cellular context can be in signal processing. Up to this point the activation of the ERK pathway by asbestos has produced no major differences between the two cell lines, both mechanistically and kinetically. However, the downstream signal obviously has completely different effectors in the biological responses produced.

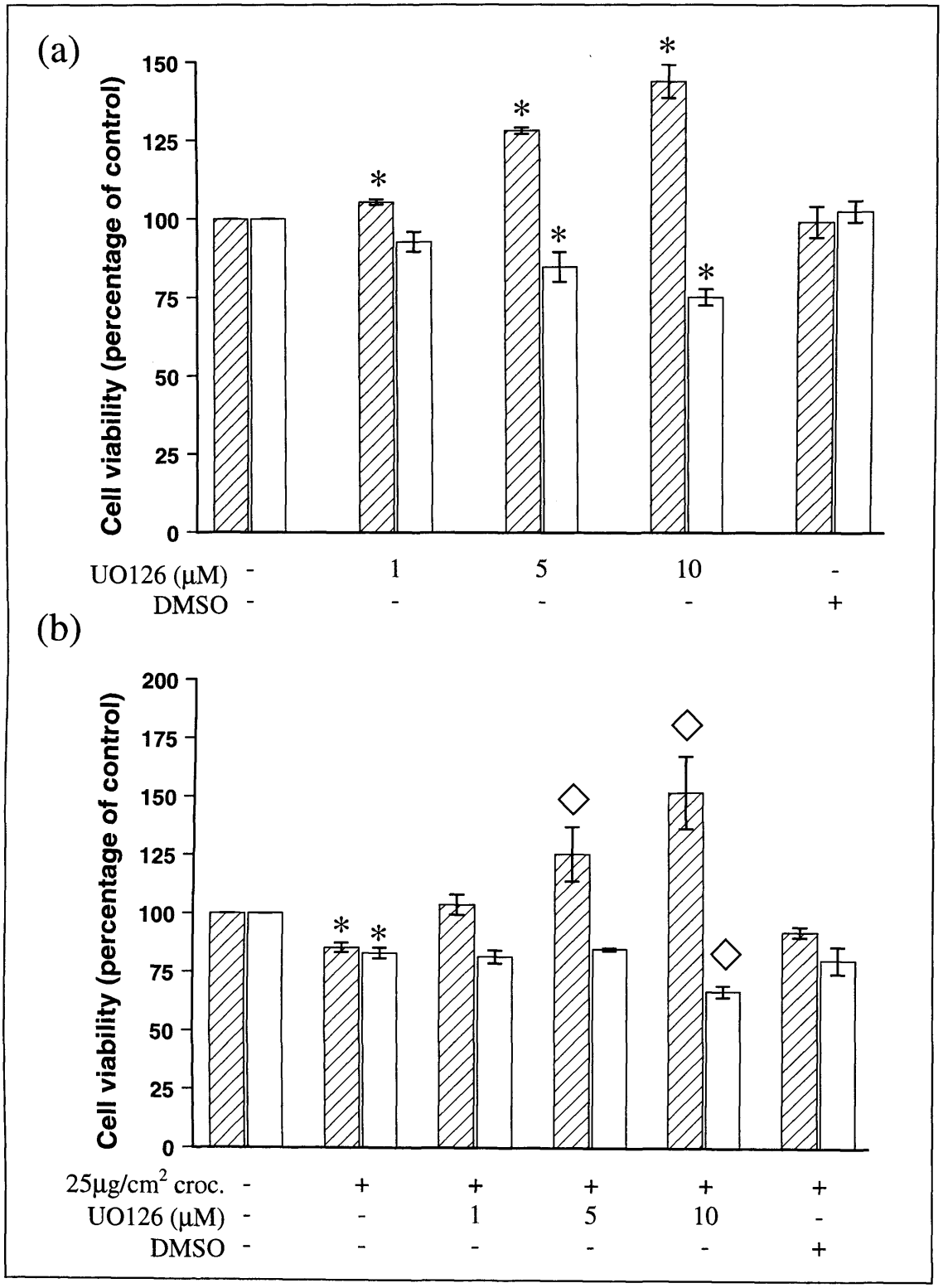


Figure 6.2 Modulation of crocidolite-induced cytotoxicity by UO126. $3x10^4$ 4/4 RM4 (hatched bars) or MET 5A (open bars) cells were plated in wells of a 96 well plate and left to settle for 24hrs prior to serum starvation for a further 24hrs. (a) UO126 or a DMSO vehicle control was added alone to cells for 26hrs, (b) Crocidolite 25μg/cm^2 was added in the presence or absence of a 2hr preincubation with UO126 at varying concentrations. Values shown are mean ± S.E.M, n=3. * $p<0.05$ compared to untreated control, ◇ $p<0.05$ compared to crocidolite alone.

6.2.3 Role of p38 in crocidolite-induced cytotoxicity

The next set of experiments were designed to investigate the p38 pathway. For these studies, the selective inhibitor SB203580 was used at concentrations that had elicited effects upon crocidolite-induced AP-1 DNA binding activity. Alone, SB203580 was marginally cytotoxic and was significant ($p<0.05$) at 100nM only, the higher doses of 250 and 500nM having lesser effects. The difference between cell lines in each case was marginal, although the 4/4 RM4 cells displayed slightly more sensitivity with each treatment (Figure 6.3a). When SB203580 was added in combination with crocidolite, cell viability was returned to near untreated control levels. Again the 4/4 RM4 cells were slightly more sensitive to its effects as either 250 or 500nM could significantly ($p<0.05$) increase cell viability compared to crocidolite alone whereas in the MET 5A cell line, this was achieved only at 500nM (Figure 6.3b). Again DMSO alone had notable effect upon cytotoxicity.

6.2.4 Role of PI3K in crocidolite-induced cytotoxicity

Due to the current lack of selective inhibitors for Akt, its role in cytotoxicity could not be directly established. However, inhibitors of PI3K are available and these are widely used as surrogate modulators of Akt activity. As in chapter 5, we used LY294002, a compound that is selective for PI3K activity. Similar doses that had produced favourable effects upon crocidolite-induced NF-κB translocation were used. In keeping with the role of the PI3K/Akt pathway's role in cell survival, its inhibition led to significant ($p<0.05$) decreases in cell viability at all doses (10, 20 and 50μM) tested. At 50μM LY294002, approximately 25% of cells were killed (Figure 6.4a). Differences between the cell lines were marginal, although the 4/4 RM4 cells were again slightly more sensitive to pharmacological manipulation. These findings were reflected under asbestos-stimulated conditions. The cytotoxicity induced by crocidolite was enhanced significantly at doses of 20 and 50μM LY294002 in 4/4 RM4 cells, and at 50μM only in MET 5A cells (Figure 6.4b). In both cell lines, the highest dose of this compound reduced cell viability to around 60%, suggesting that there is a population of cells that rely on the PI3K/Akt pathway

