"Resistance Vessel Structure & Function in an Experimental & Genetic Model of Insulin-Dependent Diabetes Mellitus"

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by

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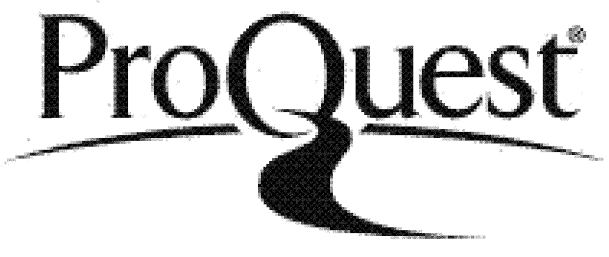

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ABSTRACT

Microvascular disease is a major complication of diabetes but little is known about how diabetes effects the precapillary resistance arteries. The work described in this thesis was designed to investigate the effects of insulin-dependent diabetes on the structure and function of the resistance vasculature, using an experimental and genetic animal model of diabetes.

Preliminary in vitro studies of normal resistance arteries showed that hyperglycaemia (40mM glucose) resulted in impaired endothelium-dependent relaxation to the vasodilator acetylcholine (ACh). This effect was shown to not be due to an increase in osmolarity.

Resistance arteries from the spontaneously diabetic BioBred rat at the onset of diabetes, and after long-term insulin treatment showed a significant increase in the media wall:lumen ratio, cross sectional area and media thickness compared to their age-matched non-diabetic controls. Significant impairment to both ACh and bradykinin (BK) was evident in the diabetic vessels, which was unaffected by pretreatment with L-arginine, or free radical scavengers. Inhibition of EDRF revealed a significant left-ward shift in the dose response curve to noradrenaline (NA) in the recent onset diabetic vessels, but not in the insulin treated. However, the shift was not as marked as that seen in the age-matched control vessels which suggests a reduction of basal EDRF release in the diabetic vessels. Incubation with a cyclo-oxygenase inhibitor indomethacin significantly improved ACh relaxation in the diabetic vessels indicating the abnormality arises from an over-production of a vasoconstricting prostanoid.

Resistance arteries from chemically-induced diabetic rats after 6 or 12 weeks of diabetes were examined, along with the response to the intra-portal transplantation of islets. Inhibition of EDRF revealed an increased response to NA in the diabetic vessels; whereas, vascular reactivity to NA in the islet transplanted and control groups remained unaltered. Endothelium-dependent relaxation to ACh and BK was not impaired in the diabetic vessels. Indeed, the ACh relaxation was significantly augmented in the untreated 6 week diabetic group indicating an increased release of EDRF early in chemical-induced diabetes, but this disappeared with a long duration of diabetes. These studies highlight differences in the response of the resistance artery structure and function to genetic or chemical-induced diabetes mellitus.

CHAPTER ONE

DIABETES MELLITUS

1.1 Historical Review of Diabetes

Diabetes mellitus is a group of complex metabolic disorders, characterised by hyperglycaemia. It was recognised as a disease entity in ancient times, with the first description of the disease dating back as far as 1500 BC. This was recorded in an ancient Egyptian script or "papyrus", which was drafted by an Egyptian medical scribe and contained advice for the treatment of frequent urination, or polyuria, which was a symptom of the disease.

Sweetness in the urine was first recorded around 400 BC in India where Sanskrit scriptures referred to "*madhumeha*" (honey urine). However, the actual term "diabetes", which in Ionic Greek means "to run through a siphon", was given to the disease much later by a Greek physician (ca.AD 30-90) called Aretaeus of Cappadoci, and again refers to the symptom of polyuria. Sweetness of the urine also was recorded by Japanese and Chinese physicians of the second and third centuries, and later the Latin word for "honey" was added to the Greek word for "siphon", describing a disease now called "diabetes mellitus" (Wong & Wu, 1932). In the 16th century, the Swiss physician Paracelsus (1493-1541) evaporated the urine from a diabetic patient and obtained a white, powdery residue which he mistook for salt, and incorrectly concluded that this was responsible for causing the symptoms of thirst and frequent urination. The sweetness of diabetic urine was "re-discovered" in 1679, when the physician Thomas Willis stated that the urine of diabetic patients tasted "wonderfully sweet". However, another 100 years passed before this sweetness first described by Willis was shown to be due to excess sugar in the urine (Dobson, 1776). Subsequently, early in the nineteenth century, improvements in biochemical analysis allowed the sugar to be identified as glucose (Chevreul, 1815). Interestingly, Johann Conrad Brunner in 1682 observed that removing the pancreas from dogs produced the characteristic signs of diabetes, although he failed to make the connection since his experimental animals rapidly recovered due to incomplete removal of the pancreas.

In 1869, a German medical student Paul Langerhans described clusters of non-acinar cells within the pancreas which differed in structure from acinar enzyme secreting pancreatic cells.

He called these clusters "islets of Langerhans", although the key hormone secreted by them, insulin, still eluded researchers. Later, the link between the pancreas and diabetes was established by Von Mering and Oscar Minkowski after a study in de-pancreatised dogs. Together they made a chance observation that total pancreatectomy led to polyuria which was caused by severe diabetes mellitus. Soon afterwards, Laguesse in 1893 suggested that the absence of islets described earlier in 1869 by Langerhans could be responsible for diabetes. It was only in 1901 that Eugine Opie demonstrated this connection between the pancreas and diabetes when he found that the islets of Langerhans were damaged in a girl who had died of diabetes. Soon after the turn of the century, several other researchers recorded a number of histologic alterations in the islets of Langerhans of diabetic individuals. Thus, by the end of the first decade of the 20th century, it was widely hypothesised that carbohydrate metabolism was controlled by an "internal secretion" of the pancreas, and the name "insulin" the Latin word for island was proposed for this hypothetical hormone (De Meyer, 1909; Scharpey-Schäfer, 1916).

There then followed many attempts to demonstrate the existence of this internal secretion by administering crude pancreatic solutions to both diabetic animals and man. The results were variable and inconclusive, but the German scientist Georg Zuelzer was able to occasionally reduce glycosuria and acidosis in human diabetic individuals (Zuelzer, 1908). These limited successes were dismissed as dangerous because blood sugar reduction often was accompanied by severe and life-threatening reactions. Nevertheless, these studies continued and in 1916 a Romanian physiologist Paulesco observed that the intravenous administration of an aqueous solution of pancreatic extract to a diabetic dog provided rapid symptomatic relief. In 1920, Paulesco went on to demonstrate that his pancreatic extract also reduced blood sugar, ketones, urea and urine volume in both normal and depancreatised dogs (Paulesco, 1921). However, inadequate funding combined with primitive experimental techniques slowed the work of European researchers such as Paulesco (Bliss, 1993).

In 1921, beginning where other researchers had left off, a Canadian surgeon Frederick Banting and his medical student assistant Charles Best, began working on diabetes under the supervision of Professor MacLeod in the department of Physiology at the University of

Toronto. They showed that crude extracts of pancreas (with previously ligated ducts) injected intravenously, could successfully reduce the blood glucose of diabetic dogs. Next, they enlisted the help of a biochemist, J.B. Collip who developed a new method of extraction, leading to a relatively pure extract being precipitated from the solution. This was shown to be a significant improvement on Banting and Best's original pancreatic extract (Banting *et al.*, 1922; Collip, 1923). Having obtained a purified extract, they went on to treat a severely diabetic boy, who was subsequently treated with insulin and lived to maturity.

The successful isolation of the blood-sugar lowering hormone, insulin, by Banting and Best in 1921 represented an important landmark in the history of diabetes. Although this discovery did not provide a cure, the work of Banting and Best revolutionised the lives of diabetic patients. Thus, instead of short and uncomfortable lives, diabetic patients could dramatically improve their quality of life, and increase their life expectancy by administering daily injections of insulin. However, the improved survival of diabetic patients revealed a set of devastating long-term complications, including damage to the small blood vessels of the retina, renal glomeruli and peripheral nerves and accelerating vascular disease in the large arteries supplying the brain, heart, and lower limbs.

In the last twenty-five years intense medical research has resulted in break-throughs in the haemodynamic, metabolic and biochemical changes associated with the complications of diabetes; insulin delivery mechanisms; self-monitoring techniques; the importance of optimal glucose control in the reduction and possible delay of the development of serious diabetic complications; diabetes prevention and islet cell/pancreatic transplantation. Details of the more recent discoveries and advancements in the understanding of diabetes mellitus will be described in later sections of this chapter.

1.1i Anatomy and Physiology of the Pancreas

The pancreas is made up of both endocrine and exocrine tissue. The exocrine pancreas consists of numerous lobular acini and ducts held together by connective tissue. The function of the exocrine tissue is to synthesise, store, and secrete digestive enzymes. Interspersed

among the digestive glands of the pancreas are small spheres known as islets of Langerhans which constitute the endocrine element of the pancreas, and represent approximately one per cent of its total mass. The islets comprise several distinct cell types consisting of a central core of insulin producing beta cells (B-cells), with a surrounding mantle of glucagon containing alpha cells (A-cells), somatostatin secreting delta cells (D cells), and pancreatic polypeptide containing cells (PP-cells). The islets are vascularised by direct arteriolar blood flow, and are innervated by sympathetic, parasympathetic, and peptidergic nerves (Fujita & Kobayashi, 1979).

The beta cells are the most abundant cell type making up 60-70% by volume of the islet. They secrete insulin which is essential for the synthesis and conservation not only of carbohydrate, but also of fat and protein. Insulin effectively lowers blood glucose by facilitating glucose transport into cells and by activating several of the enzymes necessary for the conversion of glucose to glycogen. High blood glucose levels stimulate the synthesis and release of insulin from pancreatic beta cells, and inhibits glucagon release from pancreatic alpha cells. This results in a lowering of blood glucose concentrations (Bloodworth & Greider, 1982).

1.1ii Insulin Secretion and Regulation

Insulin is encoded by a gene on chromosome 11. It is synthesised as a prohormone (pre-proinsulin) which is converted to proinsulin in the endoplasmic reticulum (Howell & Bird, 1989). Conversion of proinsulin to insulin takes place in insulin secretory vesicles through a series of steps involving the removal of C-peptide by proteolytic enzymes (Porte, 1991; Steiner *et al.*, 1990). A raised glucose concentration is the main stimulus for the release of insulin-containing secretory vesicles, although a wide variety of other hormones and drugs also may provoke insulin release. The response to glucose is initially bi-phasic, with a rapid initial "pulse" or first phase, followed by a more prolonged second phase. Glucose entry into the β-cell triggers a series of events culminating in insulin secretion. Glucose metabolism results in alteration of the ATP/ADP ratio, causing closure of K^+-ATP channels in the cell membrane resulting in its depolarization. This in turn activates voltage calcium channels in

the membrane, promoting entry of extracellular calcium, raising cytosolic calcium, which then triggers insulin release (Grodsky & Bennett, 1966).

1.1iii Insulin Action and Metabolism

Insulin has important effects on carbohydrate, fat and protein metabolism. The secretion of insulin in conjunction with other hormones such as glucagon, growth hormone, cortisol and catecholamines, is essential for the maintenance of glucose homeostasis. Insulin facilitates the transport of glucose across the cell membrane in adipose tissue and muscle. It stimulates the rate of glycogen synthesis in adipose tissue, muscle and liver, and decreases the rate of glycogen breakdown in the muscle and liver. Further action on the liver includes inhibition of gluconeogenesis.

1.2 Non-Insulin Dependent Diabetes Mellitus -(An Overview)

Non-insulin dependent diabetes mellitus (NIDDM) or Type 2 diabetes is the most frequent form of diabetes. Its prevalence in Western populations is 3-6 % compared to 0.5% for insulin-dependent diabetes mellitus (IDDM). It is a chronic disorder, of which the pathogenesis and natural history are poorly understood. It is characterised by basal hyperglycaemia, on which exaggerated post-prandial glucose excursions are superimposed. The hyperglycaemia results from a combination of impaired β-cell function and insulin sensitivity at the level of the muscle and liver (De Fronzo, 1988, 1992; Temple *et al.*, 1989). The onset of NIDDM is insidious, occurring several years before clinical presentation, and usually manifests itself in adulthood. The cause of insulin resistance in NIDDM is unclear, but many factors including obesity, advanced age, lack of exercise, and genetic components are likely to contribute to its development.

The rapid first phase of insulin release in response to glucose in subjects with NIDDM is severely diminished, and this coupled with a lack of the priming effect of the insulin on the target organs results in post-prandial hyperglycaemia. If the hyperglycaemia persists, the second phase of insulin release in response to glucose becomes attenuated. Individuals

without NIDDM, who show impaired glucose tolerance (IGT), appear to have a high risk of developing the disease in the future. Environmental influences such as excessive caloric intake, ageing, or decreased physical activity, could be the basis for this transition. However this is not always inevitable, as some individuals with IGT will subsequently return to normal.

NIDDM is associated with several other insulin secretion abnormalities, including a reduced sensitivity of glucose potentiation, disruption of the pulsatile release, and a raised plasma ratio of proinsulin to insulin, which may reflect a relative hyper-secretion of proinsulin (Temple *et al.*, 1989 & 1990). The relationship between the insulin response and the plasma glucose level in NIDDM, has been termed "Starling's curve of the pancreas", and is described as being "horseshoe-shaped". Thus, the secretion of insulin progressively increases in response to rising glucose levels up to about 7mmol/l, after which further increases in glucose are associated with a decline in insulin secretion. It has been suggested that this may indicate a toxic effect of hyperglycaemia on the functioning β-cells.

Genetic susceptibility is thought to be a factor leading to the development of NIDDM. This has been supported by data showing that the incidence of NIDDM varies amongst different ethnic groups living in the same environment, and that there is a higher concordance rate for the disease among pairs of monozygotic, identical twins than among dizygotic, non-identical twins (Newman *et al.*, 1987). The exact mode of inheritance is uncertain, although it is most likely to be polygenic in nature.

NIDDM is associated with the development of microvascular complications, which include retinopathy (especially with maculopathy rather than proliferative changes), nephropathy, and neuropathy. Indeed, studies of patients with non-insulin dependent diabetes have shown that up to 21% have evidence of retinopathy at the time of diagnosis (UKPDS 6, 1990). The presence of such complications at the time of diagnosis may be related to a pre-diabetic phase or to a long period of undiagnosed diabetes (Jaap *et al.*, 1995). Nevertheless, the major cause of increased mortality in NIDDM is macrovascular disease, the progression of which is reliant upon other contributory factors such as associated hypertension, hyperlipidaemia and obesity (Garcia *et al.*, 1974; Stamler *et al.*, 1993).

Recently, a Swedish study assessing the effects of long-term metabolic control in subjects with non-insulin dependent diabetes reported a link between hyperglycaemia and mortality, although these results should be interpreted with caution, as other cardiovascular risk factors were not studied (Andersson & Svärdsudd, 1995). The currently on-going UK Prospective Diabetes Study of non-insulin dependent diabetic patients should provide more conclusive data on the relationship between glycaemic control and mortality in the next few years, and will also determine the extent to which available therapies will be able to reduce the clinical burden of the disease.

1.3 Insulin-Dependent Diabetes Mellitus

Insulin-dependent diabetes mellitus (IDDM), or Type I diabetes develops as a consequence of the selective destruction of insulin-producing cells by an autoimmune reaction (Cahill & McDevitt, 1981; Bottazzo *et al.*, 1984; Eisenbarth, 1986). It is characterised by hyperglycaemia and glycosuria, and diagnosis is usually straightforward because patients present acute symptoms such as thirst, polyuria, and weight loss.

Structural β-cell damage appears to be irreversible at the time of onset of clinical diabetes, and treatment is focused on providing symptom relief, restoration of euglycaemia, and prevention of acute and chronic complications that contribute to the increased morbidity and mortality associated with this condition. At diagnosis, approximately 80% of islets contain no β-cells, and the islets are heavily infiltrated with lymphocytes. The limited insulin secretion in patients with IDDM leaves them prone to increased ketoneogenesis, and with no insulin treatment, such patients are likely to die in diabetic ketoacidosis (Leslie, 1993).

Interest has centred on the period preceding the clinical manifestation of IDDM known as prediabetes. There is no exact pattern and time course of events leading up to functional β-cell loss. It is possible that the autoimmune process once initiated is usually persistent leading to a progressively deteriorating course (Eisenbarth, 1986; Ziegler *et al.*, 1991). Alternatively, periods of remission and relapse in immunological and metabolic abnormalities may occur which finally progress to clinical IDDM, but in most cases, prove to be reversible or transient

(Palmer & McCulloch, 1991; Leslie & Pyke, 1991).

1.4 Aetiology and Pathogenesis of Type I Diabetes Mellitus

The development of IDDM is a multifactorial process involving an interplay of genetics (nature), environment (nurture) and their interactions with the immune system. In a diabetic individual all these factors combine in a highly complex and coordinated way, leading to the inevitable destruction of the β-cell. These factors are extensively covered in a review by Thai & Eisenbarth (1993), and are outlined briefly in the following sections.

1.4i Genetic Susceptibility

Although it is accepted that the development of IDDM occurs in genetically susceptible individuals, the exact genetic basis currently is not well defined, and may vary from family to family. For the last decade, a genetic region called the HLA (on chromosome 6p21), which affects immune response and allows lymphocytes to distinguish between "self" and "non-self", has been known to figure strongly in the susceptibility of IDDM (Morton *et al.*, 1983; Todd, 1994). There are several series of HLA antigens including HLA-A, B, Cw, D, and DR, and the overall mix of antigenic specificities is characteristic of an individual and can be recognised as 'self' by T lymphocytes. More than 90% of Caucasians with IDDM express either HLA-DR3 and/or -DR4. Only 2% of the population have *both* DR3 and DR4 alleles, but they represent about 40% of people with Type I diabetes (Segall, 1988).

Until recently, the region of the insulin gene (INS) on chromosome 11p15 is the only other site in the human genome at which variations clearly influence susceptibility to IDDM (Owerbach *et al.*, 1990; Spielman *et al.*, 1993). With the use of high-resolution genetic linkage maps (Gyapay *et al.*, 1994), and the application of fluorescence-based, automated DNA-fragment technology, human genome-wide exclusion mapping has now been greatly facilitated (Ziegle *et al.*, 1992; Reed *et al.*, 1994). A break-through study using these approaches has demonstrated that eighteen different chromosome regions show positive evidence of linkage to the disease in addition to IDDM1 (in the major histocompatibility

complex on chromosome 6p21) and IDDM2 (in the insulin gene region on chromosome 11p15). This indicates polygenic inheritance with a major locus at the major histocompatibility complex (Davies *et al.*, 1994).

In contrast to susceptibility-associated haplotypes, family and population studies have shown that DR2-bearing haplotypes provide protection from diabetes (Sheehy *et al.*, 1989; Baisch *et al.*, 1990). In the long term, the identification of genes associated with diabetes will permit a more accurate assessment of the individual's risk of developing diabetes. This in turn may lead to pharmacological approaches for preventing or ameliorating the genetic effects in these individuals.

1.4ii Environmental Factors

Although a genetic component is a major factor contributing to the development of IDDM, the existence of discordant monozygotic twins suggests a role for environmental factors in the aetiology of diabetes. Some environmental factors such as breast feeding (Mayer *et al.*, 1988), and dietary intervention (Elliot *et al.*, 1988) appear to be protective whereas, some viral infections appear to lead to the development of diabetes (Rubinstein *et al.*, 1982).

The importance of environmental modulation of the immunopathogenesis of IDDM has been demonstrated in studies of twins where concordance for IDDM in identical twins varies between 30-50% (Barnett *et al.*, 1981; Srikanta *et al.*, 1986). Furthermore, the rate of concordance varies with DR haplotype, but it still never reaches the 100% that would be expected if IDDM was purely determined by genetics.

Environmental influences have been shown to modify the incidence and prevalence of diabetes in animal models. For example, dietary modifications have been shown to influence auto-immunity. Most closely investigated has been the effect of dietary protein, particularly semi-synthetic diets on the incidence of diabetes in BioBred (BB) rats, and non-obese diabetic mice (NOD) (Elliott & Martin, 1984; Elliott *et al.*, 1988). If begun at weaning, such diets reduce the incidence of diabetes substantially (Scott *et al.*, 1985). In another study a diet

deficient in essential fatty acids prevented the onset of diabetes in the BB rat by affecting monocyte/macrophage function (Lefkowith *et al.*, 1990).

There is well documented evidence that viral infections may induce the development of IDDM. In particular the mumps, Coxsackie B4, and rubella viruses have all been linked to the development of IDDM (Campbell & Harrison, 1989; Yoon, 1990). However, the mechanisms by which viruses trigger the development of spontaneous forms of auto-immune diabetes remains to be elucidated. Several suggestions include:1) a direct interaction with islet β cells altering the antigenicity of islet autoantigens, as in the case of streptozotocin or Coxsackie B4 virus; 2) an anti-beta cell response elicited by molecular mimicry, so that the infected cell expresses a viral protein on its surface, thereby marking the cell as non-self; or alternatively 3), viruses may interfere with the immune system, altering the ratios of circulating immune cell subpopulations, leading to the destruction of suppressor cells or activation of autoreactive effector cells which initiate the autoimmune process. It is possible any number of viruses may trigger the development of diabetes, but so far, serological studies have provided no firm evidence. Moreover, a virus infection may precipitate the onset of hyperglycaemia in a subject with existing autoimmune destruction of islets without any previous evidence of IDDM.

1.4iii Immunology

Islet cell antibodies (ICA), and insulin autoantibodies (IAA) have been demonstrated in the serum from patients with IDDM (Gorsuch *et al.*, 1981). Bottazzo & co-workers (1974) first reported the presence of ICAs in patients with a polyendocrine autoimmune disorder (Schmidt's syndrome), but later ICAs were detected in juvenile diabetic patients without evidence of other endocrine disorders (Lendrum *et al.*, 1975). In the majority of IDDM patients, ICAs tend to disappear early (in a few weeks or months) after clinical diagnosis. Thus, the presence of ICAs in unaffected relatives of diabetic patients can be used as an immune marker of β cell damage (Bottazzo *et al.*, 1980), however, there may be a long interval between the first detection of ICA's in an at-risk subject before there is evidence of defective insulin secretion.

Autoantibodies to insulin (IAA) also have been detected in untreated newly diagnosed diabetic patients (Palmer *et al.*, 1983). Moreover there is a good correlation between IAAs and ICAs and these are useful for predicting the development of overt diabetes (Wilkin *et al.*, 1985; Srikanta *et al.*, 1986). A five year follow-up study, revealed that 70% of individuals with both antibodies developed diabetes, compared with a 42% incidence in those positive for ICA alone, and only 17% where IAA was present (Ziegler *et al.*,1989).

1.5 Complications associated with IDDM

Vascular disease is linked to duration of diabetes and is the main cause of increased morbidity and mortality in both insulin-dependent diabetes (IDDM), and non-insulin dependent diabetes (NIDDM). Diabetes predisposes to disease of both large (macroangiopathy) and small (microangiopathy) blood vessels.

1.5i Macroangiopathy

The term "macrovascular" disease refers to atherosclerotic disease of conduit arteries of the cerebral, coronary and peripheral circulations. Atherosclerosis is the major cause of morbidity and premature death in both IDDM and NIDDM (Kannel & McGee, 1979; Keen *et al.*, 1981). Diabetic patients on average have a higher incidence of atherosclerosis than the non-diabetic population, although the pathological features do not appear to differ. There is however a difference in anatomical distribution, with diabetic patients being more likely to have atherosclerosis affecting vessels below the knee.

The accelerated atherosclerosis of diabetes is most likely to be the result of a number of different processes, but as yet these are poorly understood. Several hypotheses have been implicated, including alterations in endothelial cell-platelet interactions and lipid and lipoprotein metabolism. Hyperglycaemia (especially for peripheral vascular disease) and increased levels of low-density (LDL) and very low-density lipoproteins (VLDL) may adversely affect the endothelium (Tzagournis, 1989). The increased incidence of hypertension in patients with diabetes also increases the risk of vascular endothelial injury leading to

macrophage and platelet aggregation, release of growth factors that stimulate the proliferation of smooth muscle cells and the deposition of lipid-laden foam cells.

1.5ii Microangiopathy

It was Nettleship in 1886 who first identified the microvasculature (small blood vessels-arterioles, venules and capillaries) as being affected by diabetes mellitus; however, it was the careful studies of Lundbaek (1953), who recognised a "triopathy" of complications, including retinal damage, renal disease and neuropathy. This "triopathy" was later more broadly termed "microangiopathy", when subsequent studies showed other microvascular beds and tissues were influenced by diabetes. These included those of the heart (Ledet *et al.*, 1979), skin (Tooke 1980; Rayman *et al.*,1985), and subcutaneous and skeletal muscle tissue (Faris *et al.*, 1983)

Diabetic microvascular disease is characterised by changes to both the cellular and basement membrane components of the vascular network. The cellular component is made up of two major cell types, the endothelial cells and the vascular smooth muscle cells. The basement membrane provides extracellular support and a permeability barrier for blood vessels. The functional and structural abnormalities of these two components in diabetes are described later in this chapter (section 1.7).

1.5iii Retinopathy

Diabetic retinopathy is the most common microvascular complication affecting nearly all insulin-dependent diabetics in the long-term. Thus, approximately 90% of diabetic patients will have background retinopathy, and 50% will have proliferative changes (Klein *et al.*, 1984) after 15-20 years. Although genetic susceptibility, hypertension and lipid levels all influence the final clinical outcome of retinopathy, hyperglycaemia undoubtedly plays a major role in its pathogenesis (Weber *et al.*, 1986; Chase *et al.*, 1989; DCCT, 1993).

Haemodynamics studies have provided evidence that the development of diabetic retinopathy

is associated with an elevation in retinal blood volume (Kohner *et al.*, 1975), and vasodilation of the retinal microcirculation (Skovborg *et al.*, 1969). There is a direct relationship between diabetic retinopathy and retinal blood flow since diabetic patients without retinopathy have a blood flow similar to that of non-diabetics; whereas, patients with increasing severity of retinopathy have step-wise increases in blood flow (Patel *et al.*, 1992). The association of increased blood flow with worsening severity of retinopathy was also reported by Feke *et al.* (1985). Moreover, studies with fluorescein have demonstrated an increased permeability of the blood-retinal barrier in patients with diabetes (Cunha-Vaz *et al.*, 1975; Waltman *et al.*, 1978). This fluorescein leakage is augmented in poorly controlled patients, and can be reduced by improved diabetic control (White *et al.*, 1982).