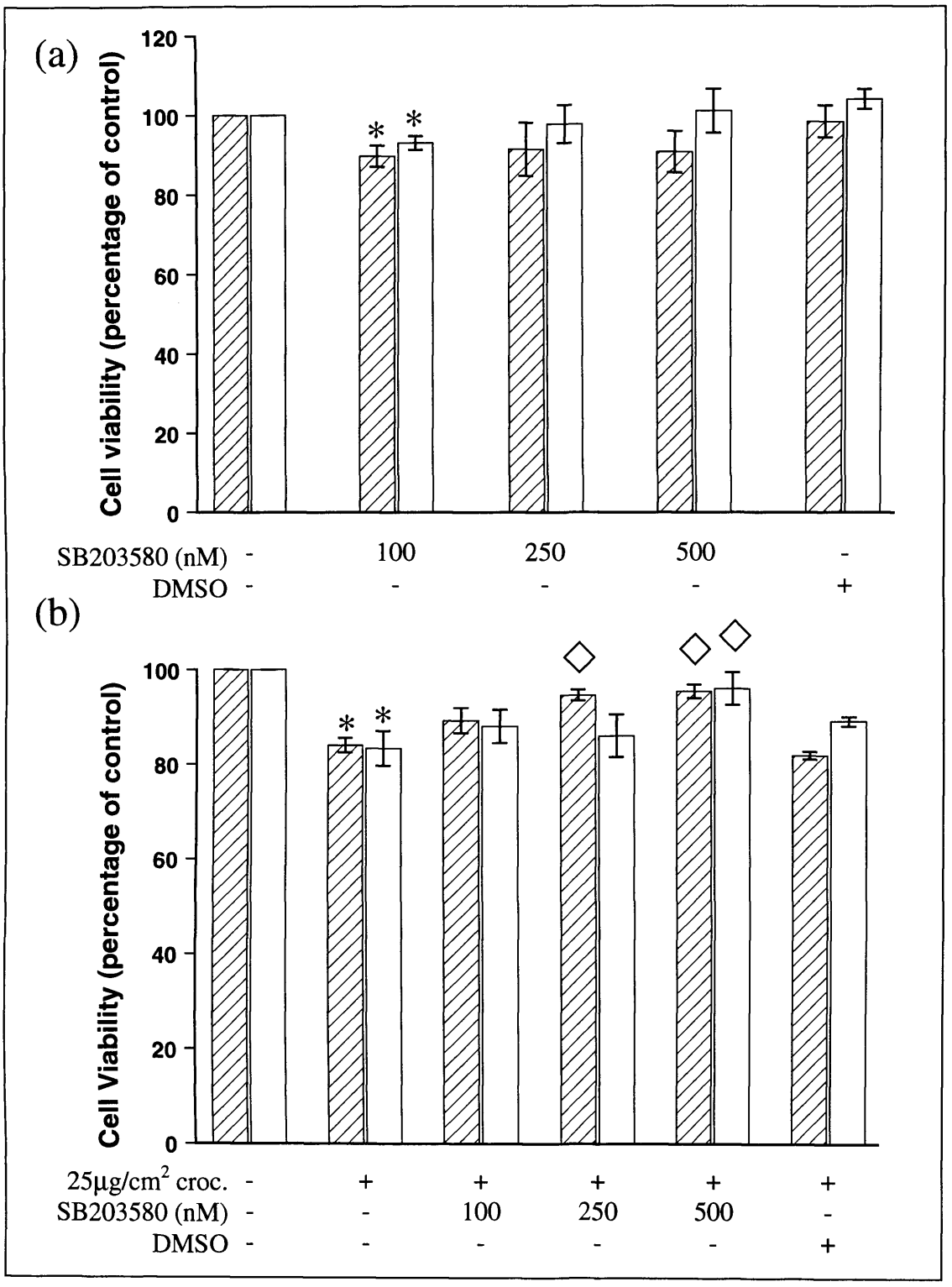


Figure 6.3 Modulation of crocidolite-induced cytotoxicity by SB203580. $3x10^4$ 4/4 RM4 (hatched bars) or MET 5A (open bars) cells were plated in wells of a 96 well plate and left to settle for 24hrs prior to serum starvation for a further 24hrs. (a) SB203580 or a DMSO vehicle control was added alone to cells for 26hrs, (b) Crocidolite 25µg/cm^2 was added in the presence or absence of a 2hr preincubation with SB203580 at varying concentrations. Values shown are mean ± S.E.M, n=3. * $p<0.05$ compared to untreated control, ◇ $p<0.05$ compared to crocidolite alone.

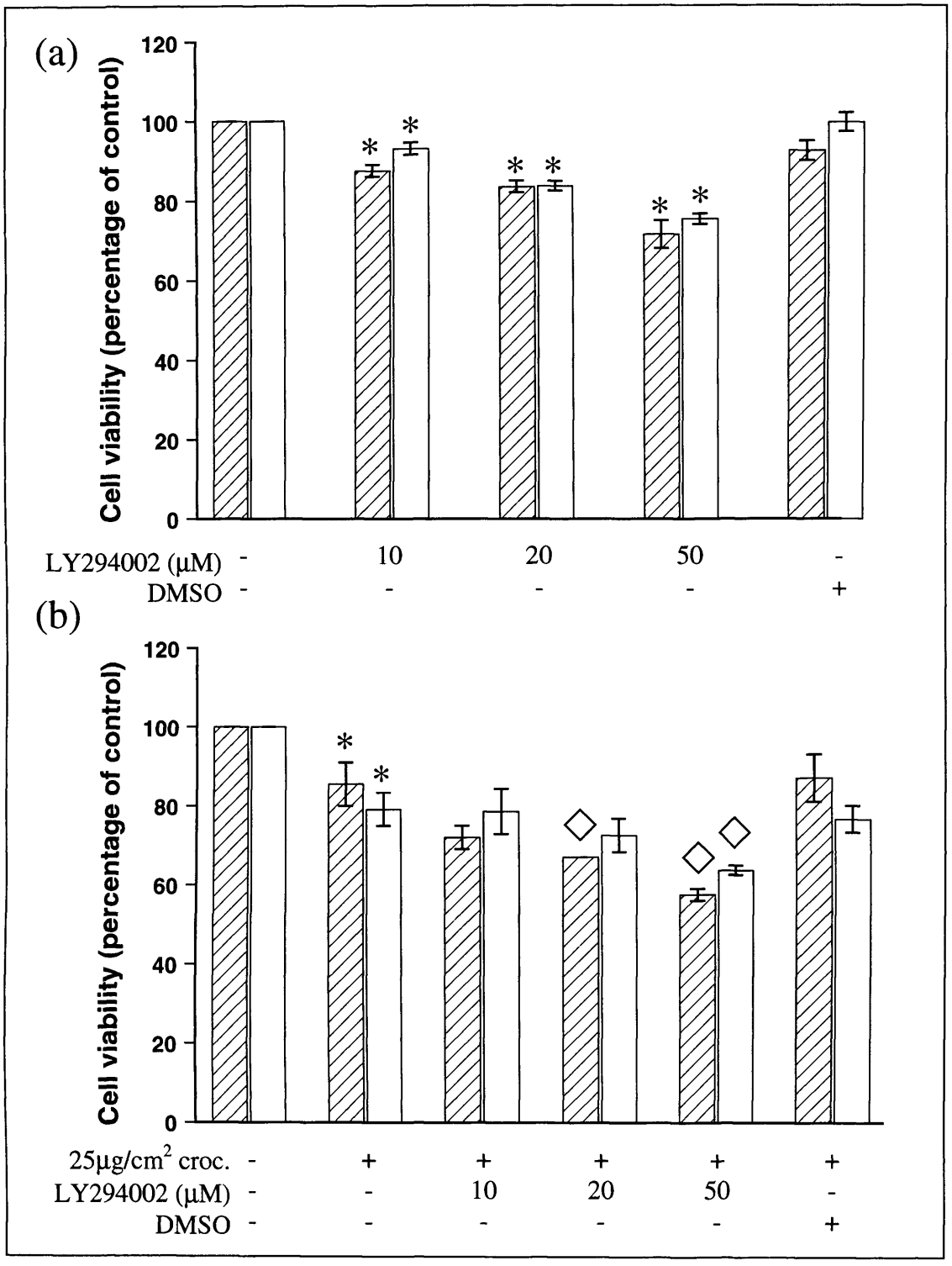


Figure 6.4 Modulation of crocidolite-induced cytotoxicity by LY294002. $3x10^4$ 4/4 RM4 (hatched bars) or MET 5A (open bars) cells were plated in wells of a 96 well plate and left to settle for 24hrs prior to serum starvation for a further 24hrs. (a) LY294002 or a DMSO vehicle control was added alone to cells for 26hrs, (b) Crocidolite 25μg/cm^2 was added in the presence or absence of a 2hr preincubation with LY294002 at varying concentrations. Values shown are mean ± S.E.M, n=3. * $p<0.05$ compared to untreated control, ◇ $p<0.05$ compared to crocidolite alone.

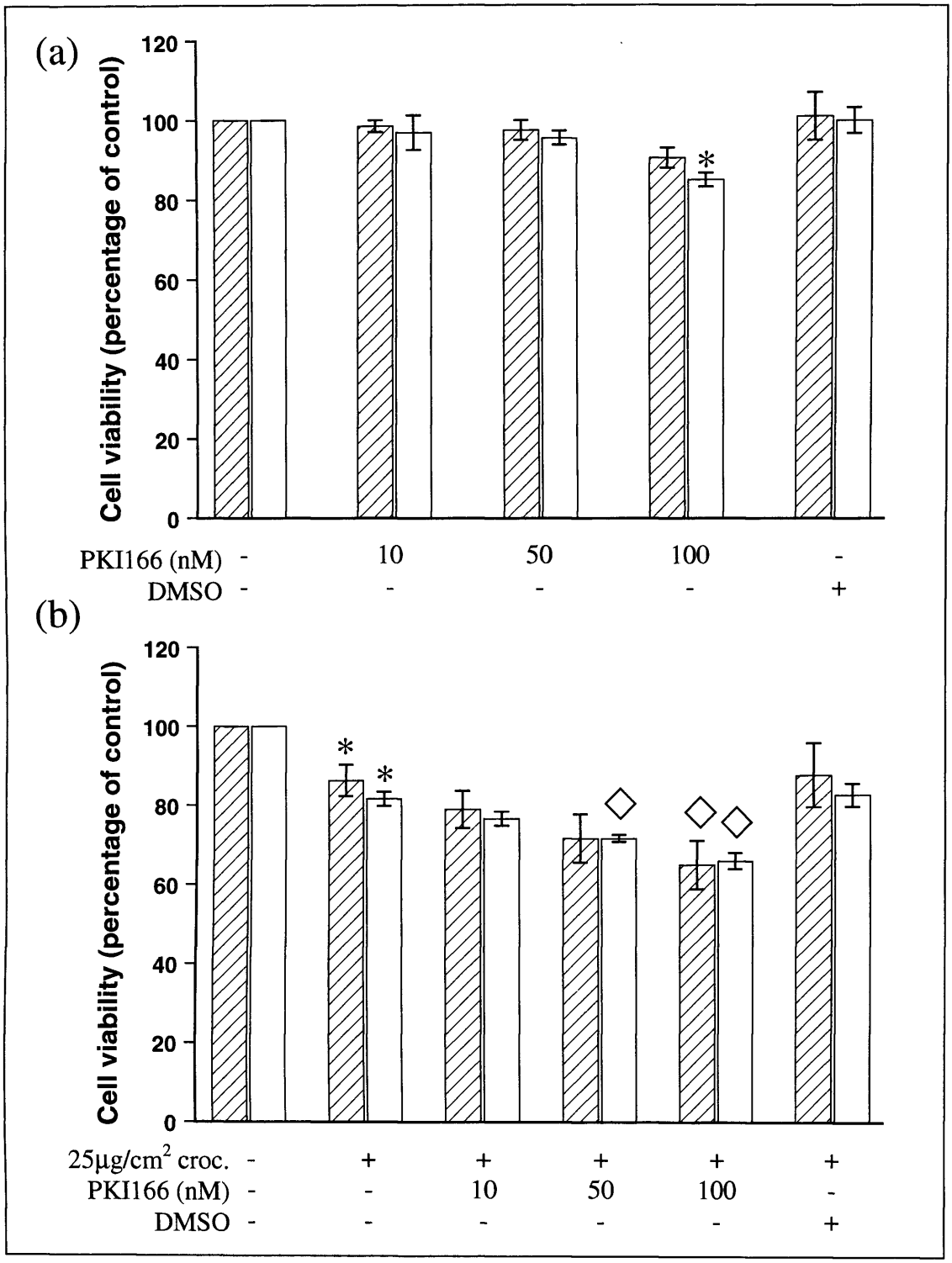


Figure 6.5 Modulation of crocidolite-induced cytotoxicity by PKI166. $3x10^4$ 4/4 RM4 (hatched bars) or MET 5A (open bars) cells were plated in wells of a 96 well plate and left to settle for 24hrs prior to serum starvation for a further 24hrs. (a) PKI166 or a DMSO vehicle control was added alone to cells for 26hrs, (b) Crocidolite 25µg/cm^2 was added in the presence or absence of a 2hr preincubation with PKI166 at varying concentrations. Values shown are mean ± S.E.M, n=3. * $p<0.05$ compared to untreated control, ◇ $p<0.05$ compared to crocidolite alone.

to survive what would otherwise be a cytotoxic asbestos insult. As before, DMSO alone had no effect on these cell lines.

6.2.5 Role of EGFR in crocidolite-induced cytotoxicity

Finally, the role of EGFR tyrosine kinase activity in crocidolite-induced cytotoxicity was examined. As before, PKI166 was utilised to selectively inhibit this activity. When this compound was added alone to the cell lines, its effects were mild, although a slight downward trend in cell viability was noted (figure 6.5a). This result was significant ($p<0.05$) in MET 5A cells at the highest dose tested (100nM). Where cells were coincubated with PKI166 and crocidolite, the effects were more dramatic. PKI166 potentiated the cytotoxicity of crocidolite from approximately 80% to below 70% in both cell lines examined (Figure 6.5b). This finding suggests that the role of EGFR tyrosine kinase activity is predominately in cell survival under these conditions. DMSO had no notable effects.

6.2.6 Effect of crocidolite on caspase 3 activity

Whilst MTT assays provide a useful insight into the general cytotoxicity of a given compound, it does not show the exact type of cell death, be it apoptosis or necrosis. In order to examine this more fully assays that can measure levels of apoptosis should be used. One such assay involves measuring the level of caspase 3 activity, which is described as an executioner of apoptosis as once it has been activated a cell is committed to undergo apoptosis. The assay itself is very simple, treated cell lysates are incubated with a peptide substrate that contains a fluorogenic motif. Active caspase 3 will cleave this compound to release the fluorophore, increases in fluorescence are therefore indicative of elevated active caspase 3. As not all cells express caspase 3 it was important to first check for its presence in these cell lines by western blotting using a caspase 3 antibody that detects both pro-caspase 3 (inactive) and the cleaved, active fragment. For each cell line two sets of untreated and crocidolite treated samples were tested for caspase 3 expression (Figure 6.6). Both cell lines did express pro-caspase 3 (Figure 6.6a), as shown by the band at 30kDa. The 4/4 RM4 samples show quite clearly that crocidolite causes cleavage of pro-caspase 3 to form caspase 3 as indicated by the band at 21kDa (Figure 6.6b). MET 5A cells did

not, however, display any cleavage of pro-caspase 3 under these conditions. Having established the presence of caspase 3, its activity could be measured.

As shown in Figure 6.7, crocidolite was capable of causing increases in caspase 3 activity in 4/4 RM4 cells at doses that had been previously shown to induce cytotoxicity. MET 5A cells did not behave in the same way, as was indicated by the western blot below. In fact, crocidolite reduced the level of background caspase 3 activity, and was significant at 25µg/cm^2.

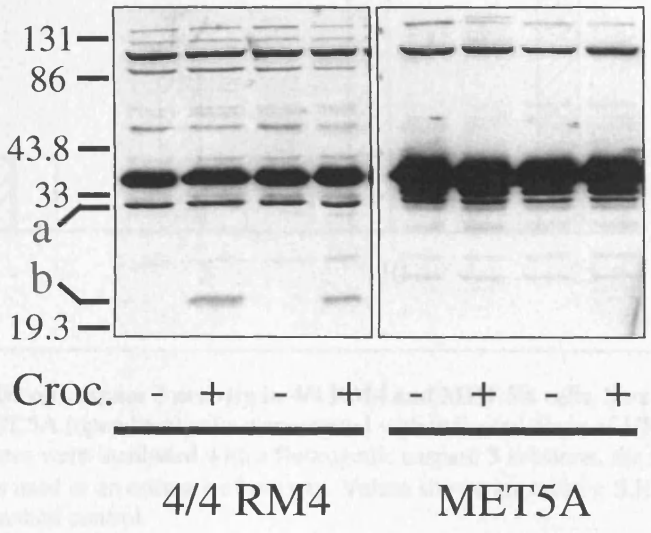


Figure 6.6 Effect of crocidolite on caspase 3 activation in 4/4 RM4 and MET 5A cells. Serum starved cells were treated with or without 25µg/cm^2 UICC crocidolite for 24 hrs. Lysates were subjected to western blotting as described in materials and methods and probed with anti-caspase 3 antibody. a) pro-caspase 3 b) caspase 3.