One of the biochemical mechanisms underlying the development of retinopathy is the formation and accumulation of hyperglycaemia-induced advanced glycation end products (AGE) leading to extensive cross-linking and basement membrane thickening. The long term administration of aminoguanidine, an inhibitor of AGE formation has been shown to prevent the progression of experimental diabetic retinopathy in the rat (Hammes *et al.*, 1995). Moreover, an *in vitro* toxicity study using bovine retinal pericytes and endothelial cells demonstrated that increasing concentrations of AGE-modified bovine serum albumin (AGE-BSA) were significantly more toxic to bovine pericytes than endothelial cells, and that AGE-BSA was mitogenic to retinal endothelial cells (Lambourne *et al.*, 1996).

Thus, hyperglycaemia has a direct deleterious effect on both pericytes and endothelial cells, and at the same time increased blood flow results in damage because of the marked increase in shear stress. As a consequence there is damage to the vessel wall, occlusion of some vessels, hypoxia and ischaemia, resulting in proliferative retinopathy. The exact interactions between the individual factors and underlying biochemical and cellular changes need to be further elucidated, although the early steps involved in the evolution of diabetic retinopathy are now closer to being defined and eventually prevented (Kohner *et al.*, 1995).

1.5iv Nephropathy

Approximately 30-40% of patients with insulin-dependent diabetes will develop overt clinical nephropathy (Krowleski *et al.*, 1985; Andersen *et al.*, 1983). Despite the increased ability to control the metabolic disturbances of diabetes mellitus, diabetic nephropathy still remains a major cause of morbidity and mortality. Nephropathy is not usually detected until at least five years duration of IDDM, after which the incidence increases dramatically, peaking at 15-20 years after diagnosis (Lestradet *et al.*, 1981; Kofoed-Enevoldsen *et al.*, 1987).

Clinical nephropathy is diagnosed when proteinuria (albuminuria) exceeds 0.5g/24 hours (Viberti & Keen, 1984). Albuminuria is due to glomerular capillary damage and reflects generalised damage to the microcirculation and large vessels. Hypertension accelerates the rate at which albuminuria increases and glomerular filtration rate declines. A period of increased urinary albumin excretion, called microalbuminuria (albumin excretion rate 20-200μg/min) usually precedes the development of proteinuria (Viberti *et al.*, 1982; Mogensen & Christensen, 1984). Even at this stage, renal damage is likely to have already started. The finding of an elevation in glomerular filtration rate (GRF) preceding the development of microalbuminuria implies that hyperfiltration plays a role in the pathogenetic process leading to diabetic nephropathy (Mogensen & Andersen, 1973; Christiansen *et al.*, 1981). Possible causes of hyperfiltration include increased renal plasma flow and filtration area in the glomerulus.

Experimental studies in diabetic rats support the relationship of glomerular hyperfiltration to the subsequent development of clinical nephropathy (Zatz *et al.*, 1985; Anderson & Brenner, 1988). Indeed, retrospective studies of patients with IDDM have shown that GFR is a predictor of diabetic nephropathy (Mogensen & Christensen, 1984; Mogensen, 1986), although in these studies the influence of long-term metabolic control was not assessed.

However, in a more recent study of adolescent IDDM patients with a disease duration of eight years, an increased GFR was found to predict both incipient and overt diabetic nephropathy, independent of the degree of metabolic control (Rudberg *et al.*, 1992). This study also

showed that an increase in albumin excretion was evident prior to a rise in blood pressure in patients who developed incipient or overt nephropathy.

Intervention therapies such as tightening glycaemic control (Feldt-Rasmussen *et al.*, 1986; DCCT, 1993), dietary protein restriction and the treatment of hypertension with hypotensive agents (Mogensen, 1982; Parving *et al.*, 1983a) have all been shown to be effective in reducing some of the functional abnormalities associated with diabetic nephropathy. Specific treatment with angiotensin-converting enzyme (ACE) inhibitors in IDDM patients with microalbuminuria has also led to a delay and a possible prevention in both increased urinary albumin excretion and deterioration in kidney function (Marre *et al.*, 1988; Mathiesen *et al.*, 1991). Studies in animal models of diabetes have suggested that ACE inhibition directly reduces intra-glomerular pressure, prevents the increase in AER, and lessens the development of glomerular histological lesions (Zatz *et al.*, 1986; Anderson *et al.*, 1989). Further evidence for the benefit of ACE inhibition in the treatment of IDDM patients was recently provided in two large randomised multi-centre trials. These studies demonstrated that treatment with the ACE inhibitor captopril, significantly ameliorated the progression of IDDM patients with microalbuminuria to clinical proteinuria (Viberti *et al.*, 1994; Laffel *et al.*, 1995).

1.5v Neuropathy

The epidemiology of diabetic neuropathy is unclear due to inconsistent definitions of what constitutes neuropathy. The prevalence and incidence of neuropathy both increase with the duration of diabetes. Impaired nerve function may be recorded in up to 60% of diabetics, with one third of these developing overt neuropathy. Diabetic neuropathy has a wide variety of manifestations which include reduced sensations in the feet leading to ulceration, atrophy of the thigh muscles, and impotence as well as relatively uncommon complications associated with autonomic neuropathy such as diarrhoea, postural hypotension and gustatory sweating. Nerve biopsies show axonal degeneration, and segmental demyelination associated with changes in the vasa nervorum. Neurophysiological studies have shown reduced motor and sensory nerve conduction velocities and resistance to the failure of conduction which normally occurs when the nerve is rendered ischaemic (Sampson *et al.*, 1990).

The pathogenesis of diabetic neuropathy remains unclear, although a combination of metabolic changes and interference with nerve blood supply are likely mechanisms. Abnormalities in metabolic pathways have been well substantiated in experimental diabetic neuropathy. Hyperglycaemia-induced polyol pathway hyperactivity associated with sorbitol accumulation (Ward *et al.*, 1971; Dyck *et al.*, 1988), and myo-inositol depletion (Greene *et al.*, 1987) is a key factor in the genesis of diabetic neuropathy. The polyol pathway consists of two basic steps; the first being the reduction of glucose to sorbitol by the enzyme aldose reductase, coupled with the oxidation of NADPH to $NADP^+$, and the second is the oxidation of sorbitol to fructose coupled with the reduction of NAD^+ to NADH by sorbitol dehydrogenase.

A study of STZ-induced diabetic rats with aldose reductase treatment, demonstrated the correction of nerve functional deficits, which was due to the neurovascular action of the inhibitors that is not dependent on the restoration of nerve myo-inositol (Cameron *et al.*, 1994a). However, another study of neural dysfunction in STZ-induced diabetic rats showed that treatment with a sorbitol dehydrogenase inhibitor improved impaired caudal nerve conduction velocity and vascular dysfunction -(assessed by regional vascular albumin permeation and blood flow). This group suggested that polyol pathway-linked diabetic vascular and neural dysfunction are more closely linked to increased oxidation of sorbitol to fructose than only due to osmotic effects of elevated sorbitol levels or redox and metabolic imbalances associated with the reduction of glucose to sorbitol by aldose reductase (Tilton *et al.*, 1995).

Other proposed mechanisms associated with the development of diabetic neuropathy include; non-enzymatic glycosylation of proteins (Brownlee *et al.*, 1988), defective protein synthesis (Spritz *et al.*, 1974), a reduction in sodium-potassium-ATP-ase (Greene & Lattimer, 1983), abnormalities of axonal flow (Sidenius & Jakobsen, 1987), and abnormalities in transport and levels of nerve growth factor (Schmidt, 1993). Studies using anti-oxidant treatment in streptozotocin diabetic rats have demonstrated the role of oxidative stress associated with glycation of structural proteins in the pathogenesis of diabetic neuropathy. In these studies abnormal nerve conduction, reduced endoneurial blood flow and oxygen tension were

prevented (Cameron *et al.*, 1994b; Cotter *et al.*, 1995). More recently, the same group went on to show that nerve conduction velocity (NCV) defects in rats with experimental diabetes could be significantly improved by treatment with a novel compound ascorbyl-GLA. This compound is a combination of an anti-oxidant ascorbate, and an essential fatty acid γ-linolenic acid (GLA). The results showed the effect of the two components was additive indicating a synergistic interaction (Cameron & Cotter, 1996).

Nerve blood flow is reduced in rats with experimental diabetic neuropathy, and oxygen supplementation (Low *et al.*, 1984) or vasodilator treatment (Cameron *et al.*, 1991) improves blood flow and nerve conduction. Similarly, there is also evidence of impaired nerve blood flow, arteriovenous shunting, and a reduction in sural nerve oxygen tension in human diabetics with neuropathy (Ward 1993). Laser Doppler velocimetry has been used in animal studies to measure nerve blood flow. In one such study Doppler flux in rats with experimental diabetes was 80% that of controls after only 4 days of diabetes, and steadily decreased and formed a plateau at 40% of control values after 4 weeks. Treatment of diabetic rats with insulin prevented the reduction in nerve blood flow (Stevens *et al.*, 1994). Similarly, laser Doppler flowmetry studies in humans with diabetes have demonstrated an early functional disturbance of skin blood flow (Tooke *et al.*,1985). Subsequently, this group went on to show in diabetic patients with and without clinical evidence of microvascular complications, that some degree of sympathetic neuropathy is almost inevitable after a long duration of insulin-dependent diabetes (Tooke *et al.*, 1987).

The strong association between diabetic retinopathy, nephropathy and neuropathy suggests a common pathogenic mechanism in the development of these complications. It is likely that multiple factors are implicated in the pathogenesis of diabetic neuropathy, and although metabolic factors are likely to be the source of all diabetic complications, vascular and haemodynamic factors that cause functional impairment of the retina and kidney must also be considered to play a significant part in the initiation of clinical diabetic neuropathy (Tesfaye *et al.*, 1994; Stevens, 1995).

1.6 Glycaemic Control and the Development of Microvascular Complications

Although the cause of microvascular disease has not been clearly defined, the development of microangiopathy is positively associated with the duration and magnitude of hyperglycaemia; but other factors such as genetic predisposition, hypertension, and dyslipidemia influence its clinical course (Cahill *et al.*, 1976).

Evidence for the association between poor glycaemic control and the risk of developing microvascular complications was gathered in the 1960's and 1970's (Johnson, 1960 and Pirart, 1978). This association was confirmed in the 1980's with the introduction of HbA_{1c} which provides information about long-term metabolic control (Wiseman *et al.*, 1984; and Bangstad *et al.*, 1989). Moreover, the relationship has been strengthened by the results of the 5 year Stockholm Diabetes Intervention Study, which showed that albuminuria, deterioration of nerve conduction velocities, and the progression of non-proliferative retinopathy were ameliorated in patients treated with an intensified insulin regime, although the development of serious retinopathy requiring photocoagulation was unaffected (Reichard *et al.*, 1991a & b; Reichard, 1992).

More recently the Diabetes Control and Complications Trial provided conclusive evidence that intensified insulin treatment achieving near-normal blood glucose levels reduces the risk of microangiopathy, and the incidence of macrovascular disease (DCCT, 1993). However, the available evidence indicates that although good control of hyperglycaemia may reduce the risk of developing diabetic microangiopathy, once structural changes have occurred the process is essentially irreversible and tends to progress irrespective of good control.

1.7 Pathophysiology of Microvascular Disease in IDDM

The pathophysiological processes underlying the deterioration of the function and structure of the microvasculature are poorly understood, but alterations in organ and tissue haemodynamics, with an increase in intra-capillary pressure and blood flow leading to

enhanced vascular leakiness have been implicated (Tooke, 1986). Such alterations have been found in diverse vascular beds, including the kidney (Ditzel & Junker, 1972; Vora *et al.*, 1992), retina (Kohner *et al.*, 1975), skin (Sandeman *et al.*, 1992; Houben *et al.*, 1992), and the forearm (Halkin *et al.*, 1991). It has been suggested that this alteration of blood flow depends on precapillary vasodilatation, and this may be the initiating factor in diabetic microangiopathy.

1.7i The Haemodynamic Hypothesis

These observations have led to the generation of a haemodynamic hypothesis for the pathogenesis of diabetic complications (Parving *et al.*, 1983b; Tooke, 1986; Zatz & Brenner, 1986). It is proposed that the presence of increased blood flow and capillary hypertension early in diabetes, leads to an injury response due to repetitive endothelial damage, eventually resulting in microvascular sclerosis and consequently, impaired vasodilatation and autoregulation. In addition, genetically determined changes in the endothelial charge barrier may cause increased permeability of the membrane, and this could be a contributing feature in the development of "malignant" microangiopathy (Deckert *et al.*, 1989).

1.7ii Functional Changes

Capillary pressure and blood flow

An elevation in capillary pressure has been demonstrated in micropuncture studies of the glomeruli in diabetic rats (Zatz *et al.*, 1986), and more recently in the nail folds of young insulin-dependent diabetic patients (Sandeman *et al.*, 1992). The increase in capillary pressure appears to be related to glycaemic control, and is largely reversed when normoglycaemia is achieved. If an increase in capillary blood flow is not accompanied by capillary recruitment and/or a fall in post-capillary resistance, an increase in capillary pressure is inevitable.

Other vascular beds subjected to the characteristic haemodynamic abnormalities associated with diabetes include the eye and the kidney. In the eye, retinal blood flow is increased, and the retinal vessels themselves are dilated in diabetic patients, leading to oedema, new vessel

formation and haemorrhage, causing visual impairment and the accelerated development of cataracts (Skovborg *et al.*, 1969; Kohner *et al.*, 1975). In the kidney, glomerular filtration rate (GFR), and urinary excretion of plasma proteins are abnormally high in newly diagnosed IDDM patients, particularly during poor glycaemic control (Mogensen, 1972; Ditzel & Junker, 1972; Parving *et al.*, 1976). Strict metabolic control for twelve months has been shown to normalise glomerular hyperfiltration in IDDM patients, the GFR returning to the previously elevated level if glycaemic control is relaxed (Wiseman *et al.*, 1985).

Thus from the experimental studies described, there is little doubt that organ blood flow is increased in the early stages of diabetes. However, this functional disturbance of the regulation of the skin microcirculation evident in patients with recently diagnosed diabetes is not reported in patients with longer disease duration. Indeed a study of diabetic patients with a mean disease duration of 13.3 years, using laser Doppler flowmetry showed impaired microvascular blood flow in response to minor skin trauma. Moreover, the reduction in hyperaemic response to skin trauma was not due to a reduction in capillary numbers, since the mean density of capillaries was similar in the diabetic group compared with the age-matched non-diabetic control group (Rayman *et al.*, 1986). Similarly, in another study using laser Doppler flowmetry, diabetic patients with a long duration of diabetes (38±6years) and no clinical microangiopathic complications, had a shorter duration of reactive hyperaemia compared with patients of a similar disease duration with complications (Tooke *et al.*, 1987). These results indicate that the reactive hyperaemia response may be a useful non-invasive method for detecting the presence or possibly predicting the risk of developing complications in diabetes. In long standing diabetes, the haemodynamic abnormalities evolve, leading to a limitation of maximal blood flow and a loss of autoregulation; both of which may be the result of the microvascular sclerosis induced by prolonged hyperaemia and microvascular hypertension.

Protein leakage and capillary permeability

An alternative to the haemodynamic hypothesis has been postulated by the Steno group who have suggested that patients with microalbuminuria (a marker of incipient diabetic nephropathy), who exhibit increased risk of microangiopathy in other organs, are

characterised by increased permeability of the endothelial lining of blood vessels, which are perhaps due to genetically determined differences in the charge barrier (Deckert *et al.*, 1989).

An elevation of capillary pressure appears to be a major factor responsible for the enhanced leakage of plasma proteins through, and their deposition in the wall of the microvasculature and mesangium (Parving *et al.*, 1976);as well as increased exchangeable sodium (O'Hare *et al.*, 1983), and reduced tissue oncotic pressure (Fauchald *et al.*, 1985). Increased permeability is a cardinal feature of diabetic microangiopathy; indeed, hyperpermeability of the capillary wall to molecules of different sizes such as dopamine (Lorenzi *et al.*, 1980), fluorescein (Porta *et al.*, 1981; Bollinger *et al.*, 1982), and albumin (Parving, 1976; Fauchald *et al.*, 1985) have been demonstrated in patients with microangiopathy. The main pathway for fluid filtration across the microvascular wall is through channels between the endothelial cells, and subsequently through a glycoprotein lattice in the basement membrane (Curry & Michel, 1980). Disruption of the endothelial layer (Katz *et al.*, 1989), or glycation of proteins in the glycocalyx and basement membrane (Brownlee *et al.*, 1984), leading to an altered structure or charge are possible mechanisms that could alter pore size and therefore fluid permeability (Deckert *et al.*, 1989).

The temporal relationship between altered permeability changes and the different stages of clinical diabetic complications remains uncertain. It has been proposed that an increased transcapillary escape rate of albumin (TER_{Alb}) reflects a more generalised vascular dysfunction involving the glomerulus and retinal capillaries as well as the intima of large vessels (Deckert *et al.*, 1989). Increased fluorescein leakage occurs into the vitreous after intravenous injection in young insulin-dependent diabetic patients with no clinical retinopathy, or mild background changes only (Cunha-Vaz et *al.*, 1975, and Waltman *et al.*, 1978). Moreover, another study assessing microvascular fluid permeability (using a strain gauge plethysmographic method) in the forearm of young insulin-dependent diabetic patients showed that the capillary filtration coefficient (CFC) was significantly higher than that seen in non-diabetic control subjects (Jaap *et al.*, 1993). These insulin-dependent diabetic patients were of short disease duration, had satisfactory glycaemic control, and no evidence of microangiopathy, suggesting that the changes reflect a *primary alteration* in microvascular fluid permeability. Further support for

this concept was provided by the demonstration of increased forearm microvascular permeability in insulin-dependent patients with incipient nephropathy, whereas fluid permeability in patients with a long disease duration and little evidence of microangiopathy was found to be no different from that in control subjects (Jaap *et al.*, 1996). The authors speculate that only those patients who have a high capillary filtration coefficient will go on to develop severe microvascular complications; although this hypothesis can only be confirmed by prospective studies following insulin-dependent diabetic patients from the time of diagnosis.

Rheological Factors

Abnormal plasma viscosity, whole blood viscosity (Barnes *et al.*, 1977), platelet function, and red cell deformability (McMillan *et al.*, 1978) have been shown to impair blood flow, and may contribute to the development of microangiopathy in diabetes. Furthermore, the degree of disturbance of blood rheology is related to glycaemic control. Platelets from diabetic patients show an increased sensitivity to aggregation induced by adrenaline, ADP, or collagen, and there is some evidence that this increased sensitivity correlates with the development of certain diabetic complications (Mustard & Packham, 1977). The inability of the less flexible cells to deform, may enhance the tangential stress upon the microvascular wall, enhancing the effects of capillary hypertension, and also may predispose the tissues to hypoxia and poor nutrition.

Increased tangential stress also stimulates the endothelial release of vasodilator substances such as endothelium derived relaxing factor (EDRF) and prostacyclin. The production of prostacyclin, prostaglandins and EDRF by the endothelial cell inhibits the aggregation of platelets on the luminal surface of the vessel, as well as modulating vascular tone. The influence of tangential stress on the function of the endothelium will be discussed in more detail in chapter 2.

1.7iii Structural Changes

Capillary Basement Membrane Thickening

Increased shear stress stimulates the endothelial synthesis and expansion of the mesangial

matrix which may be important in the development of basement membrane thickening. Increased thickness of the capillary basement membrane (CBM) is the third pathophysiological process, and is considered to be the hallmark of diabetic microangiopathy (Keen & Jarrett, 1982; Parving *et al.*, 1983b; Cagliero *et al.*, 1988).

Morphometric studies have revealed basement membrane thickening in the kidney, retina, skin, skeletal muscle, brain and heart in diabetic patients (Johnson *et al.*, 1981 & 1982; Osterby & Hansen, 1965; Tilton *et al.*, 1986; Silver *et al.*, 1977; Siperstein *et al.*, 1968). There is a direct relationship between hyperglycaemia and CBM thickness. Using tumour cells which produce basement membrane materials, it has been demonstrated that exposure to hyperglycaemia caused an enhanced synthesis of basement membrane (Ledbetter *et al.*, 1987). Similarly, exposure to an elevated glucose concentration caused an increase in the expression of collagen IV and fibronectin mRNA levels as a result of increased gene transcription in umbilical vein endothelial cells (Cagliero *et al.*, 1988). On the other hand intensive therapy to produce near-normoglycaemia has been shown to bring about a significant reduction in the width of the CBM in diabetic patients (Petersen *et al.*,1980; Raskin *et al.*, 1983).

The mechanism leading to the increase in basement membrane thickness is not merely a consequence of change in the metabolic environment. Increased capillary pressure, and/or the proliferative response of the endothelium to vascular injury may act as an important stimulus for its formation (Williamson & Kilo, 1977). Furthermore, it has been shown that CBM thickening in diabetes is progressively more marked the further the tissue sample is taken below the heart (Vracko & Benditt, 1970; Tilton *et al.*, 1981), presumably reflecting the influence of hydrostatic load (Tooke, 1986).

Another possibility is the increased hydrostatic pressure may cause an increased permeation of plasma proteins into the vessel wall, and induce a limited proliferative response with the synthesis of increased amounts of basement membrane. Histochemical analysis has shown increased membrane glycosylation which may favour the increased binding of plasma proteins, and it has been suggested that glycosylated collagen is more resistant to normal

processes of degradation (Rohrbach *et al.*, 1982).

This chapter has detailed the macro- and microvascular complications of diabetes mellitus, and has highlighted the significant abnormalities in the microcirculation of many vascular beds in diabetic patients. Microvascular disease is the hallmark of diabetes, and microangiopathy greatly increases the morbidity of IDDM patients. A number of studies have shown that the vasoregulatory function of the endothelium lining the small blood vessels, is impaired in diabetes and this phenomenon may be relevant to the pathogenesis of diabetic microangiopathy (Cohen, 1993). Altered production of endothelium-derived vasoactive factors such as endothelium-dependent relaxing factor (EDRF) may be partly responsible for these abnormalities (Tilton *et al.*, 1993; Komers *et al.*, 1994). In the next chapter, the structure and function of the normal microcirculation, and the vascular endothelium is reviewed, with particular emphasis on the role of the endothelium in the modulation of vascular tone and changes in endothelial function associated with diabetes.

CHAPTER TWO

THE MICROCIRCULATION AND VASCULAR ENDOTHELIUM

2.1 Function of the Microcirculation

The microcirculation is a network of microscopically small blood channels including the arterioles, capillaries and venules. In a "typical" microvascular bed the arterioles further differentiate into capillaries, which are structurally characterised by a vessel wall consisting of a single layer of endothelial cells, surrounded by their basement membranes which split to enclose occasional cells called pericytes. The capillaries are the major exchange vessels, and the nutrients required to sustain the cells in the tissue flow across their surface.

The main role of the precapillary vessels within the cardiovascular system is to distribute blood so that each capillary receives an appropriate blood flow at the correct pressure. This is achieved in the short-term by relaxation/contraction of the smooth muscle cells (Pohl *et al.*, 1986a), and in the long-term by the reorganisation of vascular wall cellular and extra-cellular components (Langille & O'Donnell, 1986), resulting in the adjustment of the lumen diameter and therefore the resistance to flow in each vessel.

Intravascular measurements indicate that up to 50% or more of the resistance to flow appears to lie proximal to vessels with diameters of 100μm, depending on the vascular bed studied. Most of the total resistance to flow is provided by the small arteries and arterioles known as *resistance arteries*, and changes in their internal diameter (<500μm) regulate peripheral resistance. These small arteries may be defined by two functional criteria: 1) those vessels proximal to the arterioles that contribute substantially to the precapillary resistance (Mulvany & Aalkjaer, 1990), and 2) the resistance vessels should be at a site of potential physiological control (Duling, 1991).

2.2 Structure and Function of Resistance Arteries

The structure of the resistance arteries, i.e. arteries with a lumen diameter of approximately 500μm or less (Bloom & Fawcett, 1968) is similar to that of the large arteries. The vascular wall consists of three layers, an outer tunica adventitia, a central tunica media, and an inner tunica intima (Mulvany & Aalkjaer, 1990).

2.2i Adventitia

This outer layer which is more prominent in the small muscular arteries contains connective tissue (made up of elastin and collagen), fibroblasts, mast cells, macrophages, as well as occasional Schwann cells and associated nerve axons (Lee *et al.*,(a) 1983). The sparsely arranged nerves are all contained within the adventitia and do not penetrate the media. This layer also contains the blood supply, which is made up of arterioles, venules, capillaries and lymphatic vessels, known collectively as the vasovasorum.

2.2ii Media

The tunica media of small arteries is bounded by a prominent internal elastic lamina on the luminal side, and a less well-defined external lamina which lies between the media and the adventitia. The media largely comprises smooth muscle cells which are arranged circumferentially. The number of smooth muscle layers in the media of the small arteries decreases with decreasing vessel diameter, so that vessels with a diameter of 300μm have approximately six layers (Lee *et al.*,(b) 1983), whereas, 30-50μm arterioles have a single layer of smooth muscle cells (Walmsley, 1982). There are few, if any nerve endings in the media, and it appears that the smooth muscle cells act as an electrical syncytium responding to signals generated in the adventitial layer by the perivascular nerve endings.

2.2iii Intima

The intima is composed of three layers, of which the innermost, the endothelium is in direct contact with the blood, and forms a continuous lining of the vascular tree. Surrounding the endothelium is a sub-endothelial layer consisting of elastic fibres and collagen fibrils, and the outer layer forming the internal elastic lamina which makes contact with the smooth muscle cells within the tunica media (Carlson *et al.*, 1982).

2.3 The Peripheral Circulation and its Control

The peripheral circulation is normally tightly regulated by a combination of control mechanisms. These include the autonomic nervous system, local reflexes, circulating mediators and locally produced vasoactive substances such as nitric oxide (vasodilator) and endothelin (vasoconstrictor). In its strictest sense, autoregulation is defined as the ability of the vessels to keep blood flow constant despite changes in perfusion pressure (Johnson, 1964). A wider role for autoregulation is thought to be the ability of the vessels to control blood flow to meet the metabolic demands of the tissues (Guyton &Coleman,1967). This must be done without the compensation of general body functions which rely upon the tight control of blood flow in various vascular beds, such as the maintenance of arterial blood pressure and core temperature of the body. The meeting of these specifications depends upon a complicated array of local and extrinsic mechanisms controlling blood flow through the microcirculation.

The tissue responsible for the control of total peripheral resistance, arterial and venous tone, and the distribution of blood throughout the body is the vascular smooth muscle; the contraction of which is mediated by a combination of neural and humoral factors. The vascular smooth muscle cells are small, mononucleated, spindle-shaped cells which are generally arranged in helical layers around the lumen of the vessel. They contain large numbers of thin actin filaments, and relatively small numbers of thick myosin filaments (Belloni & Sparks, 1978).