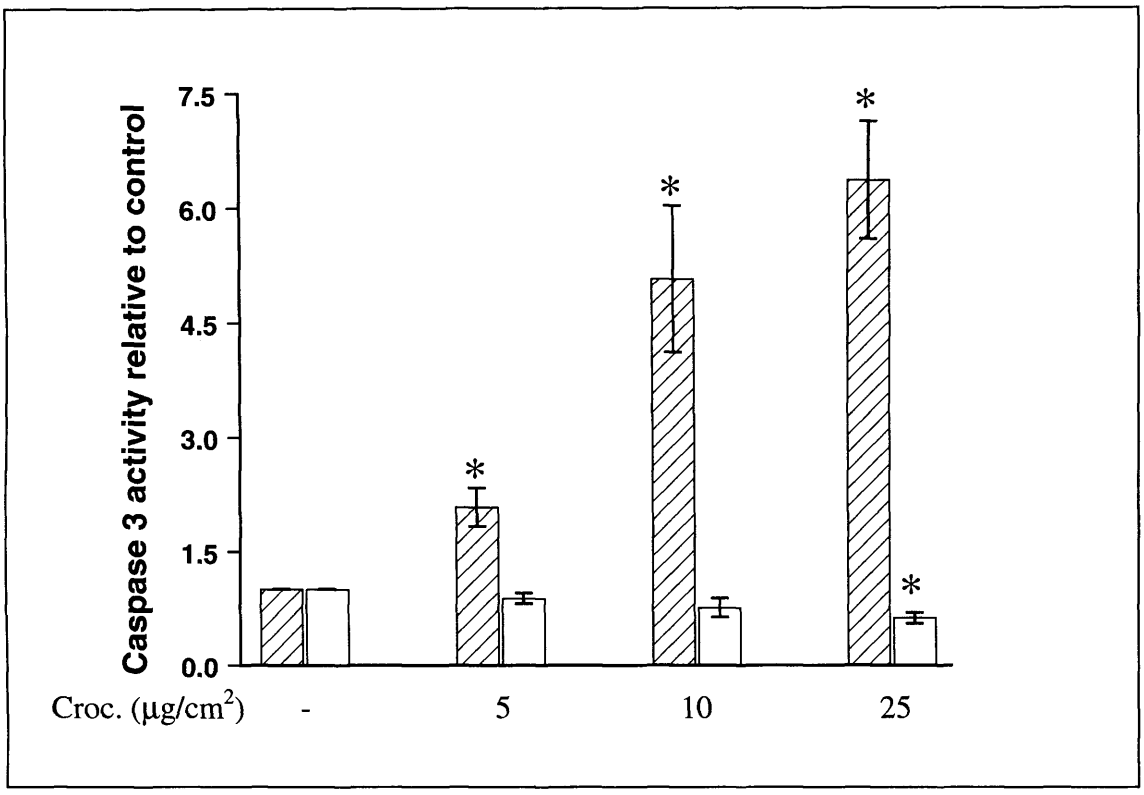


Fig 6.7 Effect of crocidolite on caspase 3 activity in 4/4 RM4 and MET 5A cells. Serum starved 4/4 RM4 (hatched bars) or MET 5A (open bars) cells were treated with indicated doses of UICC crocidolite for 24 hrs. Lysates were incubated with a fluorogenic caspase 3 substrate, the resultant change in fluorescence was used as an estimate of activity. Values shown are mean ± S.E.M, n=3. *=$p<0.05$ compared to untreated control.

6.2.6 Role of ERK/p38/PI3K in crocidolite-induced caspase 3 activity

Having established that MET 5A cells did not display caspase 3 activity in response to crocidolite, the following experiments were performed only in 4/4 RM4 cells. As before the importance of the ERK, p38 and PI3K/Akt pathways were tested, using pharmacological inhibitors. Figure 6.8a shows the effect of MEK inhibition on crocidolite-induced caspase 3 activity. As indicated by MTT assay, the ERK pathway serves a protective role in this system, as inhibition by 10μM UO126 attenuated the increase in caspase 3 activity by crocidolite to near control levels. UO126 alone had no major effect on background levels and DMSO had no significant effect on the crocidolite induced activity. Figure 6.8b demonstrates that there was also a role for the p38 pathway in crocidolite-induced caspase 3 activity, although the effect of SB203580 is less than that of UO126, with the activity reduced only to about 50% of

that observed with crocidolite alone, DMSO had no such effect. When added alone, SB203580 had no effect on caspase 3 activity. The data from previous experiments had suggested that co-incubation with LY294002 would potentiate the induction of caspase 3 by crocidolite and this was indeed the case as shown in Figure 6.9. The present experiments show that co-incubation with 50μM LY294002 nearly doubles the level of caspase 3 activity compared to crocidolite alone. In this case LY294002 alone did have an effect on background levels. As before DMSO had no effect on the induction by crocidolite.

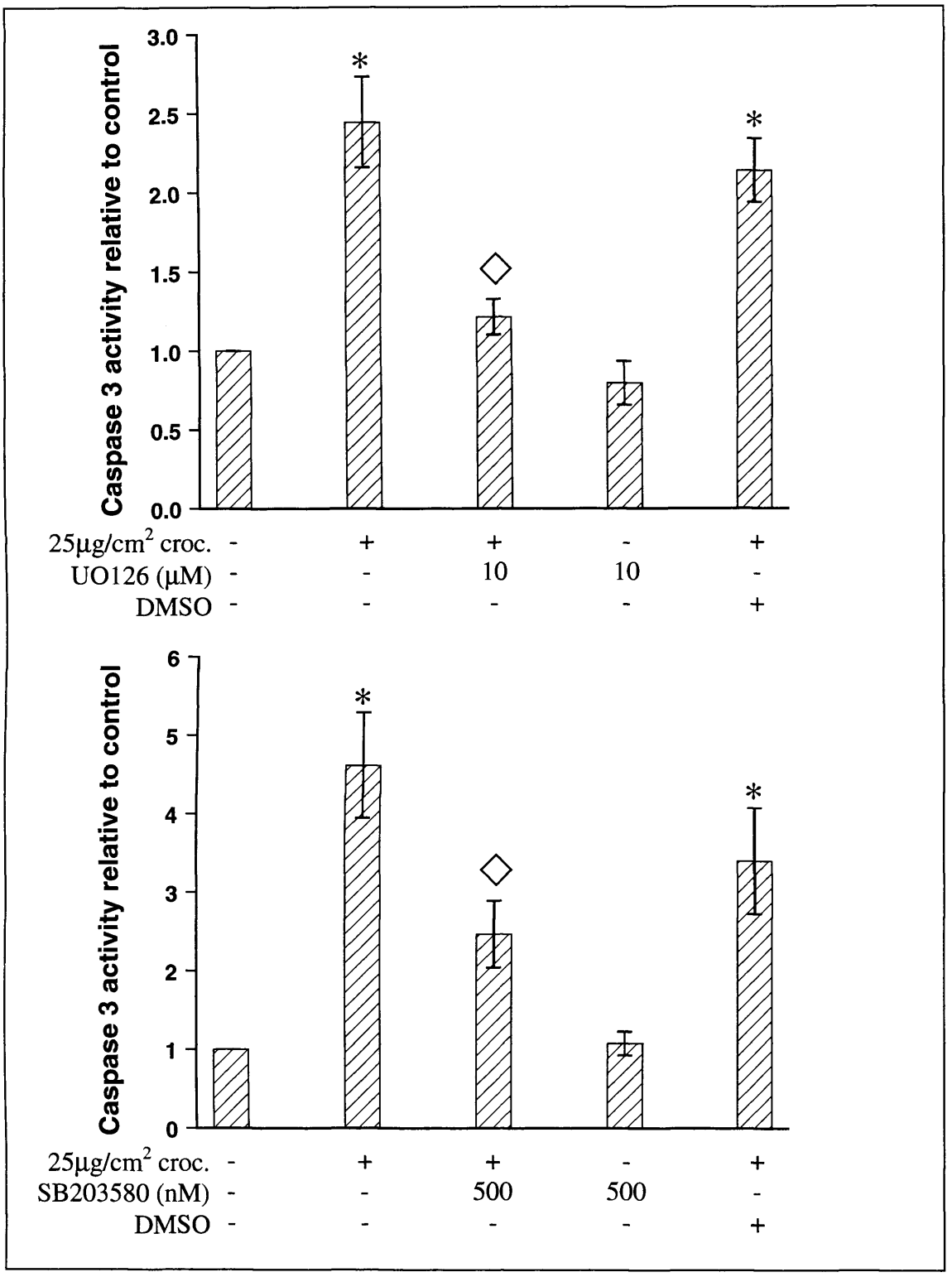


Figure 6.8 Effect of UO126 and SB203580 on crocidolite-induced caspase 3 activity in 4/4 RM4 cells. Serum starved 4/4 RM4 cells were treated with 25μg/cm^2 crocidolite in the presence or absence of a 2hr preincubation with UO126 or SB203580. Lysates were incubated with a fluorogenic caspase 3 substrate, the resultant change in fluorescence was used as an estimate of activity. Values shown are mean ± S.E.M, n=3. *=$p<0.05$ compared to untreated control, ◇=$p<0.05$ compared to crocidolite alone.