Receptors exist on the surface of the vascular smooth muscle cells and the endothelium for a variety of peptide and non-peptide neurohumoral mediators such as catecholamines, histamine, acetylcholine, serotonin, angiotensin, adenosine, and prostaglandins. It is the distribution of these receptors which partly determine the reactivity of a particular vascular bed (Vanhoutte, 1986). Once the receptors in the vascular smooth muscle membrane are activated by an agonist, excitation-contraction coupling occurs. Briefly, the receptors stimulate phospholipase C in a reaction coupled to guanine nucleotide binding proteins (G-proteins), which leads to (via a series of steps) the release of Ca^{2+} from the sarcoplasmic reticulum. The Ca^{2+} then binds to calmodulin, which stimulates the movement of actin/

myosin filaments via activation of the myosin light chain kinase resulting in the contraction of the vessel. The converse occurs during relaxation, where myosin light chain kinase is inactivated by dephosphorylation and the Ca^{2+} is actively pumped out of the vascular smooth muscle cell by the Ca^{2+} pump, and the Na^{+}/Ca^{2+} exchanger (Berne & Levy, 1993).

Myogenic activity in the smooth muscle of small arteries and arterioles appears to be a major component of the *basal tone* evident in the resistance arteries of most vascular beds (Folkow, 1949, 1952), and is independent of nervous control. This myogenic tone is the element on which all other control factors act in order to modulate local blood flow; and it creates the basis for most of the blood flow reserve that tissues are able to recruit when their metabolic needs increase (Johansson, 1989).

The tone of a vessel basically refers to a sustained state of contraction in the muscle, and it is an active process of the contractile proteins. Theories for its functional role include participation in the regulation of blood flow, capillary pressure and vascular resistance (Johnson, 1980). One or more of the following processes may be involved in producing this tonic activity including: 1) an expression of myogenic activity in response to a stretch stimulus, 2) a high oxygen tension of arterial blood, 3) the presence of Ca^{2+}, or 4) some other factor present in the plasma. It is now firmly established that the control of blood flow by the local regulation of vascular smooth muscle tone is mediated by the release of vasoactive substances from the endothelium; details of which will be discussed in subsequent sections.

Calcium is important in the myogenic mechanism for the transduction of the contractile response to stretch (Harder, 1984; Laher & Bevan, 1989; Nakayama, 1982). However, other second messengers such as protein kinase C also may be involved in the myogenic response, by altering the sensitivity of the smooth muscle contractile process to calcium (Meininger *et al.*, 1991). Pharmacological studies indicate that myogenic tone can be enhanced by the activation of protein kinase C, and attenuated by its inhibition (Laher *et al.*, 1989).

2.3i Sympathetic Constrictor Influence on Resistance Vessels

With the exception of capillaries, all blood vessels contain smooth muscle and receive vasoconstrictor fibres from the sympathetic nervous system. However, the small arteries and arterioles are most densely innervated suggesting that the sympathetic nervous system may play a major role in regulating tissue blood flow and peripheral resistance (Berne & Levy, 1993). The nerves supplying the small/resistance arteries are located in the adventitia with little or no penetration into the vascular media (Mulvany & Aalkjaer, 1990). These adrenergic nerves consist of axons interspersed at regular intervals with varicosities. Noradrenaline is the major neurotransmitter of the sympathetic nervous system, causing vasoconstriction via α-adrenergic receptors and vasodilation via β-adrenergic receptors.

The vascular response to noradrenaline ultimately depends on the amount of noradrenaline released into the synaptic cleft. This depends on the density of sympathetic innervation and on the pre-synaptic regulating mechanisms at the neuroeffector junction. These include neuronal amine re-uptake and adrenergic auto-inhibitory receptors. After release from the pre-synaptic nerve terminal, noradrenaline crosses the synaptic cleft, and combines with adrenergic receptors initiating a post-synaptic response. These receptors (α_1,α_2,β_1,β_2) have been categorised based on pharmacological specificity and physiological actions, and are described in detail in a review article by Raymond *et al.*, 1990. In general, α_2 receptors are located in pre-synaptic regions where they modulate the release of noradrenaline from nerve endings and have an autoinhibitory effect on the amount of noradrenaline released into the synaptic cleft. In contrast, post-synaptic receptors can be entirely α_1, or α_2 or a mixture of both. Vascular smooth muscle has both sub-types, and stimulation causes contraction and an increased peripheral resistance.

Likewise, β-adrenoreceptors also are known to exist in the form of two subtypes. The β_1 receptors are always located on the membrane of the effector cell, and are therefore post-synaptic. Whereas, the β_2 receptors although mainly post-synaptic, may also be found in pre-synaptic locations, and stimulation of these β_2 receptors enhances the release of noradrenaline.

2.4 The Endothelium

The endothelium is the innermost lining of the blood vessel wall, thereby forming the cellular lining of the entire circulatory system including the lymphatic channels (Bennett *et al.*, 1959). The single layer of endothelial cells which constitute the vascular endothelium, are flattened, uniform and elongated, approximately 25-50μ long, and 10-15μ wide, with their long-axis orientated in the direction of blood flow (Simionescu & Simionescu, 1977). The endothelial cell contains all of the usual cytoplasmic organelles, including a prominent oval nucleus, smooth and rough endoplasmic reticulum, ribosomes, mitochondria, Golgi apparatus, lysosomes, glycogen granules, and thin and thick filaments containing actin and myosin (Florey, 1966: Rhodin, 1968; Haudenschild *et al.*, 1975).

Its ability to act as a selective exchange surface is determined by haemodynamic factors such as: microvascular flow rate, the size and charge of the molecule concerned, the permeability of the capillary wall for the exchange product, and the surface area available for the exchange to take place (Tooke, 1987). Endothelial cells interact with their neighbours by two basic mechanisms; firstly by humoral mechanisms, i.e. the synthesis and secretion of endothelial products into the extracellular fluid; and secondly, by direct contact between other endothelial and vascular cells via communicating gap junctions (Davies *et al.*, 1988).

It is now known that the endothelium is a vast organ with multiple functions and plays a pivotal role in maintaining homeostasis of the blood vessels. The endothelium plays an important role in the regulation of vascular tone by modulating the degree of contraction of the smooth muscle that surrounds it by the synthesis and release of biologically active substances, such as endothelium-derived relaxing factor (EDRF) and endothelium-derived contracting factor (EDCF) (Vanhoutte, 1989). It also synthesises and releases anticoagulant and fibrinolytic factors; secretes growth factors; binds lipoprotein lipase and metabolises lipoproteins. It converts the inactive angiotensin I to the vasoconstrictor angiotensin II, through the action of angiotensin converting enzyme (ACE), which also inactivates the vasodilator bradykinin, and the synthesis of prostaglandins, e.g. prostacyclin.

2.4i The Discovery of Endothelium-Derived Relaxing Factor

Many endothelium-derived substances may modify vascular tone, but the most significant finding of the importance of endothelial cells in local cardiovascular control was first provided by Furchgott and Zawadzki in 1980(a), who demonstrated endothelium-dependent relaxation to the vasodilator acetylcholine (ACh). They also showed that the relaxation induced by ACh was dependent on the presence of an intact endothelium. Furchgott and Zawadzki went on to demonstrate that the relaxing effect of acetylcholine on strips of rabbit aorta was mediated indirectly by the release of a labile substance from the endothelial cells, acting on the smooth muscle cells. This unknown substance was named endothelium-dependent relaxing factor or EDRF.

The local nature of EDRF was first demonstrated using two rabbit aortic strips suspended together in a "sandwich" arrangement. In this procedure, a transverse strip freed of endothelial cells was tested when mounted separately and also when mounted intimal surface against intimal surface, with a longitudinal strip of the same width and length with endothelial cells present ("sandwich mount"). Using this technique the endothelium-free transverse strip, which alone did not relax in response to ACh, now exhibited good relaxation in response to that agent. This was attributable to the transfer of a biologically active substance from the ACh-stimulated endothelial cells of the donor vessel (longitudinal strip) to the detector vessel (transverse strip denuded of endothelium) (Furchgott & Zawadski, 1980b; Furchgott *et al.*, 1981). A different approach confirming the release of EDRF from the endothelium involved superfusing an endothelium-denuded aortic ring with the perfusate from an intact isolated rabbit aorta (Griffith *et al.*, 1984; Rubanyi *et al.*, 1985). By increasing the length and therefore the time before the perfusate reached the detector vessels, it was established that EDRF had a biological half-life of only a few seconds in oxygenated physiological salt solutions (Griffith *et al.*, 1984; Cocks *et al.*, 1985).

Acetylcholine or carbachol-mediated endothelium-dependent relaxation also has been demonstrated in small resistance arteries; from guinea pig mesenterium (Bolton & Clapp, 1986), rabbit ear (Owen & Bevan, 1985), rat (DeMey & Gray, 1985) and rabbit mesentery

(Furchgott *et al.*, 1987), as well as in human subcutaneous vessels (Mc Nally *et al.*, 1994). Furthermore these studies revealed that the potency of acetylcholine increased with decreasing vessel diameter, possibly reflecting the shorter diffusion distance from the endothelium to the outermost muscle cell layer in the smaller vessels.

2.4ii The Identification of EDRF as Nitric Oxide

The discovery of acetylcholine-mediated endothelium-dependent relaxation stimulated the development of bioassays to characterise the endothelial substance(s) responsible for this effect. Early studies indicated that EDRF may be a lipoxygenase product (Singer & Peach, 1983; Forstermann and Neufang, 1984), or a product of the cytochrome P-450 enzyme system (Pinto *et al.*, 1986; Macdonald *et al.*, 1986), or an unstable compound with a carbonyl group at or near its active site (Griffith *et al.*, 1984). However all of these suggestions of chemical structures for EDRF were discounted by subsequent studies.

In 1977, some three years before the discovery of endothelium-dependent relaxation, Katsuki and co-workers proposed that what they termed "nitrovasodilators" (including glyceryl trinitrate, nitroglycerin) activated soluble guanylate cyclase in vascular smooth muscle in response to the release of nitric oxide, and the resulting increase in cyclic GMP lead to smooth muscle relaxation. A decade later, the possible identity of EDRF as nitric oxide was suggested simultaneously by two laboratories based on the comparison of the properties of both substances (Khan & Furchgott, 1987; Ignarro *et al.*, 1987). Thus the response to both EDRF and nitric oxide were shown to be inhibited by haemoglobin, and methylene blue (Martin *et al.*, 1985), and that superoxide anions (O^{2-}) contributed to the instability of EDRF, as the scavenger of superoxide anions, superoxide dismutase, prolonged the action of EDRF (Gryglewski *et al.*, 1986a; Rubanyi & Vanhoutte, 1986).

The strong evidence that EDRF was identical to nitric oxide came from Palmer *et al.*, in 1987 who reported that endothelial cells stimulated with bradykinin released nitric oxide in amounts sufficient to account for the actions of EDRF. The release of EDRF as NO was measured directly as the chemiluminescent product of its reaction with ozone (Downes *et al.*,

1976). Palmer *et al.*, (1988b) then went on to show that N^G-monomethyl-L-arginine prevented the formation of nitric oxide by cultured porcine endothelial cells, and this effect could be overcome by incubation with L-arginine. Subsequent studies demonstrated that both EDRF and NO inhibited platelet aggregation (Radomski *et al.*, 1987a), induced the disaggregation of aggregated platelets (Radomski *et al.*, 1987b), and attenuated platelet adhesion (Radomski *et al.*, 1987c, d).

Although the identification of EDRF as nitric oxide was strongly supported by experimental evidence, some controversy about the chemical nature of NO still remains. These include wide variations in the estimated biological half-life of EDRF which range from 3 to 5 seconds (Griffith *et al.*, 1984; Rubanyi *et al.*, 1985; Gryglewski *et al.*, 1986); differential binding of EDRF and NO to anion exchange columns (Cocks *et al.*, 1985; Long *et al.*, 1987); and differential biological activity on smooth muscle preparations (Shikano & Berkowitz, 1987; Dusting *et al.*, 1988). Despite these discrepancies, it is generally accepted that EDRF is NO or a closely related species, e.g. an S-nitrosothiol (Myers *et al.*, 1990; Furchgott, 1990; Stamler *et al.*, 1992).

2.4iii The Synthesis and Release of EDRF

In 1988 Palmer and colleagues demonstrated that cultured vascular porcine endothelial cells synthesized nitric oxide from the terminal guanidino-nitrogen atom of the amino acid L-arginine. Once synthesized by the reduced NADPH-dependent dioxygenase enzyme now referred to as nitric oxide synthase (or synthetase), EDRF/NO diffuses from the endothelium to the underlying vascular smooth muscle where it exerts its vasodilatory action by activating guanylate cyclase, resulting in a rise in intracellular cyclic guanine 3'5'-monophosphate (cGMP) (Rapoport, 1983).

The nitric oxide synthase enzyme can exist in two isoforms; a calcium and calmodulin-dependent NO-synthase (Mayer *et al.*, 1989; Mulsch *et al.*, 1989), which is mainly present in endothelial cells and neurones, and a Ca^{2+}-independent inducible NO-synthase found in cytokine-activated cells, such as macrophages, and smooth muscle as well as in endothelial

cells (Forstermann *et al.*, 1991; Dinerman *et al.*, 1993).

As well as basal release, endothelial NO synthesis can be further stimulated by both receptor-dependent (e.g. acetylcholine, ATP, or bradykinin) (De Mey & Vanhoutte,1981; Cherry *et al.*, 1982) and independent agonists (e.g. Ca^{2+} ionophores, or Ca^{2+}-ATP-ase inhibitors) (Zawadzki *et al.*, 1980; Furchgott, 1981); as well as physical stimuli such as pulsatile flow, shear stress, (Pohl *et al.*, 1986a; Rubanyi *et al.*, 1986) and hypoxia (Pohl & Busse, 1989).

2.4iv Basal Release of NO/EDRF

The endothelium exerts an important basal dilator influence on blood vessels, continuously adjusting smooth muscle tone through the release of NO from the luminal side of the vessel, counterbalancing the constrictor response of noradrenaline, ATP, and neuropeptide Y released onto the adventitial side of the vessel by the sympathetic nervous system (Vallance, 1992). The overall rate of NO formation under basal (i.e. static) conditions is somewhat diminished in cultured endothelial cells, indicating the endothelial cells require a constant stimulus e.g. shear stress to maintain NO-synthetase expression. Basal release of NO is predominantly dependent on an intact endothelium, and its removal or incubation with a nitric oxide synthetase inhibitor usually leads to vasoconstriction (Palmer *et al.*, 1988b).

In vitro studies demonstrated that N^G-monomethyl-L-arginine (L-NMMA), and nitro-L-arginine methyl ester (L-NAME) are competitive inhibitors of NO synthetase (Palmer & Moncada, 1989;Vargas *et al.*, 1991). *In vivo* studies involving anaesthetized animals support the role of basal release of EDRF. Animals treated with L-NMMA develop a dose-dependent increase in mean arterial blood pressure and a reduced response to the hypotensive action of ACh (Rees *et al.*, 1989a, 1990; Whittle *et al.*, 1989; Tolins *et al.*, 1990). Moreover, Rees *et al.*, 1989 showed that the blood vessels obtained from animals treated with L-NMMA had a reduced ability to form NO. Similarly, in man intravenous administration of L-NMMA into the brachial artery caused vasoconstriction and a 50% decrease in forearm blood flow, which was reversed by L-arginine (Vallance *et al.*, 1989a,b). These observations support the role of basal formation of NO in the regulation of blood flow and pressure (Rees *et al.*, 1990).

2.4v Agonist-Stimulated EDRF Release

Although the discovery of the role of the endothelium in local vasomotor control was based on the response to acetylcholine *in vitro*, it is unlikely in the intact organism that acetylcholine ever reaches the endothelial cells. Cholinergic nerve endings are found only in the adventitia of blood vessels and acetylcholine is rapidly destroyed by acetylcholinesterase before it can activate the endothelial muscarinic receptors. It is now known that other factors mediate the release of EDRF.

Agents which have been shown to induce endothelium-dependent relaxation of small arteries are either receptor dependent, such as bradykinin (Cherry *et al.*, 1981, 1982; Altura & Chand, 1981); histamine (Van de Voorde & Leusen, 1983); thrombin (De Mey & Vanhoutte, 1982; Ku, 1982); ATP and ADP (De Mey & Vanhoutte, 1980, 1981; Furchgott & Zawadzki, 1980c; Furchgott, 1981); substance P (Zawadzki *et al.*, 1981); noradrenaline (Vanhoutte & Miller, 1989); and arachidonic acid and serotonin (Golino *et al.*, 1991), or are receptor independent substances, such as the calcium ionophore A23187 (Zawadski *et al.*, 1980; Furchgott, 1981).

The endothelial cell membrane contains many receptors reacting to a variety of these endogenous substances. These receptors stimulate the synthesis and release of EDRF by second messenger systems. It has been shown that some responses mediated by the endothelium (though not all) can be inhibited by pertussis toxin, which is a known inhibitor of a sub-group of these coupling proteins (G-proteins). Pertussis toxin ADP-ribosylates the alpha-subunits of the toxin-sensitive G-proteins at a cysteine residue, resulting in their inactivation (Flavahan & Vanhoutte, 1990). Thus at least two distinct biochemical pathways are involved in the release of EDRF. In most cases, stimulation of the endothelium will not only lead to an increase in EDRF production but also to the simultaneous release of endothelium-derived hyperpolarising factor (EDHF) and/ or prostacyclin.

2.4vi Shear Stress

The arterial endothelium forms the interface between blood flow and the vessel wall, and

consequently it is exposed to a constant force of fluid shear stress as a result of change in blood flow and pressure. An increase in flow rate (especially if pulsatile) *in vivo*, causes a parallel increase in the release of EDRF, resulting in flow-induced vasodilation which is endothelium-dependent (Vanhoutte, 1988); and it is this effect of shear stress on the blood vessel wall which is one of the main factors concerned in EDRF release.

When endothelial cells are subjected to shear stress or stretching, a diverse set of responses are induced. These include rapid changes in ionic conductance, adenylate cyclase activity, EDRF release, inositol triphosphate generation, and intracellular free calcium. Indeed these responses to mechanical stress are similar to those resulting from agonist-receptor coupling, suggesting that they may share common transduction pathways. If the forces are sustained, these rapid responses are then followed by altered gene expression, and structural re-organisation (Davies & Tripathi, 1993).

At increased flows, resistance arteries of the intact rabbit ear have shown enhanced endothelium-dependent vasodilation to ACh (Griffith *et al.*, 1987b), and subsequently greater endogenous NO activity was demonstrated in large arterioles where hydraulic resistance and shear stress were the highest (Griffith & Edwards, 1990). Studies using cultured endothelial cells have demonstrated that an increase in shear stress enhances kinin release and as a consequence, the release of EDRF from the endothelium (Busse & Lamontagne, 1991; Wiemer *et al.*, 1991). Another study of the effects of shear stress on cultured endothelial cells showed that the stimulus of laminar fluid flow causes an elevation of endothelial cGMP via an autocrine action of EDRF. This effect is mediated by a unique signal transduction pathway that is coupled to a pertussis toxin-sensitive G-protein, which requires the activity of an endothelial potassium channel (Ohno *et al.*, 1993).

2.4vii Hypoxia

As well as detecting shear stress the vascular endothelium also acts as a local sensor and effector system, regulating the supply of oxygen to the tissues. I*n vitro* and *in vivo* studies indicate that hypoxia induces vasodilation in a variety of vascular beds, by stimulating the

release of vasoactive autacoids such as EDRF and prostacyclin (Pohl & Busse, 1989; Busse *et al.*, 1983, 1984). On the other hand, depending on the experimental conditions, sudden hypoxia causes endothelium-dependent contraction of the pulmonary and coronary arteries due to the release of a diffusible substance that remains unidentified (Van Nueten *et al.*, 1980; Rubanyi & Vanhoutte, 1985; Graser & Vanhoutte, 1991).

2.5 Other Non-NO Endothelium-Derived Relaxing Factors

2.5i Prostacyclin

Prostacylcin discovered in 1976 by Moncada and colleagues (Moncada *et al.*, 1976), is a bicyclic eicosanoid generated from arachidonic acid, which is previously liberated from endothelial phosholipids by an activated phospholipase. Prostacyclin generation can be stimulated by a variety of factors including pulsatile pressure, and hypoxia. Its release can also be stimulated by endogenous mediators derived from plasma such as bradykinin (Crutchley *et al.*, 1983), substance P and thrombin (Weksler *et al.*, 1978), as well as substances liberated from activated platelets, such as serotonin, platelet-derived growth factor (Coughlin *et al.*, 1980), interleukin-1 (Rossi *et al.*, 1985), and adenine nucleotides (Pearson *et al.*, 1983; Forsberg *et al.*, 1987).

Chemically unstable, with a metabolic half-life in the blood stream of approximately three minutes, circulating levels of prostacyclin are too low to have a systemic effect (Blair *et al.*, 1982) Prostacyclin therefore acts as a paracrine hormone, and is released on the abluminal side of the vessel, where it then causes relaxation of the underlying vascular smooth muscle, by activating adenylate cyclase and increasing the levels of cyclic 3',5'-adenosine-monophosphate (cyclic AMP) (Luscher & Vanhoutte, 1990). It acts synergistically with EDRF to inhibit platelet aggregation (Radomski *et al.*, 1987a; Macdonald *et al.*, 1988).

2.5ii Endothelium-Derived Hyperpolarising Factor

Electrophysiological studies of endothelium-dependent responses have revealed that acetylcholine and other vasodilator drugs also cause membrane hyperpolarisation of the

underlying vascular smooth muscle (Komori & Suzuki, 1987; Feletou & Vanhoutte, 1988). The hyperpolarisation occurs in response to an unidentified diffusible substance, endothelium-derived hyperpolarizing factor (EDHF), which is distinct from nitric oxide (Feletou & Vanhoutte, 1988; Komori *et al.*, 1988), and is not a product of the cyclooxygenase pathway (Hoeffner *et al.*, 1989).

Unlike the constitutive enzyme NO synthetase, which produces NO continuously, a continual basal release of EDHF is unlikely (Nagao & Vanhoutte, 1991). The release of EDHF can be activated by shear stress, acetylcholine (Hoeffner *et al.*, 1989), bradykinin (Beny, 1990), adenine nucleotides, histamine (Chen & Suzuki, 1989), thrombin and substance P (Beny *et al.*, 1986). EDHF appears to act on smooth muscle by opening ouabain sensitive potassium channels (Van de Voorde *et al.*, 1992; Chen *et al.*, 1989). The release of EDHF by the endothelial cell is Ca^{2+}-dependent, and can be triggered by the calcium ionophore A23187 (Chen & Suzuki, 1990; Nagao & Vanhoutte, 1992) It is likely to be a calmodulin-dependent process, because it is inhibited by calmodulin inhibitors (Nagao *et al.*, 1992). The endothelial cell membrane receptor responsible for the acetylcholine-induced release of EDHF has been shown to be pharmacologically distinct from that releasing EDRF/NO, in that the first belongs to the muscarinic M_1 subgroup, and the second to either the M_2 or the M_3 subgroup (Lüscher & Vanhoutte, 1990).

2.5iii Anti-Platelet Activity of EDRF

Abluminally released EDRF controls vascular tone, while luminally released EDRF diffuses into the platelets when they are in close contact with the endothelial cell lining, and has anti-aggregatory and anti-adhesive effects (Azuma *et al.*, 1986; Radomski *et al.*, 1987(a); Moncada *et al.*, 1991). Vasoactive mediators produced by the aggregating platelets, including serotonin, adenosine diphosphate (ADP), and thromboxane A_2 release EDRF from vessels with an intact endothelium. The release of EDRF results in the rapid flushing away of micro-aggregates thereby preventing unwanted intravascular coagulation. The effect of EDRF on platelet activity is strongly synergistic with prostacyclin, and together they constitute a powerful anti-aggregatory barrier (Macdonald *et al.*, 1988; Radomski *et al.*, 1987a).

Both EDRF and exogenous NO cause an increase in cGMP within the cytosol of the platelets, which initiates a series of phosphorylation steps, finally leading to a fall in intracellular Ca^{2+} (Busse *et al.*, 1987; Mellion *et al.*, 1981); whereas, prostacyclin generated in the vessel wall, acts by a mechanism involving a cAMP-dependent phosphorylation process, which can be suppressed *in vitro* by indomethacin. However, the cGMP-dependent and NO-mediated suppression of platelet activation seems to be more important, because indomethacin led to only a slight reduction in the anti-aggregatory potential of thrombin-activated platelets, whereas the addition of EDRF/NO to the bioassay system causes almost complete suppression of platelet aggregation (Busse *et al.*, 1989).

Disruption of the endothelial barrier e.g. as a result of trauma, leads to a breakdown in the feedback control of platelet aggregation by EDRF and prostacyclin. Aggregation of platelets then proceeds with the continuous release of thromboxane A_2 and serotonin, both of which in the absence of the endothelial barrier and of opposing EDRF release, have unrestricted access to the smooth muscle layer thereby causing vasoconstriction. The contribution of thromboxane A_2 to the vascular phase of haemostasis is highlighted by the fact that the cyclooxygenase inhibitor aspirin inhibits platelet aggregation. Clinical trials have shown its administration in low doses is effective in preventing myocardial infarction (Peto *et al.*, 1988), and occlusion of coronary artery bypass grafts (Lorenz *et al.*, 1984).

A number of non-endothelium dependent nitrovasodilators which spontaneously release NO, e.g.sodium nitroprusside and GTN can directly increase the levels of platelet cGMP, thereby reducing their tendency to aggregate, and can therefore be used to suppress thrombotic events. However, as platelets lack the ability for the metabolic conversion of organic nitrates to a NO-containing compound, nitrovasodilators which can not spontaneously release EDRF have no significant inhibitory effect on the platelets or prevent thrombosis (Moncada *et al.*, 1991).

2.6 Endothelium-Derived Contracting Factors

Endothelial cells produce and release vasoconstrictor substances in response to a number of agents and physical stimuli, resulting in the contraction of the underlying smooth muscle.

These substances have been termed endothelium-derived contracting factors (EDCF) (Vanhoutte 1987; Luscher & Vanhoutte, 1990).