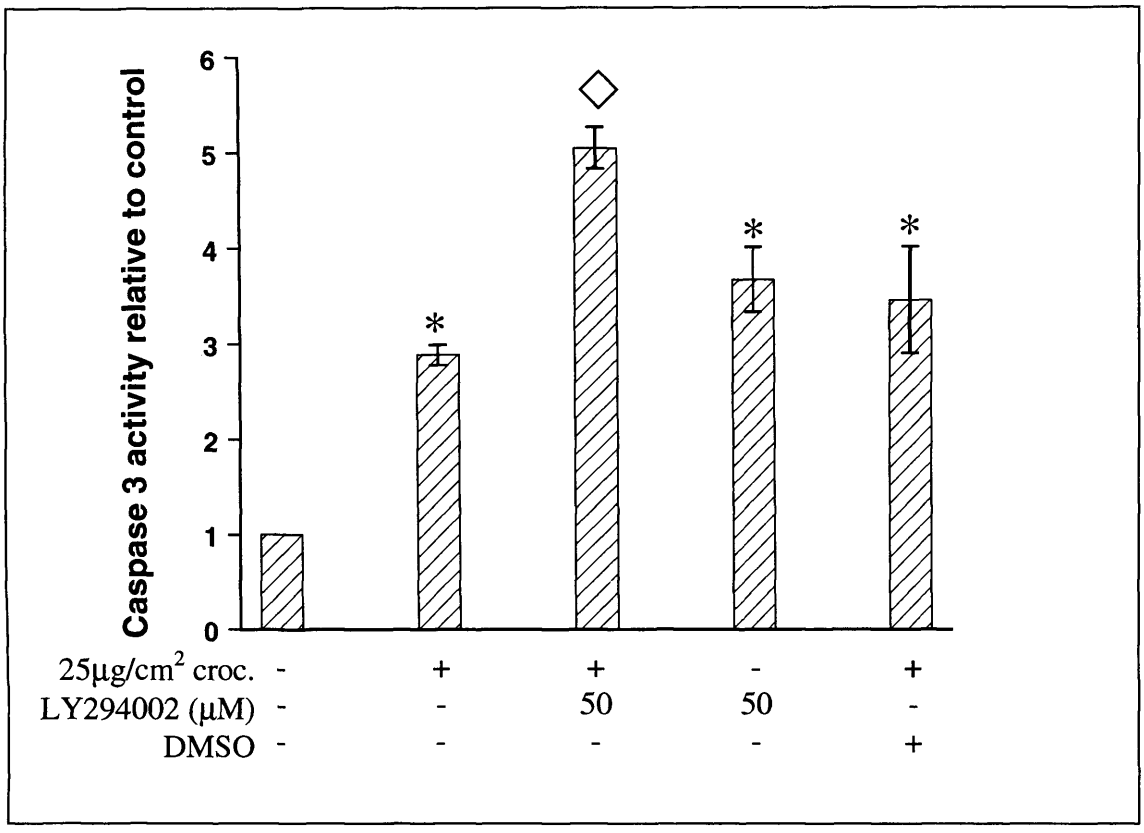


Figure 6.9 Effect of LY294002 on crocidolite-induced caspase 3 activity in 4/4 RM4 cells. Serum starved 4/4 RM4 cells were treated with 25μg/cm^2 crocidolite in the presence or absence of a 2hr preincubation with LY294002. Lysates were incubated with a fluorogenic caspase 3 substrate, the resultant change in fluorescence was used as an estimate of activity. Values shown are mean ± S.E.M, n=3. *=$p<0.05$ compared to untreated control, ◇=$p<0.05$ compared to crocidolite alone.

6.3 DISCUSSION

The cytotoxicity of asbestos fibres is well documented, with previous studies in the literature having shown that apoptosis is induced in primary rat mesothelial cells after 24 hrs exposure to 10μg/cm^2 NIEHS crocidolite (Berube et al., 1996), this was also true for rabbit and human primary mesothelial cultures under similar culture and exposure conditions (Broaddus et al., 1996).

In the present studies, MTT assays were used to gauge the effects of three types of crocidolite; firstly a preparation containing predominately long fibres with a mean length of 11.4μm (NIEHS), secondly a preparation containing shorter fibres with a mean length of 2.63μm (Clouter et al., 1998) (UICC), finally a milled preparation

containing no fibrous material as observed by electron microscopy. Perhaps surprisingly, no significant differences were noted between these particles in both a human and a rat mesothelial cell line, which is in contrast to results presented elsewhere in this thesis. In particular, the milled sample has been shown to elicit more potent responses with respect to the activation of signalling pathways, due to its large surface area and concomitant high bio-availability of iron that can catalyse the production of ROS. This finding suggests that any relationship between activation of signal transduction cascades and attainment of phenotypic endpoints is not linear, instead there may be a threshold level of signalling, above which no extra consequences arise. The apparent non-specificity of the above response may, at first, indicate that cytotoxicity is not relevant to the pathogenicity of crocidolite asbestos. However, this argument may be disregarded when one considers the situation *in vivo*. The latency period from asbestos exposure to disease is thought to arise from the biopersistence of fibres in the lung cavity which is limited to long, thin fibres that cannot be cleared by the pulmonary immune system (Donaldson et al., 1993), particles as small as those found in the milled sample would be engulfed and cleared by phagocytic cells, thus ensuring that they could not exert long term pathogenic effects.

Differences between the cell lines from the present studies were quite subtle. Whereas 4/4 RM4 cells were sensitive to the effects of these particles even at the lowest concentration tested, MET 5A cells were resistant under these conditions and required higher doses before more noticeable levels of cytotoxicity were apparent. This observation is in agreement with the literature where MET 5A cells were shown to be significantly more resistant to asbestos-induced apoptosis when compared to primary cultures (Narasimhan et al., 1998; Fung et al., 1997a). However, as the dose response curve was very shallow in the 4/4 RM4 cell line following the initial drop, both cell lines exhibited similar responses at the highest dose of crocidolite (25µg/cm^2) tested and this was about 20% cytotoxicity.

As mentioned previously, the ERK pathway has been highlighted as crucial in apoptosis caused by asbestos in primary rat mesothelial cells (Jimenez et al., 1997). It was, therefore, unsurprising that co-incubation of UO126 with crocidolite overturned

any cytotoxic effects in 4/4 RM4 cells. In fact cell viability was increased by about 50% under these conditions (similar effects were noted when this compound was added alone). Taken together, these results suggest that the ERK pathway is acting to suppress division in these cells rather than being integrally involved in promoting cell survival and proliferation, as is the case in numerous other systems. Indeed, in many of these cases down modulation of ERK pathway signalling is a prerequisite for apoptosis to occur (Wang et al., 1998; Stadheim and Kucera, 1998; Xia et al., 1995; Berra et al., 1998). Whether other factors are somehow involved is unclear at this stage, and it may be the context under which this signal is transmitted that is responsible. It would be interesting to repeat these experiments with EGF as the stimulus, as it could be that it is the activation of additional signalling cascades by asbestos that is responsible for this phenomenon. Although the plausibility of this argument seems small as UO126 had the same effect in non-asbestos stimulated conditions. When the experiments were repeated in MET 5A cells, perhaps the first and only really significant difference between the two cell lines was noted. In this model, inhibition of basal ERK signalling in the absence of crocidolite caused significant decreases in cell viability, which suggested that it had a more conventional function. The same effect was noted under asbestos stimulated conditions, although a high (10μM) dose of UO126 was required to make the difference significant. This finding might mean that the ERK pathway plays a small role in cell survival in MET 5A cells, and that other signalling cascades are more important. It is also possible that at 10μM UO126, signalling elements outside the ERK pathway are affected. These findings also highlight the multi-functionality of the AP-1 complex, as inhibition of MEK by UO126 led to reductions in AP-1 DNA binding in both cell lines, and yet the eventual endpoint was quite different.