2.6i Cyclo-oxygenase Dependent Endothelium-Derived Contracting Factor

Arachidonic acid induced contraction was first demonstrated in canine veins (DeMey & Vanhoutte, 1982), and later demonstrated in a variety of blood vessels including, rabbit aorta (Singer & Peach, 1983), canine basilar (Katusic *et al.*, 1988) and cerebral arteries (Toda *et al.*, 1988). The contractile response to arachidonic acid depends on an intact endothelium, and can be blocked by cyclo-oxygenase inhibitors such as indomethacin (Luscher, 1990). Endothelium-dependent contraction also can be evoked by neurohumoral mediators such as acetylcholine, rapid stretch, or the Ca^{2+} ionophore A23187, and again the response can be prevented by indomethacin (Katusic *et al.*, 1988). The observation that the Ca^{2+} ionophore A23187 mediates the release of EDCF suggests a Ca^{2+}-dependent process. Studies using isolated human saphenous veins indicate that endothelium-dependent contractions produced by acetylcholine and histamine are mediated by the metabolism of arachidonic acid via cyclo-oxygenase and the production of the intermediates thromboxane A_2 (TXA_2) and prostaglandin H_2 (PGH_2) (Young *et al.*, 1991).

2.6ii Superoxide Anions

In some arteries the activation of endothelial cyclo-oxygenase generates superoxide anions, which evoke contraction of the blood vessel (Vanhoutte & Katusic, 1988). Such a response has been demonstrated using the canine basilar artery, where the endothelium-derived contractions were shown to be inhibited selectively by the free radical scavenger superoxide dismutase (Katusic & Vanhoutte, 1989). The superoxide anion is a potent chemical inactivator of nitric oxide but also selectively inhibits the production of prostacyclin (Rubanyi & Vanhoutte, 1986; Katusic & Vanhoutte, 1989), and therefore may contribute to the development of increased vascular tone and vasospasm. However, superoxide radicals interact not only with EDRF but also with other tissue components, and can lead to

endothelial and vascular smooth muscle damage. Therefore, it is not uncommon to see alteration of a variety of responses in addition to endothelium-dependent relaxation when the free radicals levels are exceptionally high(Marshall & Kontos, 1990).

Excessive vascular superoxide production has been demonstrated in disease states associated with endothelial dysfunction, including hypercholesterolemia (Ohara *et al.*, 1993), and diabetes (Tesfamariam & Cohen, 1992). However, as it can be assumed that superoxide anions cause endothelium-dependent contraction by preventing an endothelial inhibitory influence rather than by acting directly on the vascular smooth muscle, and therefore can no longer be regarded as true EDCFs (Vanhoutte, 1993).

2.6iii Endoperoxides and Thromboxane A_2

Thromboxane and endoperoxides have been shown to induce endothelium-dependent contraction in other blood vessels. Thromboxane A_2 is the major vasoconstrictor product of the cyclo-oxygenase pathway, and its formation from endoperoxides is catalysed by the enzyme thromboxane synthetase (Moncada & Vane, 1979). Studies using the isolated pulmonary artery of the rabbit have demonstrated that thromboxane synthetase inhibitors reduce endothelium-dependent contractions evoked by acetylcholine (Altiere *et al.*, 1986). Similarly, in the canine basilar artery endothelium-dependent contractions to norepinephrine, nicotine and the calcium ionophore A23187, can be blocked by the thromboxane synthetase inhibitor dazoxiben (Usui *et al.*, 1987; Katusic *et al.*, 1988). On the other hand, thromboxane synthetase inhibitors failed to prevent the endothelium-dependent contractions evoked by arachidonic acid in canine veins, or by ACh and endoperoxides in canine cerebral arteries (Miller & Vanhoutte, 1985; Katusic *et al.*, 1988; Toda *et al.*, 1988).

Thus, the endothelial cyclo-oxygenase pathway can generate a variety of EDCFs, including superoxide anions, thromboxane A_2, and prostaglandin H_2, depending on the agonist and blood vessel studied. However, it is possible that the endothelial cells may produce a second messenger which initiates the formation of the endoperoxides/thromboxanes in the underlying vascular smooth muscle (Vanhoutte, 1993).

2.6iv Hypoxia-Induced Endothelium-Dependent Contraction

Sudden hypoxia/anoxia has been shown to augment the contractile responses of isolated canine coronary, cerebral and pulmonary arteries (DeMey & Vanhoutte, 1982, 1983; Rubanyi & Vanhoutte, 1985). This rapid and transient endothelium-dependent contraction depends on the diffusion of an unidentified constrictor substance from the endothelium to the smooth muscle (Rubanyi & Vanhoutte, 1985). The contractile response does not depend on the cyclo-oxygenase pathway, nor is it endothelin, which produces a slow and sustained contractile response (Vanhoutte *et al.*, 1989). The effect is exacerbated by a reduced release of basal NO by the endothelial cell, resulting in decreased activation of soluble guanylate cyclase in the underlying smooth muscle (Graser & Vanhoutte, 1991). It appears therefore that a critical reduction of soluble guanylate cyclase activation is required for the hypoxic endothelium-dependent contractions to occur (Vanhoutte, 1993).

2.6v Endothelin

Endothelin is a 21-amino acid polypeptide first obtained and chemically characterised from pig aortic endothelial cells (Yanagisawa *et al.*, 1988). There are three structurally and pharmacologically distinct endothelin (ET) isopeptides in humans and other mammalian species; ET-1, ET-2, and ET-3 (Inoue *et al.*, 1989). Endothelin-1 is an extremely potent, long-lasting vasoconstrictor produced by the endothelial cells, both *in vitro* and *in vivo,* and whose action is closely associated with the influx of Ca^{2+} (Yanagisawa *et al.*, 1988; Brain *et al.*, 1988, 1989).

The production of endothelin is stimulated by mechanical forces, vasoconstrictor hormones such as arginine vasopressin, angiotensin II, and catecholamines; as well as coagulation and platelet products such as thrombin, and transforming growth factor β, and finally by certain cytokines such as interleukin-1 (Yanagisawa & Masaki, 1989). Since circulating endothelin levels are very low, it is suggested that its release is preferentially in the abluminal direction. Endothelin can cause vasodilation through the release of NO and prostacylcin as well as vasoconstriction (Yanagisawa *et al.*, 1988; Schini & Vanhoutte, 1991).

Under physiological conditions, the basal tone maintained by the continuous release of ET may be balanced by the tonic release of NO, and consequently an imbalance between these two factors could lead to pathological elevation of vascular tone (Rubanyi & Polokoff, 1994). The pathological importance of endothelin is suggested by its plasma elevation in disorders of cardiovascular and renal function, as well as diabetes (Margulies *et al.*, 1990, 1991; Lerman *et al.*, 1991;Haak *et al.*, 1992). Thus, drugs which interfere with the synthesis of endothelin from its inactive precursor "big endothelin" such as endothelin converting enzyme inhibitors, or antagonists of endothelin receptors, could provide novel therapies to reduce vascular tone or prevent vasospasm and diabetic vascular disease (Vallance, 1992).

2.7 The Endothelium In Diabetes

Due to its strategic anatomical positioning between the circulating blood and the vascular smooth muscle layer, the endothelium not only plays an important physiological role modulating vascular tone (Furchgott & Zawadzki, 1980b), but also represents a major target organ for vascular injuries. There is evidence to suggest that disturbances in endothelial function may contribute to both large and small vessel disease in diabetes mellitus (Porta *et al.*, 1987). Altered production and release of endothelium-derived relaxing factor (EDRF), as well as prostacyclin, thromboxane A_2, or endoperoxides has been observed in various vascular beds (Tesfamariam *et al.*, 1989). Changes in the reactivity of the vascular smooth muscle cells may also contribute to the altered responsiveness of the vasculature in diabetes (MacLeod, 1985; Harris & MacLeod, 1988). (See section 2.7iii)

Long standing diabetes mellitus is associated with structural changes to the vascular wall leading to type IV collagen, laminin, fibronectin, and the proteoglycan heparin sulfate becoming the major structural elements of the extracellular matrix (Beisswenger & Spiro, 1973; Barnett, 1991). These changes can at least be partially ascribed to enhanced levels of growth factors in diabetes such as platelet-derived growth factor (PDGF), and angiotensin II (AII)(Bar *et al.*, 1989; Flyvberg, 1990). Because of the growth factor-like properties of AII and its pathological role in diabetes, it has been suggested that treatment with angiotensin-converting enzyme (ACE) inhibitors may prevent the progression and further deterioration

in diabetic microangiopathy (Viberti, 1992). This view is supported by studies showing that the treatment with ACE-inhibitors has a beneficial influence on vascular dysfunction in both humans with diabetes (Lewis *et al.*, 1993), and animal models with experimental-induced diabetes (Murray *et al*, 1994; Olbrich *et al.*, 1996).

Other growth factors reported to be elevated in diabetes, and in diabetic microangiopathy include, tissue-type plasminogen activator (tPA) (Jensen *et al.*, 1989), and von Willebrand factor (vWF) (Porta *et al.*, 1991). Recently, vascular permeability factor (VPF), or vascular endothelial growth factor (VEGF) also has been linked to the endothelial dysfunction associated with diabetes mellitus (Thomas *et al.*, 1995). The significance of both vWF and VPF acting as possible endothelial markers in determining the development and/or the progression of microangiopathy in humans with diabetes thus warrants a more detailed discussion.

2.7i Von Willebrand Factor

Von Willebrand factor (vWF) is a complex glycoprotein synthesized by endothelial cells of small and large blood vessels (Jaffe *et al.*, 1974), and is released when endothelial cells are damaged (Suffredini *et al.*, 1984). It promotes platelet adhesion to the subendothelium, especially at high shear rates (Sakariassen *et al.*, 1979), and protects factor VII, which plays a key role in the events of plasma coagulation (Ruggeri & Ware, 1992).

High plasma levels of vWF are widely regarded as an indication of endothelial damage and dysfunction in IDDM (Boneu *et al.*, 1975), and raised vWF levels have been reported in patients with diabetic nephropathy (Jensen *et al.*, 1989a,b; Stehouwer *et al.*, 1991), neuropathy (Plater *et al.*, 1996), and retinopathy (Porta *et al.*, 1981). Although it is not clear whether diabetic retinopathy or nephropathy is the more important determinant of high vWF in the study by Porta *et al.*, since patients with proteinuria were not excluded, and may have confounded the results. Subsequent studies addressing this issue have shown that there was no evidence of an association between retinopathy and the plasma concentration of vWF in Type 1 patients with normal urinary albumin excretion, and proposed that in these patients

high or increasing plasma vWF levels may be related to systolic blood pressure (Jensen,1989; Stehouwer *et al.*, 1992).

Until recently, it was not known whether endothelial dysfunction actually precedes the development of microalbuminuria. Evidence in favour of the relationship was provided by a long term study of a group of IDDM patients who were followed-up over a period of 64 months. During this time period urinary albumin excretion, and plasma vWF concentration were measured at regular intervals. Results demonstrated that endothelial dysfunction preceded the development of microalbuminuria and therefore predicts the development of microalbuminuria. These findings support the hypothesis that the vascular endothelium is an early and relevant target in the pathogenesis of diabetic microangiopathy, and patients known to have raised plasma vWF levels should receive intensive treatment of hyperglycaemia and hypertension (Stehouwer *et al.*, 1995).

2.7ii Vascular Permeability Factor

Vascular permeability factor also known as vascular endothelial cell growth factor (VEGF) is a recently identified family of secretory proteins that stimulate endothelial cell growth and also increases endothelial permeability. It is produced by the vascular smooth muscle cells, but binds almost exclusively to the vascular endothelium (Jakeman *et al.*, 1992; Gitay-Goren *et al.*, 1992), causing an increase in vascular permeability and therefore vascular leakage (Senger *et al.*, 1983). On a molar basis, VPF induces a 50,000 times greater change in permeability than histamine (Senger *et al.*, 1990). Moreover, VPF differs from other substances which alter permeability because its effect is rapid and reversible without injury or inflammation (Senger *et al.*, 1983). Changes in vascular permeability are achieved without mast cell degranulation or endothelial cell damage, but instead depend on the separation of the endothelial cell junctions (Senger *et al.*, 1986).

VPF has mitogenic effects at low concentrations on endothelial cells, but does not stimulate the growth of other cell types (Connolly *et al.*, 1989; Leung *et al.*, 1989). In addition to affecting endothelial permeability and growth, VPF also stimulates the release of von

Willebrand factor and tissue factor, and induces endothelial surface procoagulant activity (Brock *et al.*, 1991).

Activation of the renin-angiotensin system (RAS), hypoxia (Tuder *et al.*, 1995), and hyperglycaemia are potent stimulators of VPF release. Hyperglycaemia has been shown to activate protein kinase C (PKC), which increases VPF messenger RNA expression (Williams *et al.*, 1992). Moreover, PKC inhibitors prevent hyperglycaemia-induced VPF activity (Williams & Howard, 1994). Other studies have demonstrated glucose-induced VPF gene expression in cultured skin fibroblasts, but exposure to high glucose concentrations for more than 48 hours increases VPF gene expression in skin fibroblasts only in certain individuals, and this difference in response may be of relevance in the variable susceptibility of individuals to diabetic complications (Thomas *et al.*, 1995).

More recently Williams & Kimber have demonstrated that subjecting vascular smooth muscle cells to cyclical mechanical strain leads to a rise in VPF production (Kimber *et al.*, 1996). Therefore, increased VPF expression, and peptide production can be stimulated by RAS activation, hyperglycaemia, hypertension, stretch and hypoxia, ultimately resulting in endothelial dysfunction and neovascularisation.

2.7iii Experimental Studies of Vascular Reactivity in Diabetes

Studies of vascular reactivity in animal models of diabetes have yielded inconsistent results. These discrepancies may be due to a number of different factors, such as the choice of vascular bed studied, duration of diabetes, age, and whether or not the animals were treated with insulin. Therefore the experimental conditions for these studies need to be carefully considered before the results can be accurately evaluated.

Studies of both isolated large arteries and resistance arteries have reported reactivity as being increased (MacLeod, 1985; Harris & MacLeod, 1988; Pieper & Gross, 1988; Cohen *et al.*, 1990; Taylor *et al.*, 1992), or decreased (Pfaffman *et al.*, 1982; Fortes *et al.*, 1983; Oyama *et al.*, 1986; Head *et al.*, 1987) in chemically induced diabetes, and unchanged in insulin treated

rats with spontaneous diabetes (Meraji *et al.*, 1987; Durante *et al.*, 1988). Similarly the results from whole animal studies also have proved to be conflicting with one study involving the chemically induced diabetic rat model, revealing enhanced responses to angiotensin II with normal responses to noradrenaline (Christlieb, 1976). Whereas, another study of isolated perfused rat limbs, demonstrated a significant increase in catecholamine reactivity but no subsequent change in the vasoconstrictor response to vasopressin or angiotensin (Brody & Dixon, 1964).

2.7iv Endothelium-Dependent Relaxation

Animal Studies

The majority of studies of endothelium-dependent relaxation have involved the use of the aorta from rats with experimentally induced diabetes. Attenuated relaxation responses to the endothelium-dependent vasodilator acetylcholine (ACh) have been demonstrated in these animals (Oyama *et al.*,1986; Pieper & Gross, 1988; Tesfamariam, 1990; Cameron & Cotter, 1992), as well as in the spontaneously diabetic rats (Meraji *et al.*, 1987; Durante *et al.*, 1988). A specific muscarinic receptor defect appears to be an unlikely explanation since the relaxation response to the endothelium-dependent vasodilator histamine (Oyama *et al.*, 1986; Tanz *et al.*, 1989) and ADP (Pieper & Gross, 1988), also is abnormal. Relaxation produced by the calcium ionophore A23187, which in effect "by-passes" receptor-mediated calcium release, is reported to be normal in one study (Tesfamariam *et al.*, 1989) and abnormal in another (Oyama *et al.*, 1986). On the other hand, some studies reported no evidence of an impaired response to endothelium-dependent vasodilators (White & Carrier, 1986; Head *et al.*, 1987; Harris & MacLeod, 1988; Mulhern & Docherty, 1989).

Only a few studies of endothelial function have been performed using resistance vessels. Impaired endothelium-dependent relaxation to histamine (Fortes *et al.*, 1983; Takiguchi *et al.*, 1988), and ACh has been reported in isolated resistance arteries from the mesenteric bed of chemically induced diabetic rats (Takiguchi *et al.*, 1988, 1989; Taylor *et al.*, 1992, 1994a). By contrast, others failed to reveal an impaired response to acetylcholine (Fortes *et al.*, 1983), and another study reported an augmented histamine relaxation response (White & Carrier, 1986).

Comparatively few studies have examined responses to the more physiologically relevant endothelium-dependent vasodilator, bradykinin. These few studies have documented normal responses in isolated mesenteric resistance arteries from diabetic animals (Taylor *et al.*, 1992, 1995). However, one study has reported an impaired relaxation response to bradykinin in the hindlimb circulation of chemically-induced diabetic rats (Kiff *et al.*, 1991b); as well as an *in vitro* study of coronary resistance vessels of the perfused diabetic rat heart (Rösen *et al.*, 1988); and isolated mesenteric resistance arteries of diabetic rats (Fortes *et al.*, 1983).

In vivo studies of endothelial function in STZ-induced diabetic animals also have revealed abnormal endothelium-dependent relaxation (Bucala *et al.*, 1991), although Kiff and co-workers (1991) found that the depressor response to ACh was normal in the whole diabetic animal. Further evidence for variation depending on the vascular bed studied comes from a study of the renal vasculature of the STZ-induced diabetic rat, which showed an increased vasodilator response to ACh (Bhardwaj & Moore, 1988).

Human studies

Defective endothelium-dependent relaxation has been demonstrated in the smooth muscle of the surgically excised corpora cavernosa of diabetic men with impotence (de Tejada *et al.*, 1989), and in isolated subcutaneous resistance arteries obtained from gluteal biopsies taken from humans with insulin-dependent diabetes (McNally *et al.*, 1994). Moreover, impaired endothelium-dependent relaxation was shown in the forearm of subjects with non-insulin dependent diabetes (Type II) (McVeigh *et al.*, 1992). Interestingly, one study has reported normal responses to ACh in the forearm of patients with insulin-dependent diabetes, whereas the endothelium-independent response to sodium nitroprusside was impaired (Calver *et al.*, 1992); however, the patients in this study were not characterised according to their albumin excretion rate.

2.7v Endothelium Independent Responses

The literature contains a number of conflicting reports about endothelium independent relaxation in diabetes; *in vitro* the relaxation responses of isolated arteries to SNP from both

chemically induced diabetic rats, and non-diabetic controls were similar (Oyama *et al.*, 1986; Pieper & Gross, 1988; Taylor *et al.*, 1992). Similarly, most studies of human insulin-dependent diabetes have reported normal responses to SNP (Elliott *et al.*, 1993; Johnstone *et al.*, 1993; McNally *et al.*, 1994), although one study has reported an impaired response to SNP (Calver *et al.*, 1992).

2.8 Possible Mechanisms for Impaired Endothelium-Dependent Relaxation in Diabetes

The literature clearly shows that the endothelium is significantly compromised in diabetic animals and man. Several explanations have been proposed for the development of abnormal endothelial function in diabetic animals and man. The role for hyperglycaemia leading to disturbances in several biochemical pathways has already been briefly reviewed. Hyperglycaemia stimulates the polyol pathway leading to an increase in sorbitol through enhanced aldose reductase activity. Sorbitol accumulation has been implicated in endothelial dysfunction reported in the aortae of diabetic rats (Brolin & Naeser, 1988). Activation of this pathway is associated with an increased utilisation of NADPH which may lead to a reduction in cellular NADPH, an essential co-factor of the enzyme NO synthase (Hawthorne *et al.*, 1989). A decrease in the availability of cellular NADPH may have a deleterious effect on the function of the endothelium, because it is an essential requirement for the cell glutathione redox process, protecting the endothelial cells against damage by free radicals.

Other possible mechanisms, unrelated to the "glucose milieu" and involving either the tonic or agonist-stimulated EDRF production and release also have been postulated. These include a decrease in the production and release of EDRF, accelerated destruction of EDRF by free radicals (Gryglewski *et al.*, 1986a), an alteration in smooth muscle to respond to EDRF (Durante *et al.*, 1988), and a selective attenuation of a particular signal transduction pathway at the level of the G-protein (Taylor *et al.*, 1995a).

An altered balance of vasodilators and vasoconstrictors produced by the endothelium leading to enhanced generation of the vasoconstrictors such as endothelin, prostaglandin PGH_2, or

indeed the decreased synthesis of the vasodilatory and anti-aggregatory compounds prostacyclin and nitric oxide, could in theory also be contributory factors to the development of the many vascular complications associated with insulin-dependent diabetes mellitus (Poston & Taylor, 1995; Cohen & Tesfamariam, 1992).

Other conditions such as hypertension and atherosclerosis also effect endothelial function, leading to an imbalance in endothelium-dependent modulation of vascular tone with the release of contracting factors becoming predominant (Luscher *et al.*, 1992). Similar results have been reported using the aorta of STZ-induced diabetic rat (Shimizu *et al.*, 1993), and rabbit aorta (Tesfamariam *et al.*, 1989), where exposure to high glucose levels attenuated ACh-induced relaxation, but the response was reversed by treatment with cyclo-oxygenase inhibitors and/or by PGH_2-thromboxane A_2 receptor antagonists. The role for vasoconstrictor prostanoids in abnormal vascular regulation is further supported by a recent study of diabetic patients free from complications where aspirin treatment restored forearm blood flow response to reactive hyperaemia (Steel *et al.*, 1993). On the other hand, a similar study by an another group failed to demonstrate an improvement in methacholine-induced relaxation after aspirin therapy (Johnstone *et al.*, 1993).

2.9 Aims of the Thesis

Despite the overwhelming importance of microvascular damage in diabetes, most workers have used large conduit arteries to investigate endothelium-dependent vascular function in IDDM. As a consequence we have limited information about the effects of diabetes on the contractile function and endothelium-dependent relaxation of the resistance vasculature. Moreover, it is not clear whether long-term insulin treatment will help to maintain the normal structure and function of the microcirculation.

Although it is well recognised that the endothelial cells play an important role in the regulation of vascular tone by the synthesis and release of vasoactive factors, the nature of the vascular endothelium defect in diabetes is unclear. The studies described in this thesis were designed to investigate how diabetes affects the biosynthesis and action of these vasoactive

molecules in the mesenteric vascular bed. Preliminary experiments examined the effects of exposure to high and low glucose concentrations on endothelium-dependent relaxation of normal resistance arteries. This was followed by more extensive studies of endothelial function, vascular smooth muscle contractility as well as the structure of the resistance arteries in two different animal models of insulin-dependent diabetes:

The spontaneously diabetic Bio Bred (BB) rat was studied at two time points, firstly at the clinical onset of diabetes (approximate disease duration of 24-48 hours), and then after a 6-8 week period of insulin treated diabetes. Because of the discrepant reports of the effects of diabetes on endothelial function in the literature, a second animal model of chemical-induced diabetes was also examined. Resistance artery function was assessed initially after six weeks of streptozotocin-induced diabetes, and then after 12 weeks. In addition, the effects of long-term treatment with either subcutaneous insulin injection or islet transplantation on the resistance vasculature function was investigated to provide information about therapeutic approaches which may ameliorate the development of diabetic microangiopathy.

CHAPTER 3

MATERIALS AND METHODS

3.1 Experimental Design

The spontaneously diabetic Bio Bred rat was first discovered in 1974 in an outbred colony of Wistar rats from the Bio Breeding (BB) Laboratories, Ottawa, Canada (Chappel & Chappel, 1983; Nakhooda *et al.*, 1977). A programme of selective inbreeding lead to the production of a line of diabetic animals which could be maintained by daily insulin injections.
The onset of clinical diabetes in the BB rat is sudden and unpredictable, occurring between 58 to 123 days of age, with a mean age at onset of 96 days. The clinical features are similar to the human disease and include, an abrupt onset of hyperglycaemia, glycosuria, ketonuria, and weight loss, with death usually occurring within days after onset, unless insulin therapy is initiated. Microvascular complications such as retinopathy, nephropathy, and neuropathy found in human diabetes also develop in the BB rat (Nakhooda *et al.*, 1976, 1977; Marliss *et al.*, 1982; Sima *et al.*,1985; Mordes *et al.*, 1987).

Both genetic and immunological factors are involved in the aetiology of insulin-dependent diabetes in the BB rat, although their exact roles remain to be defined. The mode of inheritance of diabetes in the BB rat involves an autosomal recessive gene with 50% penetrance but in addition at least one gene associated with the rat major histocompatibility complex (MHC) appears to be involved (Butler *et al.*, 1983; Like & Rossini, 1984). Manipulation of selective breeding lines has enabled the development of a non-diabetes prone or diabetes resistant BB rat, where the incidence of diabetes is less than 1%.

The second model of diabetes was produced by chemical induction by the diabetogenic agent, streptozotocin (STZ). Because of suitability for transplantation, the WAG/Leics strain of rat was used, and diabetes was induced by a single injection of STZ. This broad-spectrum antibiotic extracted from *Streptomyces achromogenes*, possesses anti-tumour (Evans *et al.*, 1965), oncogenic (Arison & Feudale, 1967), and diabetogenic properties (Rakieten *et al.*, 1963). The agent was first reported to produce diabetes mellitus after a single intravenous injection in rats and dogs was in 1963 (Rakieten *et al.*, 1963). The mode of action of STZ

has been investigated by Junod *et al* (1967), who found histological changes in the pancreatic β cells within one hour of injection. Junod (1969) then went on to show that there was a direct relationship between the dose of STZ administered and the severity of the diabetogenic response. Until recently, it was not clear how low-dose STZ induces autoimmune diabetes; but it is now known that STZ induces an antigenic change in the pancreatic β cells (Weide & Lacy,1991).

The main treatment goal of patients with insulin-dependent diabetes is to achieve near-normal blood glucose levels in order to prevent the development of chronic complications (van Suylichem & van Schilfgaarde, 1995). One approach to achieve good glycaemic control is to transplant either a vascularised pancreatic graft or free grafts of islets. However, pancreatic transplantation does not appear to improve established retinopathy (Ramsay *et al.*, 1988; Scheider *et al.*, 1991), or nephropathy (Fioretto *et al.*, 1993), although some beneficial effect on neuropathy has been reported (Wilczek *et al.*, 1987; Van der Vliet *et al.*, 1988).

Other studies suggest that early pancreas transplantation in diabetic rodents prevents the development of microvascular complications (Orloff *et al.*,1986). However, pancreas transplantation is a major procedure and therefore alternative procedures which involve transplantation of pancreatic islets have been developed. Early reports of successful clinical islet transplants have been encouraging, although functional effects are usually short-lived (Scharp *et al.*, 1990; Warnock *et al.*, 1992).

In the in-bred rat, islet transplantation is a straight forward procedure. Islets are injected into the hepatic-portal vein, through which they reach the liver, where they become vascularised within the liver tissue. The liver appears to be the most advantageous site for transplanted islets in rodents, and it has been suggested that this organ is an immuno-privileged site, although isografts into the spleen and under the kidney capsule have also been successful (Kemp *et al.*, 1973; Sutherland, 1981; Koncz *et al.*, 1976; Van Suylichem et *et al.*, 1994). Other experiments in rodents indicate that prolonged survival of islet grafts can be achieved

by immuno-alteration of the grafts in the absence of immunosuppressive therapy (van Suylichem & van Schilfgaarde, 1995).

In addition to the reversal of metabolic abnormalities in diabetic animal recipients, such as weight loss, polyuria, polydipsia, glycosuria, and hyperglycaemia, islet transplantation has been shown to have some beneficial effects on secondary complications associated with the diabetic state (Pugliese *et al.*, 1990; Pipeleers-Marichal *et al.*, 1976; Schmidt *et al.*, 1983; Britland *et al.*, 1991; Mauer *et al.*, 1974; Gray & Watkins, 1976). However, to date, no studies have been performed to investigate whether maintaining good long-term metabolic control by islet transplantation prevents or reverses diabetic complications and endothelial dysfunction.

All the studies in this thesis were carried out on isolated mesenteric resistance vessels, with diameters of <350μm. These small arteries were selected because they are known to be involved in the regulation of the peripheral resistance (Mulvany & Aalkjaer, 1990). The mesenteric vascular bed was selected for study, because it provides an easily accessible source of resistance arteries of varying diameters. Furthermore, there is a great deal of expertise on the dissection and mounting of these mesenteric arteries in a Mulvany-Halpern myograph in the Department of Medicine & Therapeutics at the University of Leicester.

3.2 The Mulvany-Halpern Myograph

Experiments investigating the mechanical, morphological and pharmacological properties of small resistance arteries *in vitro* are commonly conducted using a myograph. First described by Bevan and Osher in 1972, the technique for investigating small resistance arteries was then further developed and modified by Mulvany and Halpern in 1976. The technique allows two segments of resistance arteries to be mounted and studied simultaneously under standardised conditions, i.e. at precise wall tensions, as ring preparations on a myograph. However, a more physiological system has recently been designed which more closely emulates the

physiological situation *in vivo*. In this system, the vessel is directly cannulated by two glass pipettes thereby allowing control of the intraluminal pressure and flow. The lumen diameter variables are then monitored by a pressure monitor and a video dimension analyser.

The Mulvany myograph consists of a stainless steel chamber with a volume of approximately 12mls, in which are housed two pairs of steel mounting heads. One head is attached via a pin leading onto the body of the myograph to a pre-calibrated force transducer which accurately measures tension. The signal from the force transducer is amplified, and recorded on to a 2-channel flat-bed chart recorder. The other steel head is attached to an adjustable micrometer which allows the heads to be moved apart in a horizontal plane. Segments of resistance arteries are attached to each pair of mounting heads via stainless steel wires which are secured by fixing screws.

The myograph is heated by water circulating through each of the outer blocks from a thermostatically controlled heater. The temperature of the heater is adjusted so that the bathing medium in the chamber can be maintained at 37°C; solutions can be added through a port on the near side. Aliquots of drugs can be added directly into the chamber or through a port in a plastic cover which can be placed over the chamber to reduce evaporation. The chamber can be emptied via the ports on the near and far sides by opening a valve connected to a vacuum pump, and this drains the contents of the bath via rubber tubing into a sealed plastic bottle. The chamber is continuously aerated with a mixture of 95% oxygen and 5% carbon dioxide via a port on the near side. (Plate 1)

3.2i Mounting

As described previously, two small arteries can be mounted on two pairs of steel heads in the myograph thus allowing vessels to be studied simultaneously. The mounting procedure is carried out in physiological salt solution (PSS) at room temperature and with the aid of a light dissecting microscope and fibro-optic light source .

A 40μm steel wire is secured to each pair of mounting heads via fixing screws. The wire is thus attached via the mounting head to the force transducer. One end of the wire is secured leaving the free end of the wire pointing towards the operator. Using a pair of small watchmaker forceps, the excised vessel is carefully threaded onto the free end of the wire via its lumen. The artery is then gently pulled along the length of the wire until the vessel is situated between the mounting jaws. The jaws are carefully brought together using the adjustable micrometer, and the free end of the wire is secured around the near fixing screw in a clock-wise direction. A second wire is then passed down the lumen of the vessel using the first wire as a guide, and both ends secured to the second mounting head via the fixing screws. The wires should now be levelled, so that the plane containing the wires is horizontal. The oxygen line to the chamber is then connected, and the circulation of heated water is commenced.

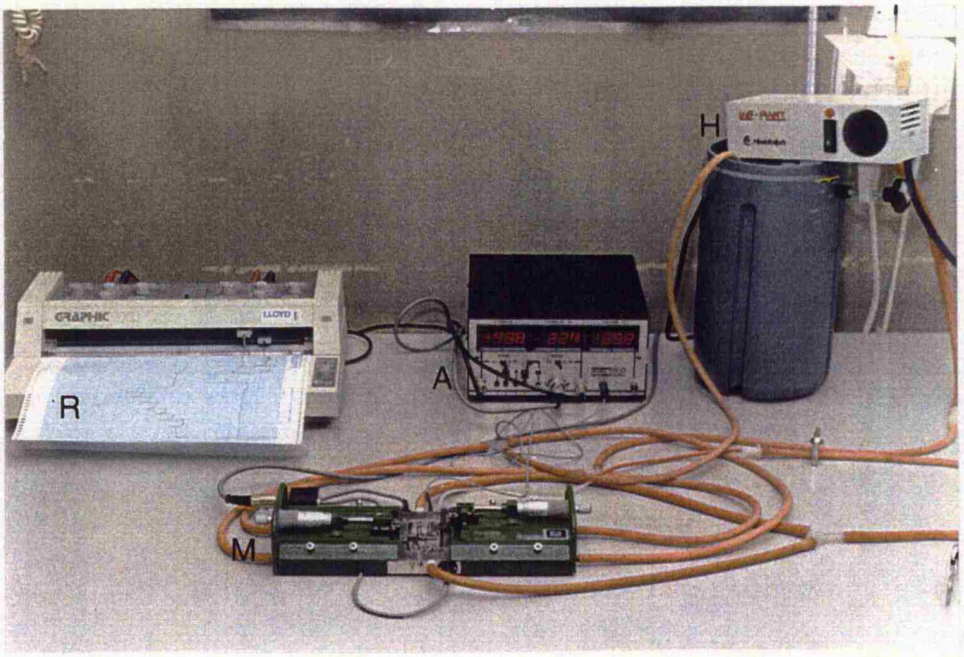

Plate 1: The Mulvany-Halpern Myograph

(M=Myograph, H= heidolph water heater/pump, R= chart recorder, A= amplifier.)

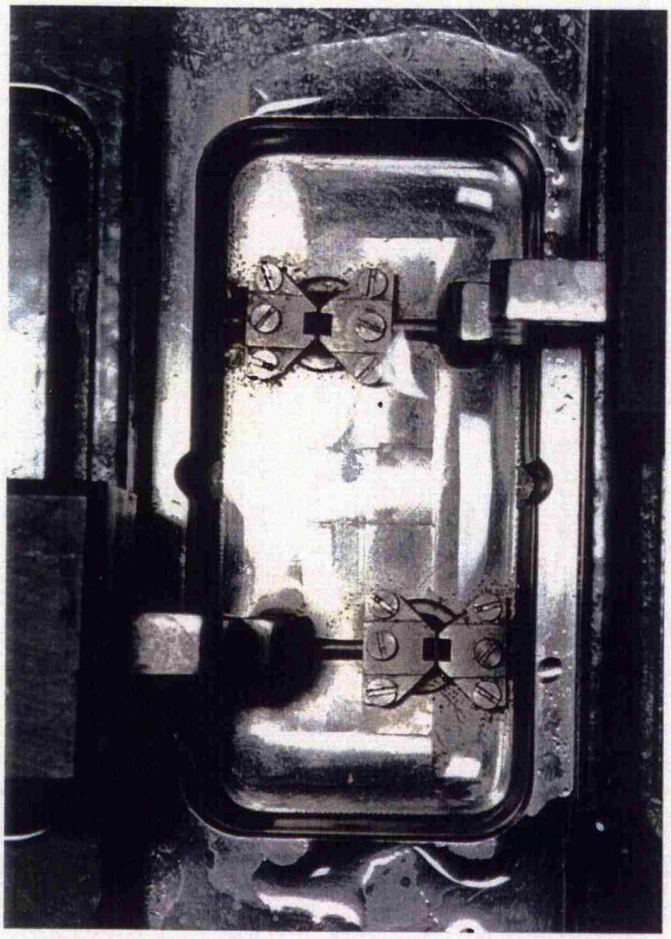

Plate 2: Close up of the myograph bath showing the two pairs of steel mounting heads. (Note the mounted resistance arteries situated between each pair of heads.)

3.2ii Morphology

Small glass windows situated beneath each pair of mounting heads permits the measurement of the dimensions of each resistance artery by light microscopy. The myograph is placed on to the stage of a Zeiss laboratory light microscope equipped with a salt water immersion objective and a calibrated filar micrometer eyepiece. The media thickness and lumen diameter can then be measured at three points along the vessel. The one disadvantage of this technique is that morphological measurements have to be made before the vessel has been placed under passive tension and set to its normalised lumen diameter, because this causes the vessel wall to become disproportionately thin around the wires. As a consequence, vessel morphology under normalised conditions has to be calculated from the measurements made at a minimal passive tension.

Each vessel was at first stretched to a minimum tension, (an approximate wall force of 0.2mN), and then three sets of measurements were made at three different positions along the length of each vessel. These included the wall thickness w (the average of six readings); wire thickness d (average of six readings); distance between the inner edges of both of the wires f_c (average of three readings); and the length of the vessel segment s. The internal circumference (Lc) can thereby be determined from these measurements as follows:

$$Lc = (2 + \pi)d + 2f_c$$

The internal or lumen diameter of the vessel can then be calculated.

$$lc = Lc/\pi$$

By entering the series of measurements into a basic "Morphology" programme, media cross-sectional area (μm^2), media volume, and wall thickness can also be determined. The wall thickness corresponding to the normalised internal diameter was calculated on the assumption that the cross-sectional area of media will remain constant (Patel *et al.*, 1969).

3.2iii Estimation of Normalised Wall Dimensions

An important factor to be considered when undertaking any pharmacological or structural study regarding isolated arteries is to ensure that procedures are carried out under standardised conditions. This can be achieved by setting the small resistance arteries to the optimum circumference or active tension which would most closely mimic *in vivo* conditions. In studies with large vessels or conduit arteries, the vessel is often set at a given tension which does not accurately reflect the size of the vessel. However, in order to standardise the conditions which will best mimic the *in vivo* behaviour of a resistance vessel, the internal circumference and segment length have to be considered, as otherwise the vessel could be irreversibly damaged or 'over stretched'. It is also known that the responsiveness of blood vessels to pharmacological agents is dependent on the extent to which the vessel is stretched.

The normalisation procedure permits the determination of the internal circumference (L_{100}) which the vessel would have if relaxed and under a transmural pressure of 100mmHg. The value of L_{100} is determined from the resting internal circumference. The relaxed vessel under minimal tension is stretched in steps using the micrometer which moves the mounting heads apart. After each stretch the output corresponding to the applied force on the chart recorder rises. Due to "stress relaxation", the force then falls. One minute after stretching the vessel, the first set of tension-length parameters from both the micrometer and the chart recorder are then keyed in to a computer pre-loaded with the BASIC programme "Normalisation" (Mulvany and Aalkjaer, 1977).

From previous measurements of the length of the vessel segment and from Laplace's law, which states that in a hollow structure the tension in the wall (T), is given by the product of the distending pressure (P) and the radius (r), the "effective pressure" at each step can be calculated. Effective pressure (P_i) corresponding to transmural pressure can be determined from equation (i)

(i) $$P_i = \text{Wall tension} / (\text{internal circumference} / 2\pi)$$

The stretching and entering of the corresponding values are repeated until the *"nth"* reading when the effective pressure (Pi) equals or is greater than 13.3kPa (ii).

(ii) $$P_i > 13.3 \text{ kPa}$$

The computer then fits a curve to the experimental points, and the internal circumference of the vessels which corresponds to an effective pressure of 100mmHg is then calculated. This circumference L_{100} corresponds to the circumference the vessel would have *in vivo* conditions and under a transmural pressure of 100mmHg. Using the micrometer, the internal circumference was then set to 90% of the effective diameter (termed the "normalised diameter") which it had at 100mmHg equivalent ($0.9L_{100}$). This configuration has been shown to allow the greatest active contraction to stimulation (Mulvany & Halpern, 1977). It has also been shown that adjusting to $0.9L_{100}$, the volumes of the wall layers remains constant (Patel *et al*, 1969). Therefore, it can be assumed that the length of the segment does not change when the vessel is stretched, and the cross-sectional area of the artery will also remain constant.

3.3 Animals & Surgical Procedures

Three strains of rats were used in different experimental studies; Wistars, WAG/Leics, and Bio Bred rats. All animals were obtained from the Biomedical Services Unit at Leicester University. The rats were maintained on a 12 hour light/dark cycle and were fed standard rat chow with free access to water.

3.3i Chemical Induction of Diabetes

Female WAG/Leics rats (8-9 weeks old and weighing approximately 150g) were randomly

allocated to three groups: diabetic, control, and insulin treated diabetic. Diabetes was induced by a single femoral vein injection of 55mgkg^{-1}STZ dissolved in a citrate buffer solution (0.01M, pH 4.2). Rats allocated to the control group received the buffer solution alone.

Anaesthesia was induced by placing the animal in a sealed perspex box saturated with an anaesthetic mixture of halothane, oxygen and nitrous oxide. Once the rat was unconscious, it was transferred to a warming mat on the operating table, and the anaesthetic maintained using a nose mask connected via rubber tubing to the anaesthetic machine. The right hind limb was taped out at an angle from the body, and an area approximately 2cm^2 in the groin region was shaved close to the nipple. A small incision was then made and scissors were used to divide the layer of fat covering the femoral vein. After the femoral vein was exposed, 55mgkg^{-1} STZ (dissolved in 1ml of citrate buffer) was carefully injected. The wound was then sutured with coated vicryl (Ethicon), and the animal allowed to regain consciousness.

Daily blood glucose levels were measured using a blood glucose test strip (BM-Test 1-44) and a Reflolux meter (Boehringer Mannheim). Rats with blood glucose readings >20mmol were considered diabetic; one group received daily subcutaneous injections of insulin (0.8 U insulin/100g body weight Ultralente, Novo Nordisk), but another remained untreated and hyperglycaemic. The dose of insulin was adjusted according to the blood sugar in an attempt to maintain euglycaemia, using the following dosing regime: blood glucose 3-4.9mmol/l - the previous day's insulin dose was halved; blood glucose 5-9.9mmol/l - no dose adjustment; blood glucose > 10mmol/l - the insulin dose was increased by 0.15U/100g body weight.

3.3ii Preparation of Donor WAGs/ Removal of Rat Pancreata

Male WAG/Leics rats (weighing 200-250g) were used as donor rats providing islets obtained by collagenase distension of the pancreas under terminal anaesthesia. The rat was anaesthetised by inhalation of a mixture of halothane, oxygen and nitrous oxide, and positioned with its head near to the operator. The abdomen was shaved and a midline incision

was made from the base of the sternum down to the pelvis. The liver was displaced by pressing down on the chest, and the three most prominent lobes were folded back using a sterile gauze swab. The duodenum was pulled up gently and spread out displaying the bile duct leading from the liver to the pancreas. At the point where the bile duct enters the duodenum, a clip was placed, and another clip was secured across the branch of the duct entering the right lobe of the liver. The animal was exsanguinated by dividing the aorta and vena cavae, and the blood packed off with a swab. 5 mls of Minimal Essential Medium (MEM) containing 1mg/ml collagenase (Sigma XI) and DNA-ase (0.1ml/ml) was drawn up into a syringe fitted with a 23G blue needle, and carefully inserted into the junction of the left and right hepatic ducts. Injection of the collagenase distended the pancreas approximately two to three times its normal size.

Then the duodenum was swung over to the right and the stomach pulled up to reveal the spleen. The spleen was removed and connecting fat was divided from the stomach. Next the pancreas was carefully dissected off the colon, the duodenum and the back wall of the abdomen leaving it suspended by its duct from the clip. The pancreas was completely removed by snipping any residual strands of tissue, and was placed in an empty universal container on ice. Three male donor rats were used to provide enough islets for transplantation for each diabetic rat and the intended reversal of diabetes.

3.3iii Purification of Islets

The technique was described first in 1965 by Moskalewski, who retrieved intact islets from guinea pig pancreas distended with collagenase. This technique was then further developed and adapted for use in the rat by Lacy and Kostianovsky in 1967. Since then, a number of improvements to the original method of islet isolation have been made, including the development of density gradients for the purification of the islets from the digest, using both Ficoll (Ballinger & Lacy, 1972; Kemp *et al.*, 1973), and bovine serum albumin (Lake *et al.*, 1987) .

After harvesting the pancreata from three rats, 10mls of warm Minimum Essential Medium (MEM comp) was poured onto the cold pancreata and placed in a water bath at 37°C to digest for fourteen minutes. Then warm MEM was decanted into a beaker and the digestion terminated by adding cold MEM (4°C). The universals were then transferred to a sterile hood and the tissue disrupted by vigorous hand shaking for one minute and the digest was poured through a mesh into a large beaker. The universals and the beaker were rinsed with MEM containing bovine serum albumin (BSA: 500mls MEM and 5mls of 35% BSA) making up a total of 100mls. Then the digest was poured in to 2x 50ml tubes and spun at 1200 rpm, 4°C for two minutes. The supernatant was decanted and the pellet resuspended in MEM + BSA and pooled into one tube. This supernatant was re-centrifuged at 1200 rpm, 4°C for two minutes. Whilst spinning, six conical bottomed test tubes were set up in the sterile hood, two per pancreas. Again, the supernatant was decanted, and the islets harvested using a bovine serum albumin (BSA) gradient technique (Lake *et al.*, 1987, 1989). 12mls of high density BSA (1.085g/cm^3) was pipetted onto each pellet and mixed thoroughly until all the digest was homogenous. The digest (+ BSA) was transferred via pipette into the six conical-bottomed tubes (2mls in each), and over-laid with 12mls of 1.069g/cm^3 BSA and 12mls of 1.056g/cm^3 BSA. Care was taken to avoid turbulence and air bubbles which might disrupt the gradients. Finally, 12mls of MEM was pipetted on top (this is very light and helps prevent clumping), so that in total there were 8mls of fluid of four different densities carefully layered one on top of the other in each of the six tubes (Plate 3).

Islets were removed from the 1.056-1.069 interface with a sterile pipette after 20 minutes centrifugation at 1970 rpm at 4°C; the supernatant was discarded, and the islets were placed in a universal, with MEM + BSA and were spun at 1200rpm, 4°C for 2 minutes. After a second wash the islets were resuspended in RPMI 1640 media, containing 10% Foetal Calf Serum + 1ml aliquot of L-glutamine (see Appendix B). This was poured into a sterile petri dish, and the islets cultured overnight in a CO_2 incubator prior to transplantation.

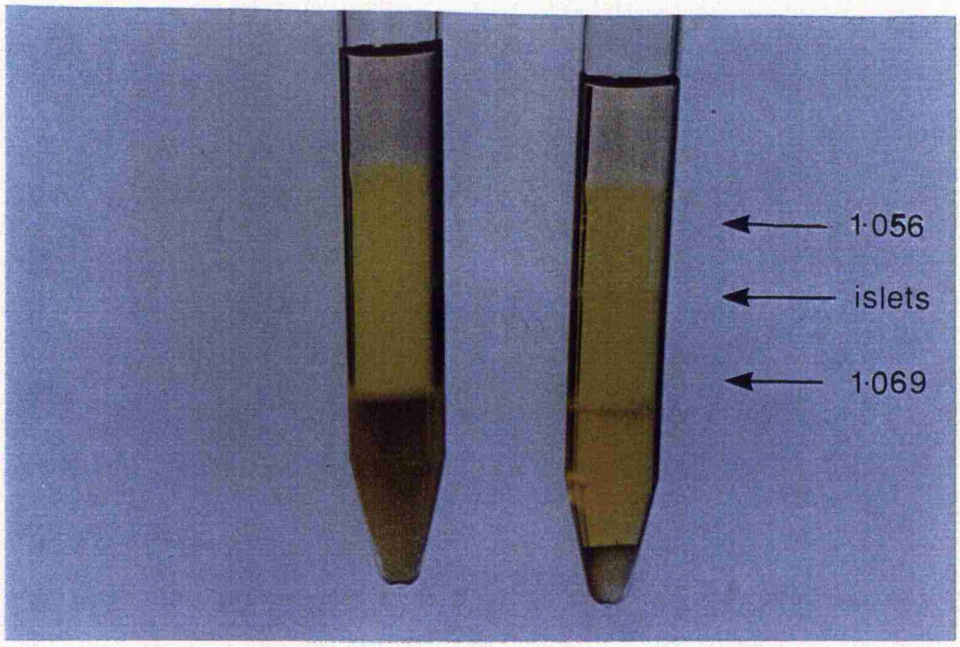

Plate 3: BSA gradient layers + MEM. Islets were removed from the 1.056-1.069 interface.

3.3iv Hand Picking of Islets

After overnight incubation in RPMI + L-glutamine, the petri dish containing the islets was examined under a dissecting light microscope, and approximately 1000 islets (diameter approx. 100-200μm) were hand-picked using a sterile glass pipette. These were spun at 1200rpm at 4°C for two minutes, the supernatant poured off and the remaining islets resuspended in 1ml of RPMI + L-glutamine and drawn up into a 1ml syringe fitted with a 23G blue needle ready for transplantation.

3.3v Preparation of Recipient Rat

Rats receiving an islet transplant were rendered diabetic by a single intravenous injection of 55mg/kg STZ up to three weeks before the transplant procedure. The rat would have received daily subcutaneous injections of insulin for two weeks, followed by cessation of insulin therapy for one week before the transplantation of islets. To ensure that the rats were truly diabetic, insulin therapy was stopped until the blood glucose levels were 20mmol/l or greater.

3.3vi Transplantation of Islets

The fully anaesthetised rat was placed on the operating table, with its abdominal area shaved and swabbed with hibitane. A midline longitudinal incision approximately 3cm in length was made so that the top of the incision just revealed the lobes of the liver. The skin and muscle layers were clipped back near the top of the wound, and using sterile cotton buds dipped in warm, sterile RPMI, the left hand lobe of the liver (in respect to the operator) was carefully raised, and the duodenal loop containing the large hepatic portal blood vessel was displayed. The 1000 islets suspended in 1ml RPMI + L-glutamine then were slowly injected into the portal vein (Plate 4). Once the syringe was emptied, blood was drawn back into the syringe and re-injected, causing any residual islets to be flushed out into the hepatic portal vein. The control group of rats undergoing a sham transplant procedure received an injection of 1ml

RPMI + L-glutamine containing no islets.

A piece of haemostat gauze was then placed over the needle puncture after withdrawal, and pressure was applied to stem the flow of blood. Once haemostasis was recovered, the bowel was replaced, the clips holding the muscle and skin were removed, and the muscle layer was sutured with dissolvable coated vicryl (Ethicon). The skin was closed with separate mersilk sutures (Ethicon). Finally, 0.1ml temgesic (an analgesic) was injected subcutaneously, and the animal was left to recover. Transplants were considered to be successful if the animal became normoglycaemic within seven days of transplantation, and maintained blood glucose levels consistently below 10mmol/l. Transplanted animals were culled if two consecutive blood glucose readings were >20mmol/l.

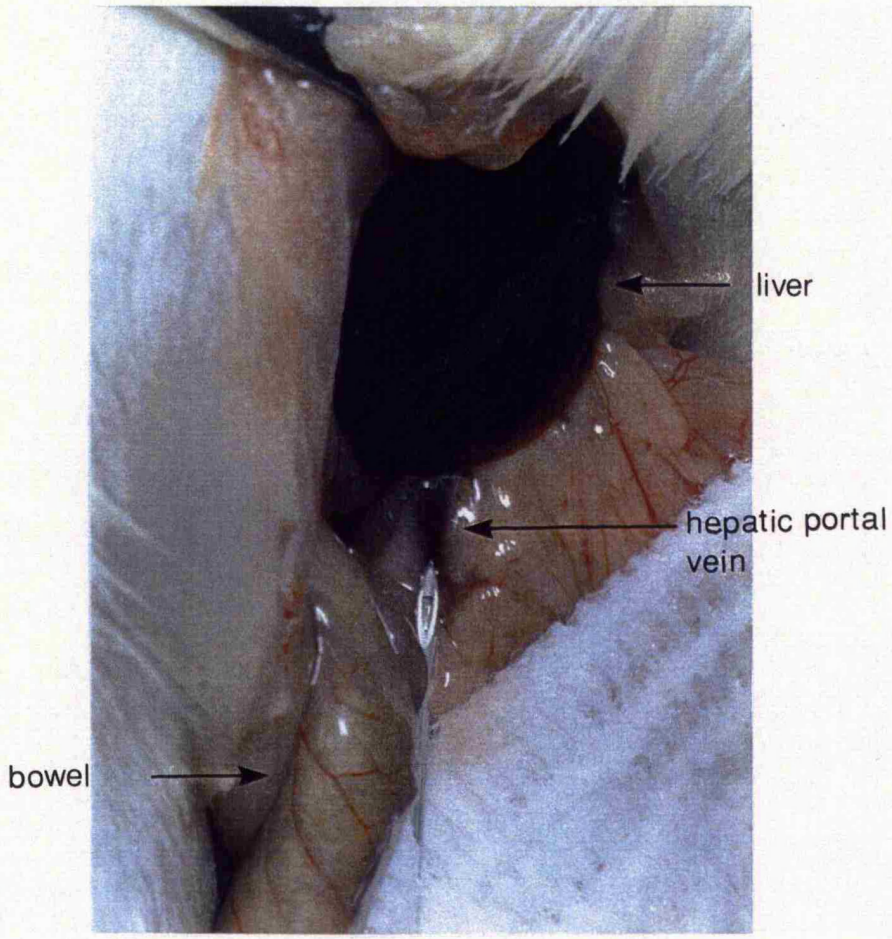

Plate 4: Transplantation of islets into the liver of a recipient diabetic rat, via the hepatic portal vein.