The selective inhibitor SB203580 was used to examine the importance of the p38 pathway in crocidolite mediated cytotoxicity. Activation of p38 by stressful stimuli or proinflammatory cytokines can drive cells into apoptosis and/or propagate the inflammatory environment. When added alone, SB203580 had little effect on cytotoxicity, although small but significant decreases were noted with 100nM treatment, the significance of this observation is unclear at this time. Under asbestos treated conditions, SB203580 was capable of returning cell viability to near normal

levels in both cell lines at concentrations of 250 and 500nM in 4/4 RM4 cells and 500nM in MET 5A cells. This result suggests a proapoptotic role for p38 under these conditions through an undetermined mechanism, which may be indirect, direct or possibly both. The former would require downstream modulation of gene expression that led to increased expression of cytokines that could then activate the extrinsic arm of the caspase system. This idea seems quite plausible as transcription of these cytokine genes could be AP-1 driven and the results presented in chapter 4 demonstrate the requirement of p38 activation in AP-1 formation induced by crocidolite. The second mechanism bypasses the need for gene transcription and would involve the activation of caspase 3 by factors downstream of p38 (Cross et al., 2000). Regardless, the recognition that p38 is important in asbestos mediated cell death is both novel and important, and as discussed previously represents a possible explanation of how the chronic inflammatory response elicited by asbestos is maintained. It is interesting to note that either p38 or ERK pathway inhibition were capable of returning cell viability to near untreated levels in 4/4 RM4 cells, suggesting that both of these pathways are required for crocidolite mediated cell death to occur. With these pathways having opposing effects in MET 5A cells, it would be interesting to observe the end result where both pathways were inhibited simultaneously.

The importance of the Akt pathway in cell survival has become increasingly apparent over recent years (Marte and Downward, 1997; Madrid et al., 2000). Clearly dysregulation of Akt has major implications in oncogenesis. We have shown for the first time in these studies that a major upstream regulator of Akt activation, PI3K, is vital in cell survival both in the presence and absence of asbestos exposure in both human and rat mesothelial cell lines. This phenomenon was demonstrated by selective inhibition of the PI3K/Akt pathway by LY294002 and this compound alone was capable of reducing cell viability by approximately 25%. This data is interesting because it shows that background activity of PI3K is required to keep mesothelial cells alive in culture, perhaps due to the serum starved conditions. When this pathway was inhibited prior to asbestos exposure, the ability of both cell lines to resist its cytotoxic properties was ameliorated, with cell viability reduced from 80% to 60%, thus indicating that the PI3K/Akt pathway is crucial in cell survival following asbestos insult. The survival signal under these conditions is probably mediated by NF-κB, downstream of PI3K/Akt as this has been described in other systems (Madrid

et al., 2000). To add to this theory, data in chapter 5 shows the importance of the PI3K pathway in crocidolite-induced NF-κB DNA binding. However, it may be that additional factors are important, for example CREB is another transcription factor that has been shown to be regulated by Akt and may also contribute to cell survival through increased expression of the antiapoptotic protein Bcl-2 (Du and Montminy, 1998; Pugazhenthi et al., 2000). Future studies in this field may help to elucidate this mechanism more fully. The findings of the present studies contribute to our understanding of what may happen when mesothelial cells are exposed to asbestos *in vivo*, and represent a mechanism whereby a population of cells in the mesothelium may escape the apoptotic process that would otherwise ensue. This subset of cells could accumulate mutagenic lesions through cell proliferation and would enable them to enter the multistage carcinogenesis paradigm.

The final compound tested for effects on crocidolite-induced cytotoxicity was PKI166. The inhibition of EGFR tyrosine kinase activity by PKI166 could lead to a variety of effects, as a number of cellular signalling modules lie downstream of it *e.g.* the ERK and Akt pathways (Gibson et al., 1999; Guyton et al., 1996). Under asbestos-exposed conditions, the overriding effect of EGFR is survival in both cell lines. This response is quite expected in MET 5A cells as both EGFR dependent signalling pathways examined (ERK and Akt) have been shown to be required for cell survival under asbestos exposed conditions. However, the extent of cytotoxicity is not quite as high as expected if EGFR is the sole upstream regulator of these pathways because the levels noted in PKI166 and crocidolite treated cells is no higher than under either ERK or PI3K/Akt pathway inhibited conditions. This finding suggests that additional factors may be involved in the activation of these pathways by crocidolite. For the ERK pathway it could involve increases in free intracellular calcium (Lee et al., 2000). Previous studies have demonstrated an increase in intracellular calcium under crocidolite exposed conditions (Faux et al., 1994b) and additionally, ERK activation by crocidolite is reduced by extracellular calcium chelation with BAPTA (S. Faux, manuscript in preparation). Alternatively crocidolite may interfere with processes that usually function to switch off members of the ERK cascade. There may be other upstream regulators of the Akt pathway, which may include the PDKs, however, PDK1 also relies on PI3K activity and therefore this mode of Akt activation would

also be affected by LY294002 (Stephens et al., 1998). Perhaps a better explanation may be offered by dysregulation of factors that switch off Akt signalling, such as the tumour suppressor PTEN, which antagonises the effects of PI3K (Maehama and Dixon, 1998). The effect of PKI166 in 4/4 RM4 cells exposed to crocidolite is quite similar, *i.e.* the cytotoxic effects are potentiated. This observation is interesting because the downstream effectors of EGFR activation in this cell line had opposing effects on cytotoxicity, that is, ERK was proapoptotic and PI3K/Akt was antiapoptotic. Therefore, suggesting that the balance of signal generated by EGFR is in favour of cell survival, so when this is blocked other signals that induce cell death take over.

Investigations involving caspases have never before been performed under asbestos stimulated conditions. Western blot analyses of caspase 3 in both cell lines indicated that it was expressed in each. However, evidence of active caspase 3 was only present in 4/4 RM4 cell lysates treated with asbestos. This finding was supported by caspase 3 activity assays, which demonstrated that crocidolite induced dose-dependent increases in this, which reached more than a six-fold increase over untreated cells at 25μg/cm^2 crocidolite. MET 5A cells displayed no such response and even showed a small reduction in this activity, the significance at 25μg/cm^2 is unclear, but may be an indication that this cell line may undergo apoptosis via a caspase independent process, which would be worth investigating in future studies. For this reason further investigations into this area were performed only in 4/4 RM4 cells. UO126 at 10μM was able to ameliorate the level of caspase 3 activity to near untreated control levels and this provides new evidence for the mechanism whereby activation of ERK leads to apoptosis. In the absence of crocidolite, UO126 had no significant effects on caspase 3 activity, indicating that the increases in cell viability noted in the MTT assays under these conditions were due to another factor.

The effect of SB203580 was less emphatic, although the level of caspase 3 activity was significantly reduced, it was only by about 50%. Addition of this compound alone had no effect. Incubation of cells with LY294002 prior to asbestos exposure increased caspase 3 activity to nearly twofold compared to crocidolite alone. When added alone this had a similar effect, and taken together these findings support evidence in the

literature showing that the PI3K/Akt pathway suppresses activation of caspase 3, possibly by phosphorylating and inactivating caspase 9 (Cardone et al., 1998). Taken together, these results suggest that evidence provided from MTT assays provide a useful gauge of apoptosis in the 4/4 RM4 line.

The lack of caspase 3 activity in MET 5A cells is intriguing and may be for two reasons. Firstly, it may be an inherent property of human mesothelial cells that rat cells do not share. Secondly, it could be indicative of the effect SV40 transformation has on these cells. It would be interesting to repeat this work in normal primary human mesothelial cells in order to elucidate the role that SV40 transformation has in this response. Some researchers argue that SV40 is integral to the pathogenesis of mesothelioma, as it has been found in 60% of these tumours in the United States (Carbone et al., 1994), the source of this infection is thought to be from contaminated polio vaccine administered between 1959 and 1961 (Stenton, 1997). Additionally, in countries such as Finland, where this contamination did not occur, mesothelioma rates are somewhat lower than expected (Hirvonen et al., 1999). Whether SV40 has a causal effect in asbestos-induced mesothelioma is a contentious issue at present and further epidemiological studies are required before it can be resolved.