3.4 Indirect Blood Pressure Measurement

Systolic blood pressure was measured in conscious restrained rats by the tail cuff method using plethysmography and a physiograph recorder (IITC Model 59, Life Science Instruments, Woodland Hills, California). This method uses a highly sensitive photoelectric cuff with a built in sensor which detects changes in light brightness produced in the rat tail during systole and diastole. These changes are then relayed to a control unit which contains a filter and an amplifier. The amplifier was connected to a dual channel chart recorder to provide permanent paper recordings of the tail pulse waves and the cuff pressure. Before rat blood pressure measurements were taken, the pressure on the chart recorder had been previously calibrated using a mercury sphygmomanometer.

Rats were placed in pre-warmed perspex restraining tubes and in a cabinet heated to 27-29°C. After a fifteen minute equilibration period, a tail cuff containing a photoelectric sensor was secured on to the tail plate of the tube by means of plastic screws. The rat was then left for a further five minutes to acclimatise. In order to detect a pulse, a minimal pressure of approximately 40-60mmHg was applied to the cuff. Once a steady pulse was achieved, the cuff was manually inflated until the pulse disappeared. Then the cuff was slowly deflated and the reappearance of the tail pulse was noted. The pressure at the onset of the pulse corresponds to the appearance of the tail pulse. When manually inflating, a typical cycle would usually take around 15-20 seconds. The cuff was inflated three times with a three minute elapse between readings; the mean of these three readings was recorded.

3.5 Rat Dissection

All rats were sacrificed by stunning followed by cervical dislocation. The mesentery with its feeding vasculature was removed by a mid-line laparotomy. The excised section was placed in a beaker of cold (4°C) physiological salt solution. The hind limbs were removed at the pelvic joint for tibia length measurement. Once removed the leg was severed above the knee

joint, and the muscle and skin of the tibia were mechanically stripped. The length of the tibia from the condyles to the tip of the medial malleolus was measured using a vernier calliper. The heart was excised from the thoracic cavity, blotted dry and weighed. Heart weight / tibial length ratio was used as an index of cardiac hypertrophy (Yin *et al.*, 1982).

3.5i Fine Dissection and Preparation of Resistance Vessels

The excised mesentery was placed in a petri dish coated with sylgaard and bathed in cold physiological salt solution (4°C). Without stretching the vessels, the mesentery was pinned out and the "test segment" selected. This was a point in the gut, routinely identified for fine dissection of mesenteric small arteries and was approximately 5cm from the posterior end of the colon. The resistance arteries were then selected according to their branching pattern, with the first order vessels branching directly from the superior mesenteric artery, and the second and third order vessels representing successive branches from the first order branch. Once identified, two of the third order branches of the superior mesenteric artery of diameters <350μm were dissected out with the aid of a light microscope, and cleaned of connective tissue and fat, using trabecula scissors and watchmaker forceps. All experiments in this thesis were performed using third order mesenteric resistance arteries.

3.6 Experimental Protocols Using the Myograph

Every study in this thesis investigating resistance artery structure and function involved a series of experiments conducted on a myograph. All mesenteric arteries routinely dissected from the sacrificed rats underwent the same procedures preceding the commencement of the pharmacological dose response curves. Once mounted on the myograph, the temperature of the bath was raised, and the vessels were allowed to equilibrate for thirty minutes, bathed in physiological salt solution (PSS-See Appendix B) at 37°C, which was gassed with 5% CO_2 and 95% O_2 in order to maintain a pH of 7.4. Morphological measurements of lumen diameter and wall thickness then were obtained. After a further thirty minute equilibration

period, the length-tension characteristics of each vessel were determined using the normalisation procedure, as previously described (section 3.1iii).

The vessels were then left in PSS for sixty minutes, with the bathing medium being regularly changed every twenty minutes, after which an activation procedure was performed. All resistance arteries were subjected to this initial activation procedure before commencing the experimental protocols. The procedure involved three stimulations of high potassium PSS, followed by one stimulation of high potassium PSS containing 10^{-5}M noradrenaline. Each contraction was maintained for two minutes before rinsing with PSS to baseline. After the fourth stimulation, the vessels were rinsed three times with PSS and left to recover at baseline for fifteen minutes, before the dose contraction and relaxation studies were performed.

3.6i Contractility of Vessels

Vascular contractility was then assessed by exposing the vessels to logarithmic increasing doses of noradrenaline (NA) over the range 10^{-8}M-$3x10^{-5}$M, and the resulting contractile responses recorded. Twenty minutes prior to the curve, cocaine (10^{-6}M) was added to the bathing medium in order to prevent neuronal re-uptake of noradrenaline. The vessels were then rinsed to baseline with PSS and allowed to recover for fifteen minutes.

3.6ii Relaxation Responses

Endothelial cell function was assessed by observing the response to cumulative doses of acetylcholine (ACh), bradykinin (BK), and sodium nitroprusside (SNP). Endothelial dysfunction was confirmed by abnormal relaxation response to acetylcholine and/or bradykinin without an alteration in the relaxation response to the endothelium-independent vasodilator sodium nitroprusside.

The acetylcholine relaxation responses were performed as follows; vessels were rinsed in

fresh PSS and then maximally contracted with a bolus dose of noradrenaline (10^{-5}M). Once a plateau in the contraction response had been reached, logarithmic increasing doses of acetylcholine or bradykinin, over the range 10^{-9}M to 10^{-5}M were added to the bath. Each dose of acetylcholine/bradykinin was left in the bath for at least two minutes or until the relaxation response had plateaued. Endothelium-independent relaxation responses were assessed by recording the response of maximally contracted arteries to cumulative doses of the nitric oxide donor sodium nitroprusside over the range 10^{-9}M-10^{-4}M.

3.6iii Endothelium Denudation

Some vessels were studied after the endothelium had been removed by a technique first described by Osol *et al.*, (1989). This was achieved by mechanical removal by passing a human hair through the lumen of the vessel mounted under tension in the myograph. (Prior to use the hair was washed in ethanol and rinsed with PSS.) The hair was then repeatedly drawn through the lumen of the vessel, causing removal of the endothelial cells. The successful removal of the endothelial lining from the vessels was then later confirmed by histological examination using a scanning electron microscope.

3.7 Solutions

All vessels were dissected and mounted in cold (4°C) PSS, the composition of which is shown in appendix B. The experimental protocols in the myograph were also carried out in PSS which was heated to 37°C and gassed with 95% O_2 and 5% CO_2, to maintain a pH of 7.4. The PSS containing high potassium was made by replacing NaCl with an equimolar concentration of KCl (see appendix B).

The solutions used in the preparation and transplantation of islets are also described in detail in appendix B. These include BSA gradients of varying densities, MEM comp, MEM + BSA, MEM for collagenase, and RPMI.

3.7i Drugs

All drugs directly involved in the myograph protocols were freshly prepared on the day of the study and dissolved in distilled water, except indomethacin and SQ 29548 which were dissolved in ethanol. Previous studies performed in this laboratory have shown that this concentration of ethanol has no deleterious effect on the contractile or relaxation responses of the vessels. Streptozotocin, noradrenaline hydrochloride, cocaine hydrochloride, acetylcholine chloride, bradykinin, sodium nitroprusside, L-arginine, superoxide dismutase, catalase, and N^G-nitro-L-arginine (L-NOARG) were obtained from the Sigma Chemical Company, Poole, Dorset, U.K. The thromboxane A_2 inhibitor SQ 29548 was a generous gift from Bristol-Myers Squibb Pharmaceuticals Ltd.

3.8 Histological Studies

At the end of each protocol, vessels were rinsed thoroughly with PSS to baseline, and fixed overnight in 10% formalin. Arteries were then carefully removed from the wires the following day and stored in formalin at 4°C, until histological examination.

3.8i Processing Procedure for Examination of Resistance Artery Endothelium - Using Scanning Electron Microscopy (SEM)

The artery was cut along its longitudinal axis, to expose the endothelial surface. After washing in phosphate buffer (4°C) for one hour, arteries were fixed in aqueous 2% osmium tetroxide for one hour, washed with distilled water at 4°C for a further 60 minutes before being dehydrated for SEM using the following protocol.

Solution	**Time**
50% ethanol in distilled water	10 mins.
70% ethanol in distilled water	10 mins

90% ethanol in distilled water	10 mins
100% ethanol	2x 10 mins.

Arteries were then placed in 100% acetone, which acted as an exchange medium, before transferral to the critical point drier (Polaron). The vessels were critical point dried at a pressure of 200psi, and at a temperature of 31.5°C. After drying, the arteries were mounted on to aluminium stubs using double- sided adhesive tabs, and coated with 1.5mm of gold in a polaron (E5150) sputter coating unit. Specimens were examined with electron microscopy for the presence or absence of endothelium.

3.8ii Procedure for the Examination of Media Thickness by Light Microscopy

Formalin fixed vessels were washed in phosphate buffer for one hour (4°C), before being post-fixed with aqueous 2% osmium at 4°C for one hour. Arteries were then washed for two hours with phosphate buffer before dehydration and embedding in araldite resin as described below.

Solution	**Time**
50% ethanol in distilled water	10 mins.
70 % ethanol in distilled water	10 mins.
90 % ethanol in distilled water	10 mins.
100% ethanol	10 mins.
100% ethanol (dried over Na_2SO_4)	10 mins.

The vessel was then placed in Propylene Oxide (1,2-Epoxy Propane) which acted as an exchange medium, before being infiltrated with Araldite resin (Agar AIDS).

Solution	Time
1,2-Epoxy Propane	20 mins.
1,2-Epoxy Propane	20 mins.
50:50 1,2-Epoxy Propane: Araldite	24 hours.

The 1,2-Epoxy Propane was allowed to evaporate for a minimum of three hours before embedding in a further change to pure Araldite resin, containing one drop of benzyldimethylamine (BDMA) catalyst /gm of Araldite for 24 hours. Following this, the tissue was embedded in fresh Araldite resin containing BDMA catalyst in a coffin mould (Agar Aids), and polymerised at 600°C for 48 hours. Transverse sections (1μm thick) were cut from the hardened blocks using a glass knife, and stained with 0.1% toluidine blue, for light microscopy and photography.

3.8iii Examination of Liver Tissue Removed from Islet Transplanted Rats

Livers removed from islet transplanted rats were fixed in 10% formalin and stored at 4°C prior to histological examination by the department of Pathology, University of Leicester. The fixed tissue was dehydrated through graded alcohols before being transferred through xylene to paraffin wax (on a Shandon Hypercentre). Sections 4μm thick were mounted on slides, deparafinised, hydrated, and stained with Haematoxylin (BDH) and eosin.

Immunohistochemistry - Other 4μm sections were mounted on silane (Sigma) coated slides, and stained using the immunohistochemical technique to the presence of insulin. Each slide was processed through xylene and alcohols to phosphate buffered saline prior to staining using the ABC method out lined below.

The sample was blocked in 5% hydrogen peroxide	10 mins
Repeated washing	5 mins

Phosphate Buffered Saline (PBS), pH 7.6	5 mins
Normal Rabbit Serum 1:20 DADO	10 mins
Primary antibody 1:50	o/n 4°C
Phosphate Buffered Saline washing	20 mins
2° Ab.-Rabbit anti-mouse immunoglobulins, Diotinylated 1:4000 DADO	30 mins
Phosphate Buffered Saline washing	20 mins
3° Ab. -ABC (Vecstastain Elite ABC Kit)	30 mins
Phosphate Buffered Saline washing	20 mins
DAB chromogen (Sigma) with hydrogen peroxide substrate	5 mins

The final stage of this process involved the sample being counter-stained in haematoxylin for 30 secs.

3.9 Data and Statistical Analysis

As two resistance arteries were mounted and studied simultaneously on the myograph the results generated in response to contractile and relaxing agents were averaged. Results are expressed as the mean ± standard error of the mean (s.e. mean). Contractile responses to noradrenaline were expressed as active tension (milli Newtons/mm), which was calculated from the measured force (milli Newtons) divided by twice the vessel length (millimetres). The active media stress (mN/mm^2) of the vessels was also calculated, by dividing the active tension (mN/mm) by the normalised wall thickness (μm), to take into account the differences in media wall thickness of each of the vessels. The coefficient of variation was calculated (as an indicator of intra-animal reproducibility) for maximum responses and sensitivity to noradrenaline within each experimental group and was found to be less than 20% and <8% respectively.

Relaxation responses to ACh, BK, and SNP are expressed as a percentage decline of the maximum noradrenaline-induced contraction. The sensitivity (EC_{50}) i.e. the concentration of the drug required to produce 50% of the maximum response, was calculated using a curve fitting program for NA, ACh, BK, and SNP concentration-response curves. Since EC_{50} values are not normally distributed, all values were converted to -logEC_{50}s or pD_2 before parametrical statistical tests were applied. Multiple comparisons were made between means of the experimental groups by a one-way analysis of variance (ANOVA). Where p values <0.05 were obtained, individual pairs of means then were compared using the Bonferroni multiple comparisons test. Comparisons within groups were determined by the Student's paired t-test. The coefficient of variation (CV) was also calculated for maximum responses and sensitivity to each of the vasodilators: acetylcholine (ACh max:CV<15%, pD_2:CV<5%), bradykinin (BK max: CV<35%) and sodium nitroprusside (SNP max: CV<10%, pD_2: CV<7%). Where appropriate retrospective power calculations were carried out to determine how many subjects were needed in each experimental group for an 80% chance of detecting a biologically significant difference at the $p<0.05$ level.

CHAPTER 4

THE EFFECT OF HYPER-AND HYPOGLYCAEMIA ON THE ENDOTHELIUM-DEPENDENT RELAXATION OF RESISTANCE ARTERIES FROM NORMAL RATS

4.1 Introduction

Clinical and animal model data indicate that chronic hyperglycaemia appears to be the central initiating factor for all types of diabetic microvascular disease. Studies of insulin-dependent diabetic patients have revealed evidence of impaired endothelium-dependent relaxation (de Tejada *et al.*, 1989; Elliot *et al.*, 1993; Johnstone *et al.*, 1993; Mc Nally *et al.*, 1994), and similarly, impaired responses also have been demonstrated in the majority of animal studies of diabetic vascular function in both the chemically induced diabetic rat (Taylor *et al.*, 1992; 1994 a,b; Pieper & Gross, 1988; Cameron & Cotter, 1992), and the spontaneously diabetic rat model (Durante *et al.*, 1988; Meraji *et al.*, 1992).

A close relationship between hyperglycaemia and the development and progression of diabetic microangiopathy recently has been demonstrated by the Diabetes Control and Complications Trial. This showed that improved glycaemic control in patients with insulin-dependent diabetes delayed both the onset and slowed the progression of retinopathy, nephropathy, and neuropathy (DCCT, 1993). Therefore of the many metabolic disturbances associated with diabetes, hyperglycaemia *per se* is most likely to be the cause of endothelial dysfunction. Indeed, aortae from normal non-diabetic rabbits incubated in elevated glucose medium displayed a marked impaired endothelium-dependent relaxation response (Tesfamariam *et al.*, 1990, 1991, 1992; Tesfamariam and Cohen, 1992).

At the time of this particular study (1992), studies of the effects of hyperglycaemia on the function of blood vessels from normal non-diabetic animals had involved conduit arteries, which have little or no role in the regulation of the microcirculation. Consequently this study was performed to investigate the effects of acute hyperglycaemia, on endothelium-dependent relaxation of resistance arteries dissected from normal rats. In order to determine whether any endothelial dysfunction was directly associated with raised glucose concentrations *per se*, or was merely due to a hyper-osmotic effect, resistance arteries were also incubated in equimolar concentrations of mannose.

4.2 Experimental Protocol

Female Wistar rats (170-190g) were culled and two third order mesenteric resistance arteries (internal diameter <350μm) were mounted in a myograph. The vessels were left to equilibrate for thirty minutes in PSS before undergoing normalisation (see the process described in 3.1iii). After normalisation, the vessels were incubated in PSS for a further 60 minutes before stimulation by the standard activation procedure. A cumulative dose response curve to noradrenaline (10^{-8}M-3x10^{-5}M) was then performed in the presence of cocaine (10^{-4}M) (see section 3.5i). The vessels were rinsed three times with PSS, and allowed to recover at baseline for 15 minutes. The vessels were then maximally re-contracted with a bolus dose of NA (10^{-5}M), and once a plateau had been reached, relaxed with cumulative doses of acetylcholine (ACh 10^{-9}M-10^{-5}M). After the dose response relaxation curves were completed, the bath was rinsed three times with PSS and the vessels allowed to recover for fifteen minutes. Following these initial dose response curves carried out in PSS (containing 4mM glucose), groups of vessels (n=8 in each group) were arbitrarily selected for study using one of the following experimental protocols:

(1) Vessels were incubated in one of the following solutions: **i)** PSS containing 4mM (to act as a time control) **ii)** PSS containing 20mM glucose. **iii)** PSS containing 4mM glucose + 16mM mannose. **iv)** PSS containing 40mM glucose; and **v)** PSS containing 4mM glucose + 36mM mannose. ACh relaxation response curves of maximally contracted vessels with a bolus dose of NA (10^{-5}M) were performed at 60, 120, 240, and 360 minutes after the medium had been changed.

(2) In a second series of studies dose relaxation responses to the endothelium-dependent vasodilators acetylcholine, and bradykinin (BK) were performed at 60, and 120 minutes after changing to PSS containing 4mM glucose, 16mM mannose + 4mM glucose, and 20mM glucose. Then the effect of incubating the vessels for sixty minutes with the nitric oxide synthetase inhibitor N^G-nitro-L-arginine (L-NOARG10^{5} M) on endothelium-dependent relaxation to ACh and BK was investigated. L-NOARG was added at 120 minutes after the final dose response curve to BK (10^{-9}M-10^{-5}M). Finally, a dose relaxation response to the

endothelium-independent vasodilator sodium nitroprusside (SNP 10^{-9}M-10^{-4}M) was performed.

(3) In the third protocol, a six hour study on the effects of incubating vessels in a hypo-osmolar medium (PSS containing 2mM glucose) was carried out. Dose relaxation responses to ACh, and BK were performed at 60, 120, 240, and 360 minutes, with a dose response to SNP also being carried out at 360 minutes.

4.3 Results

4.3.1 Analysis of Results

Differences in the maximum relaxation and sensitivity responses to ACh, and BK were determined using analysis of variance (ANOVA) for repeated measures with Dunnetts correction for multiple comparisons. In order to use this statistical test rectangular matrices of data were necessary, and where this was not the case (as n-numbers were limiting), ordinary ANOVA was used. Comparisons of the maximum endothelium-independent relaxation response to SNP were always made at one time point, i.e t=360, in this case either ordinary ANOVA, or the unpaired t-test was used.

4.3.2 Endothelium-Dependent Relaxation

i) Acetylcholine

There was no significant change in the maximum relaxation response or sensitivity to the endothelium-dependent vasodilator acetylcholine in the resistance arteries incubated for 6 hours in PSS containing 4mM glucose (figure 1), 4mM glucose + 16mM mannose (figure2), 20mM glucose (figure 3), or 4mM glucose + 36mM mannose (figure 4). However the maximum relaxation to ACh was significantly impaired after both 4 hours (240 minutes) and 6 hours (360 minutes) incubation in 40mM glucose compared with the maximum relaxation of vessels in the control medium of 4mM glucose at t=0 (t=240: 64±12% versus 88±3%, n=7, $p<0.05$ and t=360: 54±10%, versus 88±3%, n=7, $p<0.01$) (figure 5). The sensitivity to ACh of the vessels incubated in 40mM glucose was however not significantly different at any time point. (Tables 1 & 2)

The effect of nitric oxide synthetase inhibition

In three experimental groups where the vessels were incubated in PSS containing 4mM glucose, 4mM glucose + 16mM mannose, and 20mM glucose, incubation with L-NOARG significantly impaired the relaxation response to ACh (4mM glucose:81±6% versus +L-NOARG 55±9%, n=8, $p<0.01$; 4mM glucose + 16mM mannose: 70±7% versus +L-NOARG 41±11%, n=8, $p<0.01$; and 20mM glucose: 68±8% versus 36±9%, n=10, $p<0.01$). The ACh sensitivity after incubation with L-NOARG was unaltered in the vessels incubated in 16mM mannose. However, the vessels incubated in 4mM glucose and those incubated in PSS containing 20mM glucose did display a significant shift in sensitivity to ACh after the addition of L-NOARG (7.64±0.1 versus +L-NOARG 7.2±0.13, n=8, $p<0.05$ & 7.37±0.1 versus 6.95±0.22, $p<0.05$, respectively). Surprisingly a significant shift to ACh was also in vessels incubated in 4mM glucose evident at t=120 (7.09±0.15 versus t=0 7.64±0.1, n=8, $p<0.01$). (Tables 3 & 4)

A power calculation was carried out to determine what numbers of animals should be in an experimental group in order to detect a 20% change in maximum relaxation to ACh and a 0.5μmol/l change in sensitivity. According to the power calculation for an 80% chance of detecting these biologically significant differences at the $p<0.05$ level, the number of subjects needed for study should be at least 7. The group sizes used were therefore adequate.

The effect of incubating vessels in a hypo-osmolar medium

Incubating vessels in PSS containing 2mM glucose for 6 hours did not significantly effect the endothelium-dependent vasodilator response to acetylcholine. (Figure 6) Although the maximum relaxation response appeared to decrease with time, this did not achieve statistical significance. ACh sensitivity also remained constant throughout the study. (Tables 5 & 6)

ii) Bradykinin

The effect of nitric oxide synthetase inhibition

Incubation with L-NOARG did not alter the maximum relaxation response or the sensitivity to BK in vessels incubated in 4mM glucose, 16mM mannose, or 20 mM glucose. (Tables 7 & 8)

The effect of incubating vessels in a hypo-osmolar medium

The maximum relaxation response to the endothelium-dependent vasodilator bradykinin was significantly impaired at 120 minutes compared to the relaxation response at zero minutes (26±5% versus t=0: 44±8%, n=8, $p<0.05$). However, the relaxation responses at 240 and 360 minutes in 2mM glucose were not significantly different from the zero time point. The BK sensitivity was unchanged throughout the 6 hour incubation in PSS containing 2mM glucose. (Figure 7) A similar response was observed in the vessels incubated in the control medium (PSS containing 4mM glucose), although there was no significant changes in maximum relaxation or sensitivity to bradykinin over the 6 hour incubation period. (Figure 8 & Tables 9 & 10)

Power calculations revealed that it would be necessary to have at least 15 animals in each group to have an 80% power of detecting a 20% change in maximum relaxation to BK at the $p<0.05$ level. However, in this case the group sizes were a little too small to be certain too detect a significant difference in maximum relaxation. It is possible that this is why no significant difference was determined. Similarly in order to detect a change in a 0.5μmol/l change in sensitivity to BK, the power calculation showed that 8 animals should be studied. The numbers in the experimental groups were 6 & 7, therefore again, the group sizes were slightly too small to be certain to detect a significant difference in sensitivity.

4.3.3 Endothelium-Independent Relaxation

i) Sodium Nitroprusside

The effect of nitric oxide synthetase inhibition

Incubation with the nitric oxide synthetase inhibitor L-NOARG had no effect on the maximum relaxation response to the endothelium-independent vasodilator SNP. The maximum relaxation response of the vessels incubated in 20mM glucose was significantly greater compared to those incubated in 4mM glucose (99±0.43%, n=10 versus 95 ±1%, n=10, $p<0.05$), although this is of little biological significance. Similarly, the maximum relaxation response of vessels incubated in 20mM glucose also was significantly greater than that observed in vessels incubated in 4mM glucose +16mM mannose (99±0.43%, n=10 versus 96±1%, n=10, $p<0.05$). No change in SNP sensitivity was observed. (Figure 9 &Table 11)

The effect of incubating vessels in a hypo-osmolar medium

There was no significant change in the maximum relaxation response to SNP in the vessels incubated in PSS containing 2mM glucose compared to that found in vessels incubated in 4mM glucose. However, there was a slight reduction of response in the vessels incubated in 2mM glucose, but this change did not achieve statistical significance (95±1%, n=8 versus 91±2%, n=7, p=0.08). Similarly there was no change in the SNP sensitivity. (Figure 10 & Table 12)

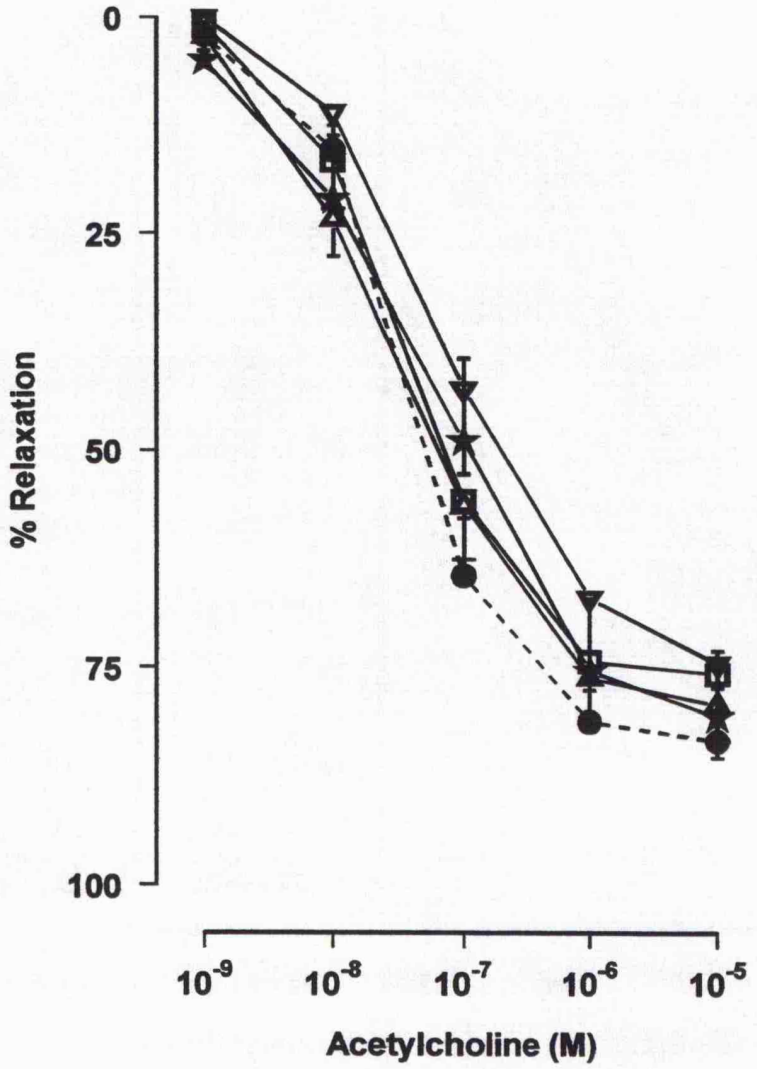

Figure 1 The maximum relaxation response to ACh of resistance arteries incubated in PSS containing **4mM glucose** at: 0 (□), 60 (●), 120 (△), 240 (★), and 360 (▽) minutes.

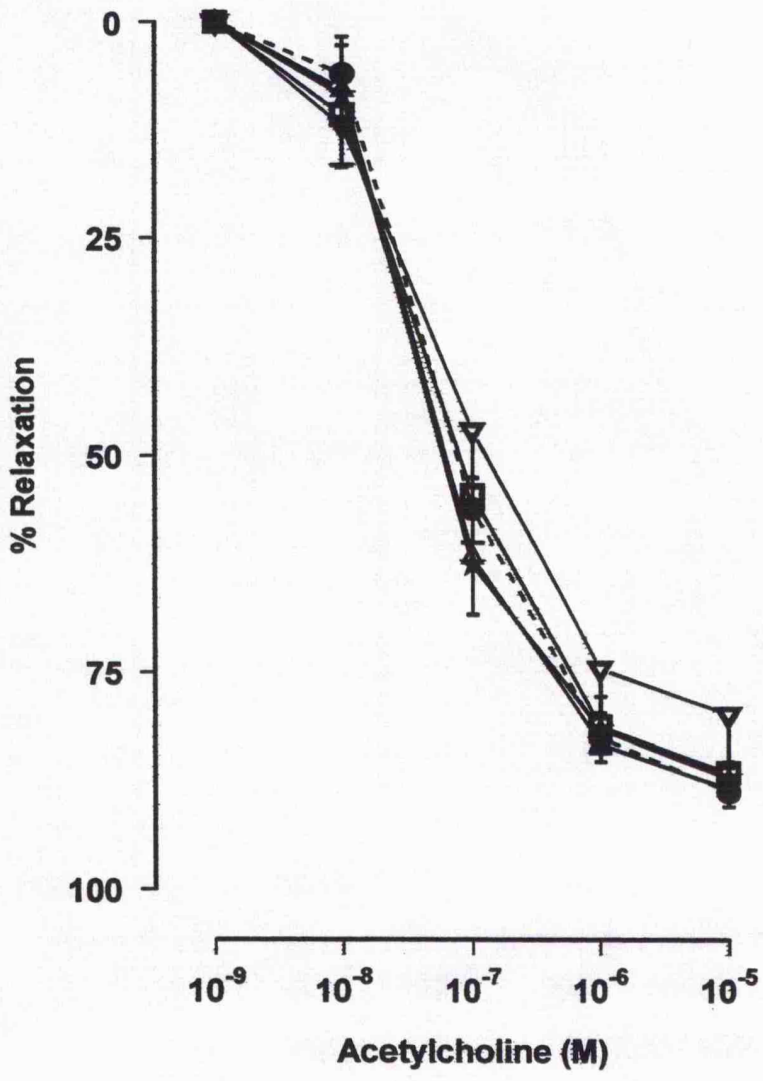

Figure 2 The maximum relaxation response to ACh of resistance arteries incubated in PSS containing 4mM glucose + **16mM mannose** at:60 (●),120 (△), 240 (★), and 360 (▽) minutes, compared to that obtained in 4mM glucose at t=0 minutes (□).

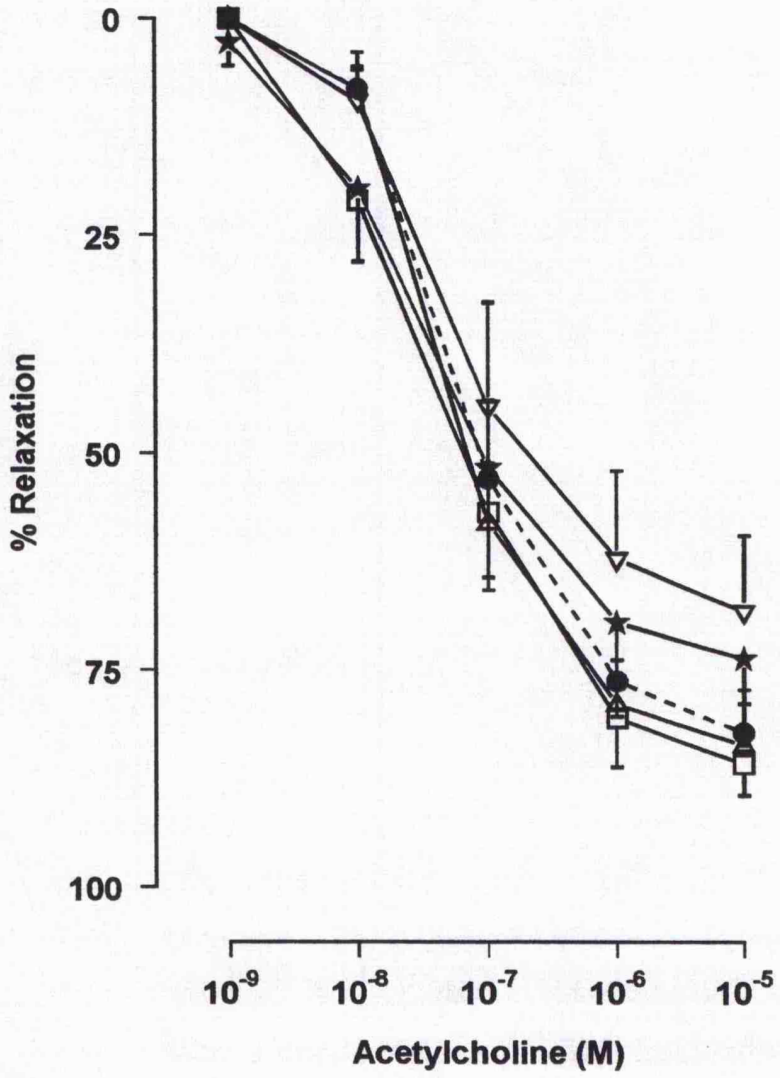

Figure 3 The maximum relaxation response to ACh of resistance arteries incubated in PSS containing **20mM glucose** at:60 (●),120 (△), 240 (★), and 360 (▽) minutes, compared to that obtained in 4mM glucose at t=0 (□) minutes.

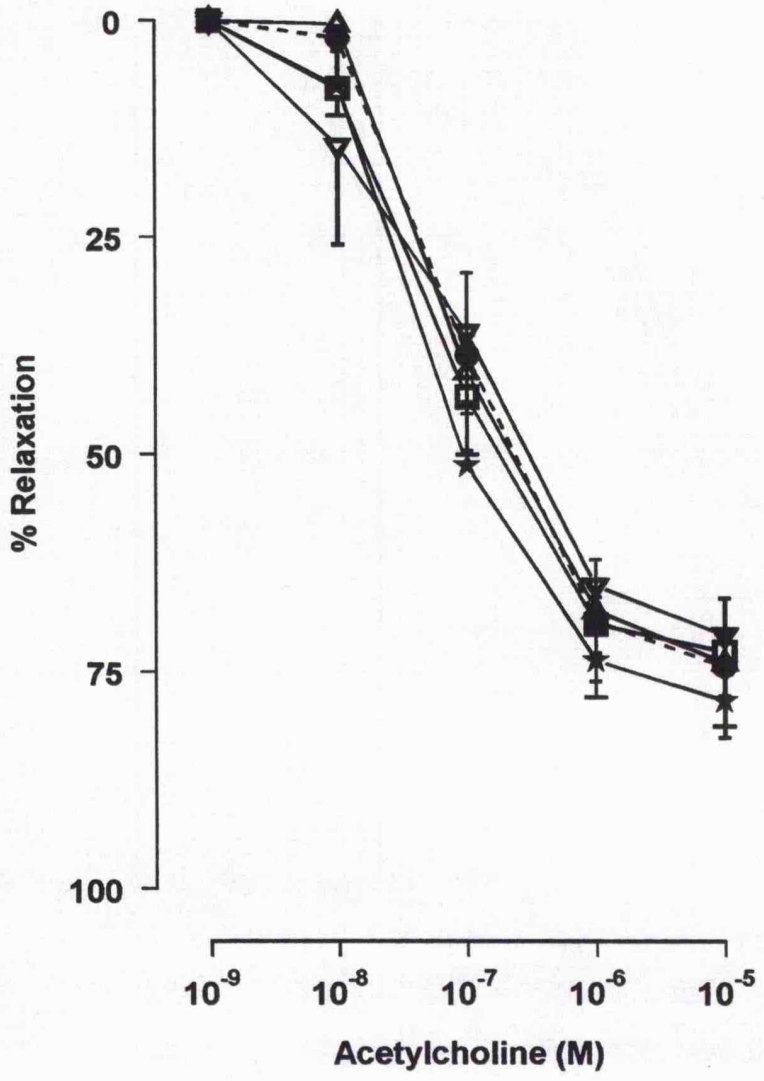

Figure 4 The maximum relaxation response to ACh of resistance arteries incubated in PSS containing 4mM glucose + **36mM mannose** at:60 (●), 120 (△), 240(★), and 360 (▽) minutes, compared to that obtained in 4mM glucose at t=0 (□) minutes.

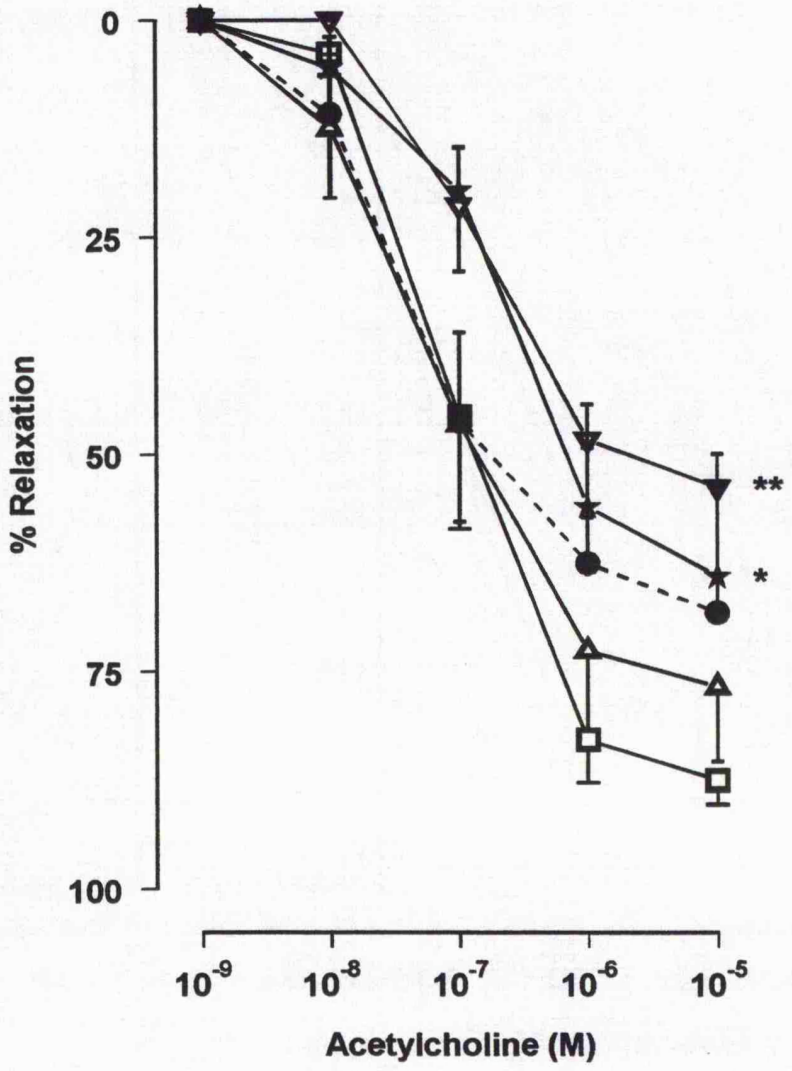

Figure 5 The maximum relaxation response to ACh of resistance arteries incubated in PSS containing **40mM glucose** at: 60 (●),120 (△),240 (★), and 360 (▽) minutes, compared to that obtained in 4mM glucose at t=0 (□) minutes. *$p<0.05$ (t=240 versus t=0); and **$p<0.01$ (t=360 versus t=0).

Table 1 Maximum relaxation response to ACh (expressed as % decline of maximum NA contractile response) of resistance arteries incubated in PSS containing: 4mM glucose; 4mM glucose + 16mM mannose; 20mM glucose; 4mM glucose + 36mM mannose, or 40mM glucose.

		Time (minutes)				
PSS (containing)	N=animals	0 (4mM Glucose)	60	120	240	360
4mM Glucose	8	77±7%	84±6%	79±6%	81±8%	75±9%
4mM Glucose + 16mM Mannose	7	87±3%	89±3%	88±2%	87±2%	80±6%
20mM Glucose	7	86±4%	82±5%	84±5%	78±7%	68±9%
4mM Glucose + 36mM Mannose	7	73±9%	74±8%	74±9%	78±8%	71±7%
40mM Glucose	7	88±3%	82±6%	77±9%	64±14%*	54±10%**

*p<0.05 (t=0 versus t=240), **p<0.01 (t=0 versus t=360) using ANOVA for repeated measures with Dunnetts correction for multiple comparisons.

Table 2 Sensitivity to acetylcholine (expressed as -log EC_{50}/ pD_2s)of resistance arteries incubated in PSS containing: 4mM glucose; 4mM glucose +16mM mannose; 20mM glucose; 4mM glucose +36mM mannose, or 40mM glucose.

		Time (minutes)				
PSS (containing)	**N= animals**	**0 (4mM Glucose)**	**60**	**120**	**240**	**360**
4mM Glucose	9	7.31±0.1	7.36±0.13	7.35±0.12	7.38±0.19	6.95±0.11
4mM Glucose + 16mM Mannose	7	6.85±0.2	7±0.09	7.01±0.07	7.03±0.13	6.99±0.08
20mM Glucose	7	7.13±0.13	7.05±0.11	6.99±0.09	7.24±0.24	7±0.11
4mM Glucose + 36mM Mannose	7	7.11±0.11	6.86±0.07	6.79±0.04	7.0±0.08	6.92±0.17
40mM Glucose	7	6.94±0.12	7.01±0.17	7.01±0.23	6.74±0.07	6.8±0.18

Table 3 Maximum relaxation response to ACh (expressed as % decline of the maximum contractile response) of resistance arteries incubated in PSS containing: 4mM glucose; 4mM glucose + 16mM mannose; or 20mM glucose.

		Time (minutes)			
PSS (containing)	**N=animals**	**0 (4mM Glucose)**	**60**	**120**	**240 + L-NOARG**
4mM Glucose	8	81±6%	79±9%	85±5%	55±9%**
4mM Glucose + 16mM Mannose	8	70±7%	68±8%	61±9%	41±11%**
20mM Glucose	8	68±8%	67±10%	62±10%	36±9%**

**p<0.01 Vessels incubated in L-NOARG versus t=0 (4mM Glucose) using ANOVA for repeated measures with Dunnetts correction for multiple comparisons.

Table 4 Sensitivity to acetylcholine (expressed as -log EC_{50}/ pD_2s) of resistance arteries incubated in PSS containing: 4mM glucose; 4mM glucose + 16mM mannose; or 20mM glucose.

		Time (minutes)			
PSS (containing)	**N=animals**	**0 (4mM Glucose)**	**60**	**120**	**240 + L-NOARG**
4mM Glucose	8	7.64±0.1	7.44±0.08	7.09±0.15**	7.2±0.13*
4mM Glucose + 16mM Mannose	8	7.43±0.14	7.39±0.11	7.39±0.13	7.82±0.43
20mM Glucose	9	7.37±0.1	7.24±0.06	7.1±0.12	6.95±0.22*

*$p<0.05$,** $p<0.01$ versus t=0 (4mM glucose) using ANOVA for repeated measures with Dunnetts correction for multiple comparisons.

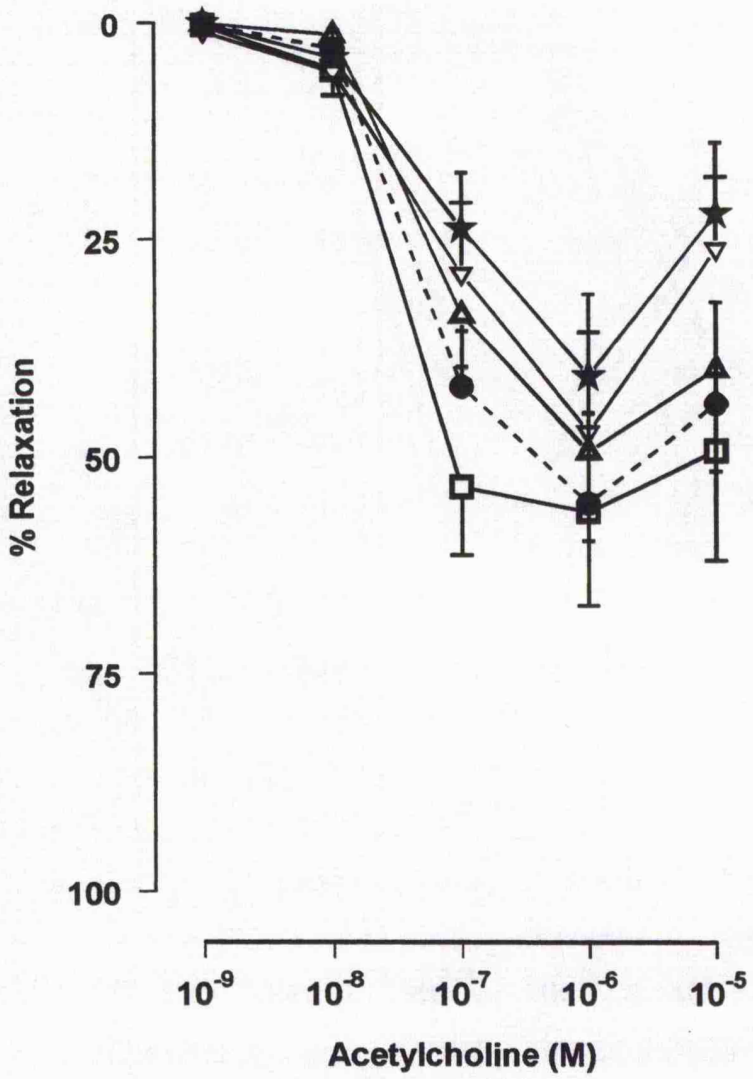

Figure 6 The maximum relaxation response to ACh of resistance arteries incubated in PSS containing **2mM glucose** at: 60 (●), 120 (△), 240 (★), and 360 (▽) minutes, compared to that obtained in 4mM glucose at t=0 (□) minutes.

Table 5 Maximum relaxation response to ACh (expressed as % of maximum NA contractile response) of resistance arteries incubated in PSS containing: 4mM glucose, or 2mM glucose.

		Time (minutes)				
PSS (containing)	**N= animals**	**0 (4mM Glucose)**	**60**	**120**	**240**	**360**
4mM Glucose	9	77±9%	84±6%	79±6%	81±8%	75±9%
2mM Glucose	8	61±9%	57±9%	51±11%	41±8%	47±10%

Table 6 Sensitivity to acetylcholine (expressed as -log EC_{50}/ pD_2s) of resistance arteries incubated in PSS containing either 4mM glucose, or 2mM glucose.

		Time (minutes)				
PSS (containing)	**N= animals**	**0 (4mM Glucose)**	**60**	**120**	**240**	**360**
4mM Glucose	9	7.31±0.1	7.36±0.13	7.35±0.12	7.38±0.19	6.95±0.11
2mM Glucose	8	7.4±0.13	7.35±0.16	7.13±0.09	7.31±0.09	7.43±0.16

Table 7 Maximum relaxation response to BK (expressed as % decline of the NA maximum contractile response) of resistance arteries incubated in PSS containing: 4mM glucose; 4mM glucose +16mM mannose, or 20mM glucose.

		Time (minutes)			
PSS (containing)	**N= animals**	**0 (4mM Glucose)**	**60**	**120**	**240 +L-NOARG**
4mM Glucose	8	48±10%	40±9%	44±10%	31±9%
4mM Glucose + 16mM Mannose	8	45±6%	42±10%	42±7%	29±10%
20mM Glucose	6	44±11%	48±15%	57±13%	54±14%

Table 8 Sensitivity to bradykinin (expressed as -log EC_{50}/ pD_2s) of resistance arteries incubated in PSS containing: 4mM glucose; 4mM glucose+16mM mannose; or 20mM glucose.

		Time (minutes)			
PSS (containing)	**N= animals**	**0 (4mM Glucose)**	**60**	**120**	**240 + L-NOARG**
4mM Glucose	5	6.69±0.15	6.71±0.23	6.58±0.25	6.44±0.1
4mM Glucose + 16mM Mannose	6	6.62±0.24	6.65±0.26	6.35±0.22	6.44±0.04
20mM Glucose	5	6.47±0.22	6.77±0.27	6.38±0.11	6.85±0.13

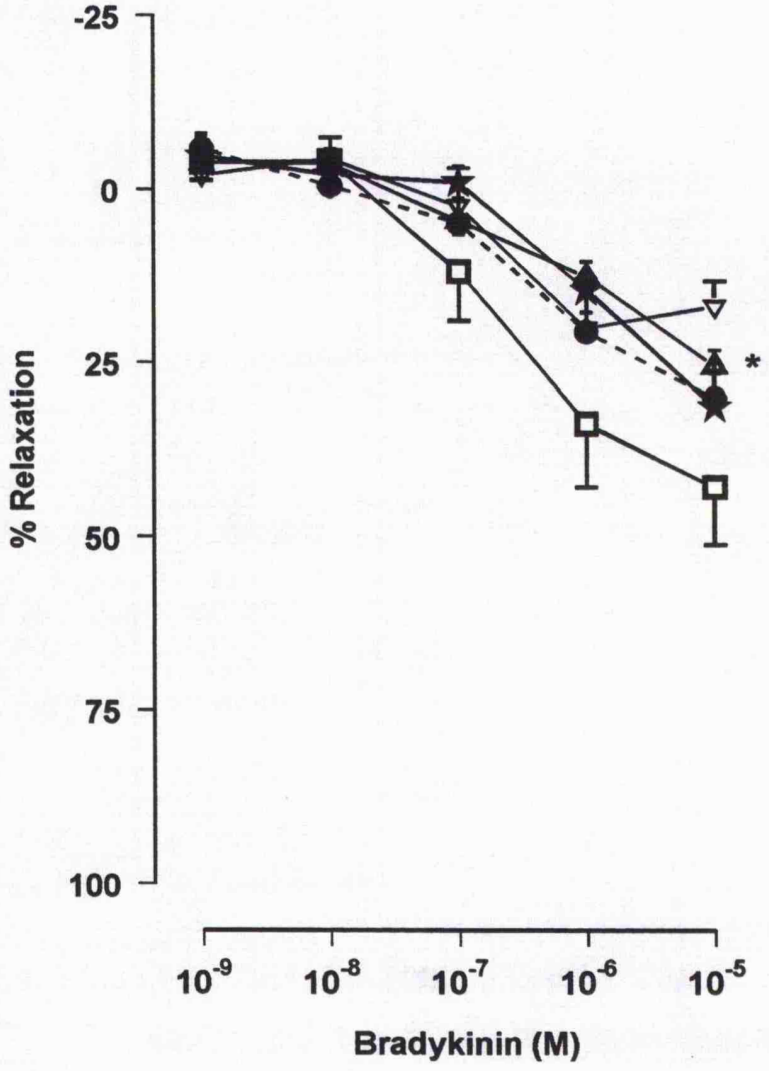

Figure 7 The maximum relaxation response to BK of resistance arteries incubated in PSS containing 2mM glucose at: 0 (●),120 (△), 240 (★), and 360 (▽) minutes, compared to that obtained in 4mM glucose at t=0 (□) minutes. *$p<0.05$ (t=120 versus t=0).

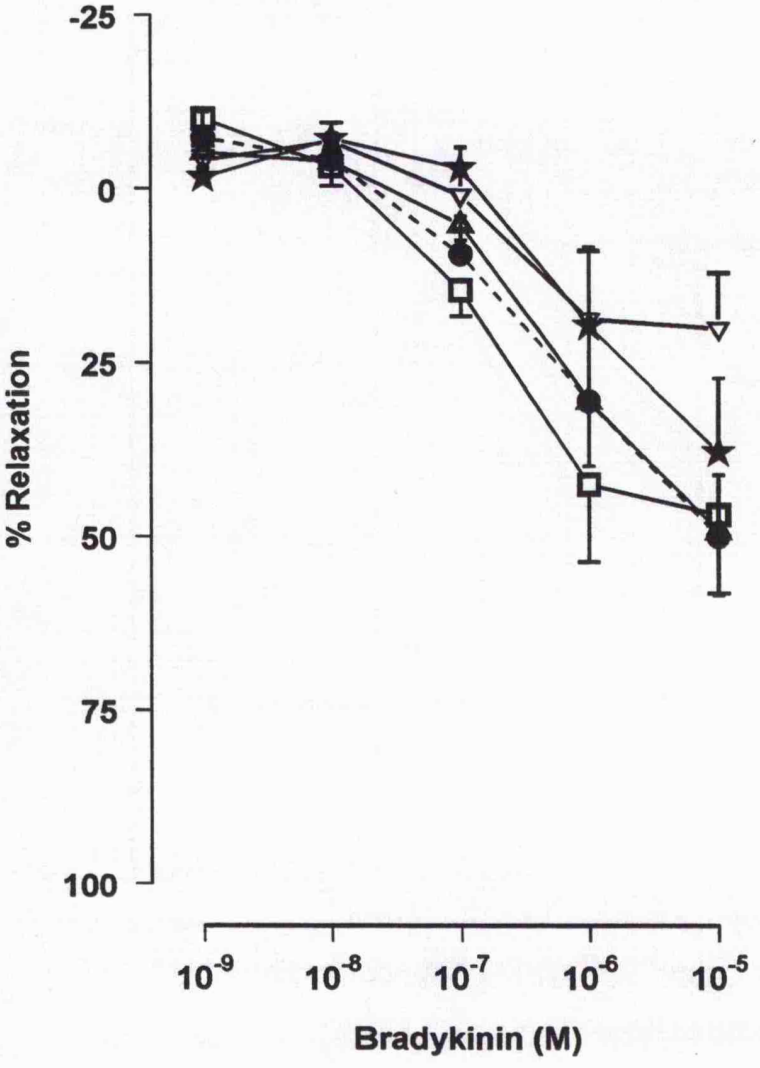

Figure 8 The maximum relaxation response to BK of resistance arteries incubated in PSS containing **4mM glucose** at: 0 (□),60 (●), 120 (△),240 (★), and 360 (▽) minutes.