The results presented here begin to illustrate the complex sequence of events that lead to the formation of malignant lesions in the mesothelium. In particular, the that role cell signalling cascades play seems to be crucial in mediating the cells' response to this insult. These studies did not investigate the kinetics of proliferation following asbestos exposure and must be considered in future work. Sustained cell proliferation, which has been observed in asbestos exposed mesothelial cells (Kane and MacDonald, 1993) is a universal factor in human cancers (Preston-Martin et al., 1990). Cancer appears to result from genetic errors induced or fixed during the process of cell division and increased mitogenesis increases the risk of multiple genetic defects occurring including mutations, translocations and amplification of oncogenes (Shigenaga and Ames, 1993). If any of these pathways could be shown to be integral to this process *in vivo*, then they surely represent important targets for pharmacological intervention. A summary of the findings of the present studies is presented below (Figure 6.10).

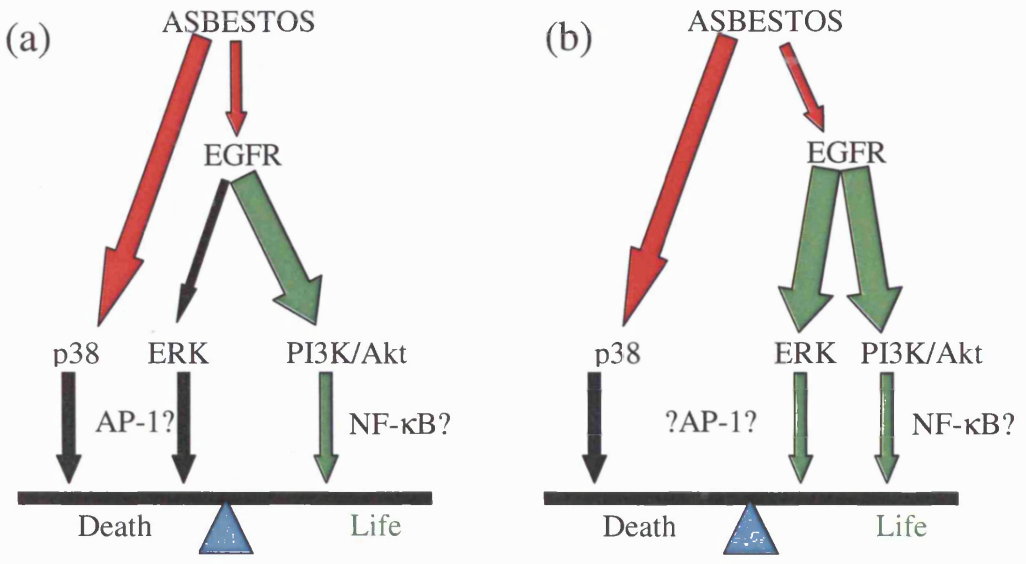


Figure 6.10 The role of signalling pathways in the balance between life and death under asbestos exposed conditions in (a) 4/4 RM-4 and (b) MET 5A cells.

CHAPTER 7

GENERAL DISCUSSION

CHAPTER 7

7.1 General discussion

The aims of these studies were to investigate the effects of crocidolite asbestos on cell signalling pathways that have been implicated in carcinogenesis in other systems. In recent years researchers have become to realise the importance of aberrant cell signalling throughout various stages of many diseases, not least cancer. Therefore, these studies targeted early events in the pathogenic process *i.e.* the frank exposure of mesothelial cells to asbestos fibres. This method is not without its limitations, as with all *in vitro* studies, but the previous documentation of asbestos fibres in the parietal pleura does validate this direct approach (Boutin et al., 1996). In addition, the targets of this research are becoming increasingly recognised as important prognostic factors in many human cancers. This observation is important because it indicates that the study of early events is relevant to cancer development and progression over many years.

Whilst the effect of crocidolite on the ERK pathway has been previously reported (Zanella et al., 1996; Jimenez et al., 1997) in primary rat pleural mesothelial cells, no studies have investigated this pathway in human mesothelial cells. The data presented here suggests that human mesothelial cells exhibit a very similar response following asbestos exposure in terms of kinetics and intensity of the signal generated. Furthermore, the factors involved in this phenomenon are also the same, *i.e.* oxidative stress and EGFR tyrosine kinase. In both cell lines, asbestos had a similar effect on DNA binding of the transcription factor, AP-1. At this stage, it seemed as though using a rat mesothelial cell model *in vitro* was well suited for investigating human mesothelioma. In the final series of experiments, outlined in chapter 6, it became clear that the phenotypic consequences of ERK pathway activation were drastically different in the two cell lines. Namely, that inhibition of the ERK pathway by inactivating MEK1/2 in the 4/4 RM4 cell line abated the ability of crocidolite to induce cell death whereas the opposite was true in MET5A cells. The former finding was unsurprising due to the work by Jimenez *et al.* (1997), although the latter did raise many interesting questions. At present the reason for these observations is unclear, and further investigations are required before a satisfactory answer is reached.

In addressing this problem, a role for SV40 must be considered. This virus was used to transform the human mesothelial cell line, MET5A, and with its emerging popularity as a possible cofactor in human MM (Bocchetta et al., 2000), these results become yet more important. SV40 has a number of effects that are mediated through the expression of genes in the early region of its genome, especially large T-antigen (Tag) and small t-antigen (tag). Tag can bind and inhibit p53 and Rb (Ali and DeCaprio, 2001), whereas tag can contribute to malignancy through repression of phosphatase 2A activity thereby altering the phosphorylation state of its target proteins (Rundell and Parakati, 2001). The effect of SV40 may give the cells a growth advantage and afford resistance to apoptosis. This phenomena has been observed *in vitro* with MET5A cells where following exposure to asbestos these cells were shown to be resistant to apoptosis in comparison to primary cultures of rat pleural mesothelial cells (Fung et al., 1997a). It would therefore be both interesting and worthwhile to investigate this matter further by comparing the effect of the ERK pathway in SV40 infected and non-infected cell lines to see if this was a genuine species-specific response or a direct effect of SV40 transformation. These studies may be performed using primary mesothelial cells, as previous work in our laboratory has shown that these cells isolated from patients without mesothelioma can be cultured, albeit not beyond passage 8. Furthermore, it would also be interesting to see if the lack of caspase 3 activity in asbestos treated MET5A cells was linked to the presence of SV40.

The apparent lack of JNK activation in this *in vitro* model is also intriguing. As shown in chapter 4, there seemed to be some induction of this pathway at early time points, but this could not be investigated further due to time limitations. With this observation being noted only in MET 5A cells again the possibility of SV40 infection being involved is a possibility. One must also consider the limitations of *in vitro* experimentation at this point. The activation of pulmonary macrophages by asbestos is well documented and is characterised by the production of pro-inflammatory cytokines such as TNF-α and IL-1α (Dubois et al., 1989; Tsuda et al., 1997). The effect of TNF-α on the JNK pathway has been shown in other cell types (Liu et al., 1996; Yuasa et al., 1998) and therefore it is conceivable that *in vivo* JNK activation would occur in mesothelial cells during asbestos exposure not from fibres directly but

from elevated TNF-α. These effects could not be measured under the experimental conditions used in these studies and co-culture of macrophages exposed to asbestos with mesothelial cells may answer these questions. A recent study sought to investigate this matter further by treating MET5A cells with TNF-α and IL-1β both alone and in combination over a 72 hr period. Microarray analyses were then performed on the samples generated. Although the data analysis is incomplete, this work has given some very interesting preliminary data and is expected to provide the basis for a large body of future work.

The activation of the p38 pathway in the absence of concomitant JNK activity highlights previous reports which demonstrate that whilst these pathways are regulated by many common stimuli it is possible to channel a signal through them individually. Interestingly, one of these reports used freshly fractured silica as the stimulus and this particulate shares a number of physical and chemical characteristics with asbestos (Ding et al., 1999). Taken together with this work it seems plausible that cells of the pulmonary system may utilise a common method of dealing with foreign matter. Furthermore, the studies by Ding *et al.* (1999), showed that the ERK pathway was also activated by silica and that together these pathways were important in AP-1 activation (Ding et al., 1999). Although p38 was shown to be involved in asbestos-induced cytotoxicity, the general consensus in the literature is that the role of p38 is in regulating immune responses through the control of cytokine expression is at least as important (Herlaar and Brown, 1999; Laporte et al., 2000; Rutault et al., 2001). This role of p38 was not investigated in these studies and provides another area worthy of attention. If chronic immune activation is indeed important in asbestos-induced carcinogenesis then blocking this response pharmacologically may provide a beneficial treatment strategy.

The studies of the Akt pathway evolved from attempting to elucidate the upstream signals responsible for NF-κB translocation to the nucleus. Emerging data from the literature describe the EGFR/PI3K/Akt/NF-κB as being a crucial modulator for cell survival under potentially genotoxic conditions. Therefore, the exposure of mesothelial cells to asbestos provides a near perfect environment for this scenario. This appears to be mediated by ROS at two levels. The first would result in genotoxic

lesions possibly through the formation of oxidised guanine bases such as 8-hydroxydeoxyguanosine, which would have to be removed prior to mitosis (Faux et al., 1994a), or malondialdehyde-DNA adducts (Howden and Faux, 1996). This DNA damage would, in most cases, be repaired, or if the damage were too great then the cell would undergo apoptosis. Alternatively a lesion could occur through removal of cell cycle regulating genes. Two of these regulatory genes are located at the INK4a/ARF locus on chromosome 9 (Xu et al., 1999). One of the genes encoded at this locus is a cyclin-dependent kinase inhibitor, p16. INK4a/ARF has been shown to be homozygously deleted in 70% of MM tumour samples in a Finnish study (Xu et al., 1999). Taken together these events may be enough for the initiation of cellular transformation, but the Akt (and ERK) pathway(s) may well regulate the progression of the transformed phenotype. ROS could also play a part, through activation of these signalling elements as described in chapter 1 thus allowing the survival of transformed cells. Therefore asbestos can fulfil the role of both initiator and promoter in the multistage carcinogenesis paradigm.

Whilst these pathways have been discussed and investigated individually, it must be noted that in the *in vivo* setting the interplay between them will have important functional consequences. There have been many reports in the literature of regulation of these pathways by others. For example, work by Berra *et al.* (1998), showed that inhibition of the ERK pathway causes activation of the p38 pathway and the induction of apoptosis, an effect that could be antagonised by overexpression of constitutively active PI3K (Berra et al., 1998). These and other studies demonstrate that these pathways do not act alone in promoting or inhibiting apoptosis and survival. Instead they form an integrated network, which requires coordinated changes to reach a phenotypic outcome.

The study of these pathways under asbestos exposed conditions would have been greatly aided by the use of transfection methodologies using constructs containing constitutively active or kinase dead mutants. This is a preferable method of investigating the specific role of a particular kinase within a defined system because the possibility that pharmacological inhibitors have additional, unknown, targets cannot be discounted. This is demonstrated by the recent finding that SB203580 can inhibit PDK1 at micromolar concentrations, although not at the nanomolar levels used

in this study (Lali et al., 2000). Previous studies have shown that rat pleural mesothelial cells are very resistant to transfection (Moritz et al., 1993) and this is also true for MET5A cells (S.P. Faux; unpublished observations). Perhaps with the emergence of new and more efficient transfection techniques, it may be possible to conduct these studies in the future.

Having defined these early signalling events *in vitro*, it is now important to investigate these pathways *in vivo*. Whilst a large-scale animal study may not be possible at the present time, it would certainly be interesting to examine the status of the key elements of these pathways in mesothelioma tumour samples. This is important to truly validate whether or not events noted in this work, *e.g.* Akt phosphorylation, are still evident at the late stage of the disease and if they have prognostic significance. Some of this work has already been performed by other groups to try and relate, for example, the level of EGFR expression and prognosis (Ramael et al., 1991). Another target worthy of attention is PTEN, which is mutated or even deleted in a number of human cancers (Li et al., 1997). As mentioned previously, this protein antagonises the actions of PI3K and this function was discovered after the observation that it was a candidate tumour suppressor gene. If this marker is decreased in mesothelioma samples then it could potentially be used prognostically.

A role for EGFR in mesothelioma is now beyond doubt. The ability of crocidolite to upregulate expression of EGFR has been shown both in these studies and previously by other laboratories (Pache et al., 1998; Faux and Houghton, 2000). Furthermore, EGFR expression is increased in MM (Ramael et al., 1991). The next logical step, therefore, is to examine whether or not tumour cells can be killed with selective EGFR tyrosine kinase inhibitors and this has work has already been started with promising initial data. Janne *et al.* (2002), have shown that serum starved mesothelioma cell lines are killed by ZD1839, an EGFR tyrosine kinase inhibitor, with an IC_{50} of >10μM. Furthermore, this compound was able to reduce the ability of these cell lines to grow in soft agar and undergo ERK1/2 and Akt phosphorylation following treatment with EGF (Janne et al., 2002). These studies could be the prelude to a clinical trial. A relatively recent approach to chemotherapy evolved from a desire to circumvent the ability of some tumours to resist conventional cytotoxic drugs. The

resistance of these cells is thought to arise from, at least in part, survival signals generated by EGFR dependent pathways *e.g.* ERK and Akt. These survival signals are especially important because the environmental conditions within a tumour are fairly inhospitable, typified by hypoxia due to inadequate vascularisation (O'Byrne et al., 2001). Therefore, the combination of cytotoxic chemotherapy alongside specific biological inhibitors of, say, EGFR may prove to be extremely beneficial in the treatment of mesothelioma.

To conclude, these studies have investigated a number of early events that occur in mesothelial cells following exposure to crocidolite, an extremely potent and specific inducer of human MM. These events are not isolated but instead form part of the integrated network of cellular communication that decides the fate of a cell, in particular, the balance between death and proliferation. It is hoped that further studies will build on these findings and lead to the formulation of strategies that may both prevent and/or treat this cancer that is almost invariably fatal at the present time.

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APPENDIX

APPENDIX

List of Publications

Faux, S.P., Houghton, C.E., Swain, W.A., Edwards, J.G., Sharma, R.A., Plummer, S.M. and O'Byrne, K.J. (2001) EGF-R induced activation of NF-κB in mesothelial cells is important in cell survival. Proc. American Association for Cancer Research Meeting 2001, New Orleans, USA, March 24-28, 2001.

Houghton, C.E., Swain, W.A., Holloway, K.A. and Faux, S.P. (2001) Activation of nuclear factor-κB (NF-κB) in mesothelial cells by asbestos is important in cell survival. *Experimental Lung Research,* **27**, 88-89.

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Swain, W. A., Patrick, G., and Faux, S. P. (2001). Cell signalling pathways initiated in mesothelial cells by asbestos fibres. Toxicology **164**, 117.

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Swain, W. A., O'Byrne, K. J., Houghton, C. E., Edwards, J. G., and Faux, S. P. (2002). EGFR activation of the PI3K/Akt pathway plays a role in human mesothelial cell survival following asbestos exposure. 93rd AACR conference. **43**, 352. Abstract

Swain, W.A., O'Byrne, K.J., Houghton, C.E., Edwards, J.G. and Faux, S.P. (2002) EGF-R activation of the PI3K/Akt pathway by asbestos plays a role in human mesothelial cell survival. Proc. 3rd International Conference on Oxygen/Nitrogen Radicals: Cell Injury and Disease, Morgantown, USA, June 1-5, 2002. Abstract.

Swain, W. A. and Faux, S. P. (2002). Activation of p38 MAP Kinase by Crocidolite in Mesothelial Cells is Dependent upon Oxidative Stress. *Annals of Occupational Hygiene* **46**, 136-139.

Faux, S.P., Houghton, C.E., Swain, W.A., Edwards, J.G. and O'Byrne, K.J. (2002) Activation of NF-κB by asbestos through EGF-R signaling in mesothelial cells is important in cell survival. *Annals of Occupational Hygiene,* **46**, 85-89.

Swain,W.A., O'Byrne, K.J and Faux, S.P. Activation of p38 MAP kinase by asbestos in mesothelial cells is mediated by oxidative stress. Submitted to Carcinogenesis August 2002.

Swain,W.A., O'Byrne, K.J., Houghton, C.E., Edwards, J.G and Faux, S.P. Epidermal growth factor receptor activation of the PI3K/Akt survival pathway in human mesothelial cells exposed to asbestos. Submitted to Journal of Biological Chemistry July 2002.