Table 9 Maximum relaxation response to bradykinin (expressed as % decline of the maximum NA contractile response) of resistance arteries incubated in either PSS containing 4mM glucose, or 2mM glucose.

		Time (minutes)				
PSS (containing)	**N=animals**	**0 (4mM Glucose)**	**60**	**120**	**240**	**360**
4mM Glucose	8	49±10%	50±9%	51±13%	38±11%	24±10%
2mM Glucose	8	44±8%	31±7%	26±5%*	33±4%	28±4%

*$p<0.05$ (t=120 versus t=0) using ANOVA for repeated measures with Dunnetts correction for multiple comparisons

Table 10 Sensitivity to bradykinin (expressed as -log EC_{50}/ pD_2s) of resistance arteries incubated in either PSS containing 4mM glucose, or 2mM glucose.

	Time (minutes)				
PSS (containing)	**0 (4mM Glucose)**	**60**	**120**	**240**	**360**
4mM Glucose	6.69±0.12 (6)	6.86±0.39 (6)	6.39±0.17 (8)	6.27±0.16 (4)	6.53±0.56 (2)
2mM Glucose	6.52±0.14 (7)	6.52±0.15 (6)	6.43±0.1 (5)	6.38±0.08 (3)	7.11±0.01 (2)

Number of animals shown in brackets.

Statistical analysis was performed using ANOVA.

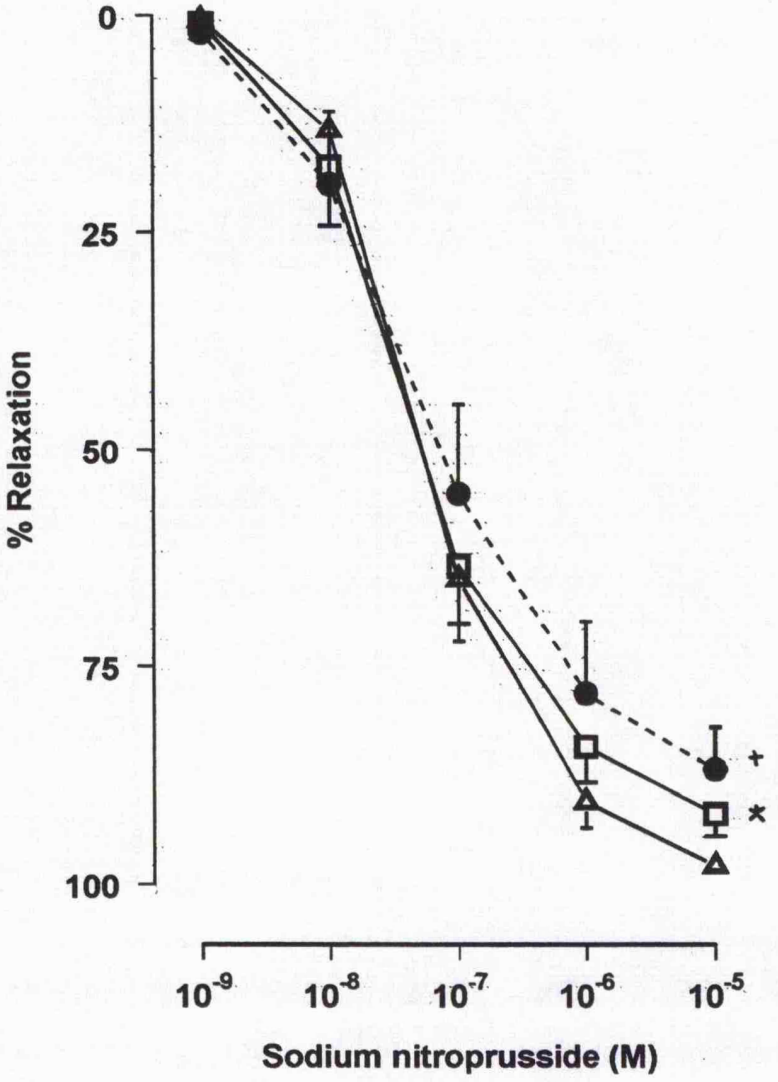

Figure 9 The maximum relaxation response to SNP of resistance arteries incubated in PSS containing: **4mM glucose** (□), 4mM Glucose + **16mM mannose** (●), or **20mM glucose** (△), at t=240minutes (+ L-NOARG). * $p<0.05$ unpaired t-test, 4mM glucose versus 20mM glucose; + $p<0.05$ unpaired t-test, 16mM mannose versus 20mM glucose.

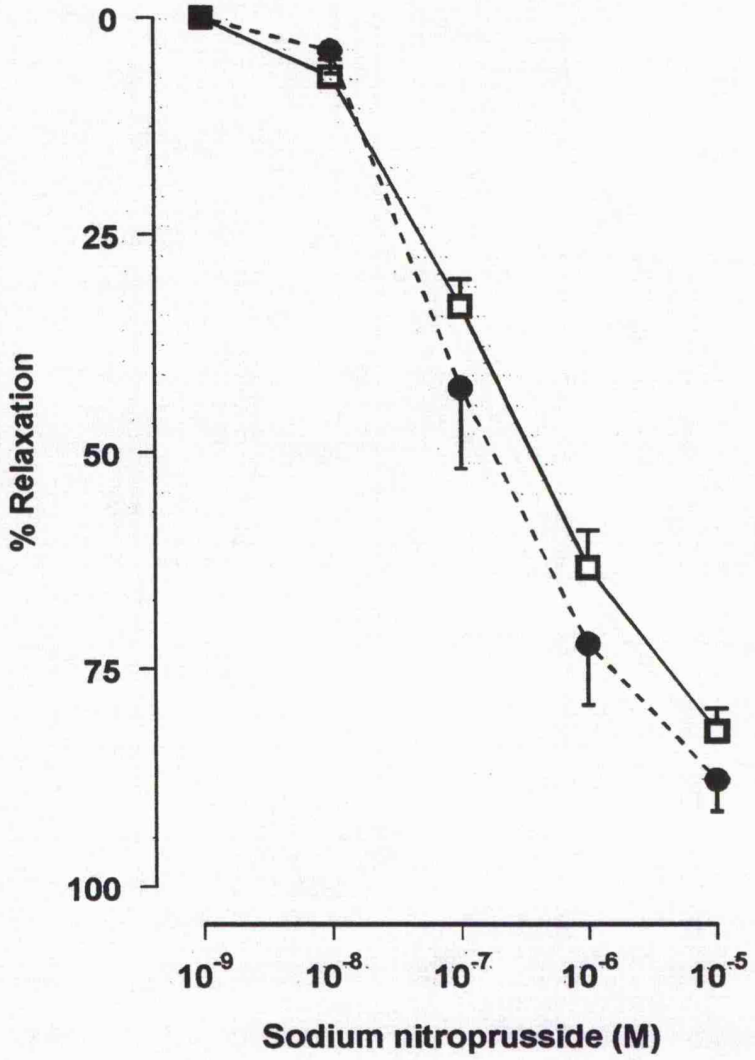

Figure 10 The maximum relaxation response to SNP of resistance arteries incubated for 360 minutes in PSS containing **2mM glucose** (□), or **4mM glucose** (●).

Table 11 Maximum relaxation response (expressed as % decline of maximum NA contractile response) and sensitivity to SNP (expressed as -log EC_{50}/ pD_2s) of resistance arteries incubated in PSS containing:4mM glucose, 4mM glucose +16mM mannose, or 20mM glucose at t=240 minutes (+L-NOARG).

PSS (containing)	N = animals	Maximum Relaxation (%)	Sensitivity (pD_2)
4mM Glucose	10	95±1	6.84±0.17
4mM Glucose + 16mM Mannose	10	96±1^{+}	6.74±0.23
20mM Glucose	10	99±0.43*	6.91±0.12

*$p<0.05$ Unpaired t-test, 4mM glucose versus 20mM glucose.

$^{+}p<0.05$ Unpaired t-test, 16mM mannose versus 20mM glucose.

Table 12 Maximum relaxation response (expressed as % of maximum NA contractile response) and sensitivity to SNP (expressed as -log EC_{50}/ pD_2s) of resistance arteries incubated in PSS containing 4mM glucose, or 2mM glucose at 360 minutes.

PSS (containing)	N= animals	Maximum Relaxation (%)	Sensitivity (pD_2)
4mM Glucose	8	95±1	6.49±0.15
2mM Glucose	7	91±2	6.56±0.08

4.4 Discussion

These studies demonstrate that acute exposure to hyperglycaemia causes progressive impairment of endothelium-dependent relaxation. This was marked in the resistance arteries incubated in PSS containing 40mM glucose where the maximum response to the vasodilator acetylcholine became significantly impaired at 4 & 6 hours. No change in the ACh relaxation response was observed in vessels incubated in PSS containing 36mM mannose, indicating that the attenuation by high glucose was not dependent on the increase in osmolarity.

Others have demonstrated that exposure to high glucose impairs endothelium-dependent relaxation of isolated rat mesenteric resistance arteries (Taylor & Poston, 1994). A significant attenuation of the ACh response was observed in vessels incubated in 20mM glucose and 45mM glucose after 2 hours. Interestingly, this study also showed an impaired relaxation response to the endothelium independent vasodilator sodium nitroprusside (Taylor & Poston, 1994). However, the design of this study was different from my study in that the basal responses of the vessels were not recorded, and the authors assumed that they would be similar in each experimental group. Furthermore the osmolarity of the high glucose PSS was maintained by reducing the sodium concentration to unphysiological levels, by substituting choline chloride for sodium chloride.

Attenuation of endothelium-dependent relaxation also has been consistently demonstrated in studies of normal rabbit aortae exposed to 44mM glucose for 6 hours (with a less marked effect at 2 and 3 hours) (Tesfamariam *et al.*, 1990 & 1991). Moreover, these studies showed that the response to the endothelium-independent vasodilator sodium nitroprusside and the calcium ionophore A23187, were unaffected, indicating that the ability of the vascular smooth muscle to relax is not inhibited by elevated glucose (44mM). This is supported by further studies which showed that cyclic 3',5'-guanosine monophosphate (cGMP) levels in these arteries were normal (Cohen, 1993). The high glucose induced attenuated relaxations were prevented by pretreatment with the cyclo-oxygenase inhibitor indomethacin, suggesting an increased generation of vasoconstrictor prostanoids (Tesfamariam *et al.*, 1990), and associated free radicals (Tesfamariam & Cohen, 1992). However the results from studies involving

conduit arteries should be interpreted with caution, since they may not reflect changes in the microcirculation (Cohen, 1993).

The relaxation response of the endothelium-dependent vasodilator bradykinin was not impaired by incubation in PSS containing 20mM glucose, but unfortunately a dose response curve to BK was not performed after incubation in 40mM glucose. As a consequence, it is impossible to predict whether an impaired response would have occurred with exposure to a more hyperglycaemic medium. However, studies of isolated arteries from the chemically induced diabetic rat have shown an impaired response to acetylcholine with normal relaxation responses to bradykinin (Taylor *et al.*, 1992; Lash & Bohlen, 1991). Similarly, impaired endothelium-dependent relaxation response to acetylcholine in association with a normal response to bradykinin also has been demonstrated in subcutaneous resistance arteries from humans with insulin-dependent diabetes (McNally *et al.*, 1994). This divergent response to the two endothelium-dependent vasodilators suggests an abnormality at the G-protein receptor level: Moreover, in both bovine mesenteric arteries (Ljusengren *et al.*, 1990), and rabbit aorta (Cohen & Tesfamariam, 1992), ACh-induced relaxation is linked to an endothelial pertussis toxin-sensitive G_i-protein, whereas BK-mediated relaxation is linked to a different G-protein, G_q which is largely pertussis-toxin insensitive (Taylor *et al.*, 1991; Liao & Homcy, 1992)..

Incubation with the competitive nitric oxide synthetase inhibitor N^G-nitro-L-arginine (L-NOARG) caused a significant inhibition of acetylcholine-induced relaxation in the vessels incubated in PSS containing 4mM glucose, 4mM glucose + 16mM mannose, and 20mM glucose. However, a complete blockade of endothelium-dependent relaxation did not occur, which may indicate incomplete inhibition of nitric oxide synthetase, or implicate a separate component to ACh -mediated relaxation, possibly by the release of the endothelium-derived hyperpolarising factor (EDHF), which has been clearly demonstrated in resistance arteries (Garland & McPherson, 1992).

The maximum relaxation to the endothelium-dependent vasodilator bradykinin also was not significantly inhibited by incubation with L-NOARG in PSS containing 4mM glucose, 4mM glucose + 16mM mannose or 20mM glucose. This is in keeping with the suggestion that

vasodilator responses to BK involves the participation of more than one mechanism (Cowan & Cohen, 1990); namely, the release of prostacyclin, which relaxes vascular smooth muscle via a mechanism which causes an increase in cyclic 3', 5' adenosine monophosphate (cAMP) in addition to releasing EDRF. On the other hand it appears that there is a negligible contribution of prostacyclin to endothelium-dependent relaxation in the majority of blood vessels (Yang et *al.*, 1991).

Incubation in a low concentration of glucose such as 2mM glucose had no deleterious effects on the endothelium-dependent relaxation response to acetylcholine and bradykinin, or the endothelium independent relaxation response to sodium nitroprusside. These findings may therefore suggest that acute hypoglycaemia *per se* does not have a significant effect on the function of the endothelium or the vascular smooth muscle of these resistance arteries. However, it is not known whether a longer exposure to a hypoglycaemic medium would have a detrimental effect on the vascular endothelium.

Hyperglycaemia appears to damage tissues by causing both acute, reversible changes in cellular metabolism and cumulative, irreversible alterations in stable macromolecules. Some of the reversible abnormalities include increased free radical generation and decreased scavenger activity (Kashiwagi *al.*, 1994); increased *de novo* synthesis of diacylglycerol, resulting in an increase in protein kinase C activity (Lee *et al.*, 1989); and activation of the polyol pathway, leading to accumulation of sorbitol through enhanced aldose reductase activity (Brolin & Naeser, 1988), reducing the availability of cellular NADPH, an essential co-factor of NO synthetase (Cameron & Cotter, 1992).

One study using isolated mesenteric resistance arteries has shown that incubation with the aldose reductase inhibitor ponalrestat prevented the development of impaired ACh relaxation in 20mM glucose, and significantly improved the impaired response to ACh in PSS containing 45mM glucose (Taylor & Poston, 1994). Moreover, two *in vivo* studies also have demonstrated that treatment with aldose reductase inhibitors improves endothelial dysfunction in chemically-induced diabetic animals (Cameron & Cotter, 1992; Tesfamariam *et al.*, 1993). On the other hand another study reported that treatment of diabetic rats with the inhibitor

ponalrestat for a 10 week period did not significantly improve the endothelial function displayed in the isolated perfused mesenteric circulation (Taylor *et al.*, 1994b), suggesting that factors other than increased stimulation of aldose reductase contribute to the impaired endothelial function in diabetes.

Also, it has been suggested that diabetes increases oxygen free radical activity, which inactivates nitric oxide, resulting in reduced endothelial relaxation, and that this reaction causes the formation of highly reactive hydroxyl radicals which damage endothelial cells (Gryglewski *et al.*, 1986a). Indeed, several studies of experimental diabetes indicate an important role for oxidising free radicals in the impaired endothelium-dependent relaxation, showing that anti-oxidant treatment improves endothelial dysfunction (Hatorri *et al.*, 1991; Tesfamariam & Cohen, 1992; Rösen *et al.*, 1995; Keegan *et al.*, 1995), and can prevent nerve conduction and perfusion abnormalities (Cameron *et al.*, 1993; Cameron & Cotter, 1994b). The origin of these oxidising free radicals causing an increased oxidative stress on the vasculature in diabetes remains unclear but may depend on the auto-oxidation of glucose (Wolff & Dean, 1987); lipid peroxidation (Jennings *et al.*, 1987b; Lyons, 1991); as well as the oxidation of glycated proteins (Bucala *et al.*, 1991).

Studies of cultured endothelial cells and tissues from diabetic animals exposed to hyperglycaemic conditions have demonstrated an increased *de novo* synthesis of diacylglycerol and a subsequent elevation in protein kinase C activity (Lee *et al.*, 1989; Williamson *et al.*, 1992; Craven and De Rubertis, 1989), which could have implications in the pathogenesis of diabetic complications. In one study, the relationship between protein kinase C activation and impaired receptor-mediated endothelium-dependent relaxation in diabetes mellitus was investigated using isolated aorta from normal rabbit exposed to elevated glucose (Tesfamariam *et al.*, 1991). This study suggested that hyperglycaemia alters endothelial function leading to an increased production of vasoconstrictor prostanoids as a result of protein kinase C activation. These results lend support to the theory that increased endothelial generation of vasoconstrictor prostanoids by elevated glucose may be responsible for the development of diabetic vascular complications.

In conclusion, the results from my study demonstrate that exposure of normal mesenteric resistance vessels to a high glucose medium (PSS containing 40mM glucose) significantly impairs acetylcholine-mediated endothelium-dependent relaxation, and this effect was not due to the increase in osmolarity, since exposure to the osmotic equivalent medium (PSS containing 4mM glucose + 36 mM mannose) did not attenuate the endothelium-dependent relaxation response to ACh. Incubation with the nitric oxide synthetase inhibitor, L-NOARG significantly impaired the relaxation response to ACh, although complete inhibition of the relaxation response was not achieved, indicating that the endothelium also releases other relaxing factor(s), possibly the endothelium-derived hyperpolarising factor (EDHF), as well as EDRF. However, the relaxation response to another endothelium-dependent vasodilator, bradykinin was not inhibited by incubation with L-NOARG, supporting the view that bradykinin mediates endothelium-dependent relaxation via several mechanisms, which include the release of prostacyclin as well as the release of EDRF from the endothelium. The differences in the response to the vasodilators ACh and BK in high glucose indicates that specific mechanisms involved in EDRF release may be differentially affected possibly at the level of the receptor or G-protein.

In this study, there was no evidence of abnormal vascular smooth muscle activity or sensitivity to NO, despite the slightly enhanced relaxation response to sodium nitroprusside displayed by the vessels incubated in high glucose (20mmM). Although, this enhancement did reach statistical significance, it was deemed to be of little biological significance as the standard deviation of the data in each of the experimental groups was minimal. We can also conclude from this study that incubating normal resistance arteries in a hypoglycaemic medium such as PSS containing 2mM glucose, does not in the short term, have a detrimental effect on either endothelium-dependent or independent relaxation.

Thus it appears that this study suggests a central role for hyperglycaemia in the development of the vascular dysfunction associated with experimental diabetes, and that this impairment demonstrated here in normal resistance arteries exposed to short-term hyperglycaemia could result from dysfunction in endothelial cell receptor-mediated signal transduction. In the next two experimental chapters, isolated resistance arteries from two different animal models of

diabetes were examined using a combination of pharmacological agents in an attempt to more closely examine the exact nature of the endothelial dysfunction associated with insulin-dependent diabetes.

CHAPTER FIVE

THE EFFECT OF DIABETES DURATION AND INSULIN TREATMENT ON RESISTANCE ARTERY STRUCTURE AND FUNCTION IN THE BIOBRED SPONTANEOUSLY DIABETIC RAT

5.1 Introduction

It is established that the endothelium has an important role modulating vascular tone (Furchgott & Zawadzki, 1980b), and it has been suggested that abnormal endothelial function may be a contributory factor to large and small vessel disease in diabetes mellitus (Porta *et al.*, 1987). Indeed, impaired endothelium-dependent relaxation responses have been demonstrated in the large blood vessels of animals with experimentally induced diabetes (Oyama *et al.*, 1986; Kamata *et al.*, 1989; Tesfamariam, 1990 and Mayhan *et al.*, 1991). Similarly, defective endothelium-dependent relaxation has been demonstrated in the smooth muscle of the corpora cavernosa in diabetic man (de Tejada *et al.*, 1989), and isolated subcutaneous resistance arteries from juvenile onset insulin-dependent diabetics (McNally *et al.*, 1994).

One particular study of mesenteric resistance arteries from streptozotocin-induced diabetic rats showed that as well as impaired ACh-induced endothelium-dependent relaxation, an increased sensitivity to noradrenaline was also demonstrated (Taylor *et al.*,1992). However, other studies suggest enhanced noradrenaline sensitivity in arteries from chemically induced diabetic animals, independent of impaired relaxation to acetylcholine (Pieper & Gross, 1988; Cohen *et al.*, 1990; Taylor *et al.*,1994a,b), which contrasts to observations of impaired contractile responses of isolated subcutaneous vessels from humans with insulin-dependent diabetes (McNally *et al.*,1994).

An alternative approach to the chemically-induced diabetic rat model for the investigation of the diabetic vasculature is in the Bio Breeding rat which develops diabetes spontaneously, and shares many of the clinical features evident in human diabetes, such as hyperglycaemia, glycosuria, ketonuria and loss of weight, followed by death within a few days if insulin treatment is not initiated (Mordes, 1987).

The majority of studies using the BB rat have shown a specific impairment of endothelial dependent relaxation in conduit arteries such as the aorta (Meraji *et al.*, 1987; Durante *et al.*, 1988). However, no studies have been performed using resistance arteries, and so there is limited information on the effects of diabetes on the structure and function of the

microvasculature or the effects of insulin treatment on the diabetic vasculature. Accordingly, the contractile and relaxation responses of mesenteric resistance arteries from BB rats at the onset of diabetes, and after long-term insulin treatment (6-8 weeks), compared to their age-matched non-diabetic controls were studied.

5.2 Experimental Protocol

BioBred Wistar rats were obtained from an inbred colony maintained at the Biomedical Services department, where the overall incidence of overt diabetes is approximately 50-60%, with insulin-dependent diabetes developing between 60-120 days. The BBs were diagnosed as diabetic when blood glucose levels were >20mM, and they showed signs of glycosuria and weight loss. Non-diabetic BB rats were used as age-matched controls. All animals were weighed daily, and blood glucose levels measured twice weekly. Once diabetic, insulin therapy was initiated to maintain euglycaemia and daily subcutaneous injections were administered according to weight and blood glucose levels.

Two separate diabetic groups were selected for study along with their age-matched non-diabetic controls. The first group of BB rats were recent onset diabetics that had not received insulin therapy, and were culled 24-48 hours after diabetes had been diagnosed. These rats were between 11-16 weeks old. The second group of BB rats were insulin treated and had received insulin for up to 6-8 weeks after onset of diabetes. Two groups of age-matched non-diabetic rats acted as controls. Systolic blood pressure was measured by tail cuff plethysmography in conscious, preheated rats before sacrifice (Bunag, 1973).

On the day of sacrifice, the treated diabetic rats did not receive their usual morning insulin injection. Rats were culled, and mesenteric resistance arteries dissected and mounted in a myograph. Morphological measurements were made, and the vessels normalised. Before the contraction studies were started, the vessels were incubated in PSS for a further 60 minutes. Vessels then underwent the activation procedure. Following this, a cumulative contraction-response curve to noradrenaline (NA 10^{-8}M-$3x10^{-5}$M) in the presence of cocaine (10^{-6}M) was performed as previously described. The vessels were rinsed three times with PSS, and

allowed to recover at baseline for 15 minutes. The vessels were then re-contracted with a bolus dose of NA (10^{-5}M), and the relaxation response to cumulative doses of acetylcholine (ACh 10^{-9}M-10^{-5}M) was observed. After a fifteen minute wash-out period, the arteries were again maximally contracted with NA (10^{-5}M), and subsequently relaxed with cumulative doses of bradykinin (BK 10^{-9}M-10^{-5}M). Following this, the vessels were brought to base line with three rinses of PSS. Finally, after another 15 minutes, endothelium independent relaxation was assessed in maximally contracted arteries with increasing doses of sodium nitroprusside (SNP 10^{-9}M-10^{-4}M)..

Time control studies were not carried out for this particular study as these had been previously conducted in the series of glucose-incubation experiments described in the previous chapter (using mesenteric resistance arteries from normal Wistar rats). These studies demonstrated that maximum responses and sensitivity to NA and ACh remain unchanged over a six hour time period in normal PSS. Moreover, previous published time control studies from our laboratory have shown that there was no difference in either the sensitivity or maximum response to noradrenaline and acetylcholine between dose response curves performed at baseline and after incubation in PSS for 30,60, and 120 minutes (Bennett *et al.*, 1992).

5.3 Results

5.3.1 Analysis of Results

Statistical analysis of the physical characteristics of the rats and the resistance artery morphology was determined using the Students paired t-test for comparisons within experimental groups, and the unpaired t-test for comparisons between groups. Contraction and relaxation dose response curves were compared using one-way analysis of variance (ANOVA). If $p<0.05$, individual pairs of means were then compared using the Bonferroni multiple comparisons test.

A power calculation based on the responses to the vasodilator sodium nitroprusside revealed that it would be necessary to have at least 6 animals in each group to have an 80% power of

detecting a 20% change in maximum relaxation to SNP at the $p<0.05$ level. Similarly for an 80% chance of detecting a 0.5μmol/l change in sensitivity to SNP at $p<0.05$ level, it would be necessary to have at least 7 animals in each group. The group sizes used in this study were therefore adequate. Within group power calculations could not be determined, although it is known that the number of animals required for an 80% chance of detecting a biologically significant difference would be less than the number needed for a between group comparison.

5.3.2 Physical characteristics

The systolic blood pressures of both the untreated recent onset diabetic BB rats, and the insulin treated diabetic rats were not significantly different to those of the age-matched non-diabetic controls (recent onset: 136±4 mmHg versus 128±5 mmHg, n=13; insulin treated: 119±5 mmHg versus 125±7 mmHg, n=6). The final body weight of animals was also not significantly different to those of the age-matched non-diabetic controls (recent onset: 263±17g, n=13 versus controls: 241±6g, n=13; insulin treated: 333±18g, n=15 versus controls: 304±23g, n=13).

Blood glucose was significantly higher in the untreated recent onset diabetic animals compared to their age-matched non-diabetic controls (recent onset: 20.6±1mmol/l, n=16, versus controls: 3.8±0.2mmol/l, n=9, $p<0.0001$). Moreover, despite daily subcutaneous insulin therapy, blood glucose levels also remained significantly elevated in the treated group of animals when compared to their controls (insulin treated diabetics: 14.9±1.8mmol/l, versus controls: 3.5±0.2mmol/l, n=15, $p<0.0001$). Finally, there was no significant difference in heart weight to tibia ratios between the diabetic animals and their age-matched controls (recent onset: 1.93±0.1, n=12 versus controls 2.03±0.04, n=11), and (insulin treated: 2.43±0.09, n=13 versus controls 2.27±0.08, n=12) (Table 13).

i) Resistance Vessel Structure/Morphology

The lumen diameters of arteries taken from the two groups of diabetic animals were comparable to that seen in the non-diabetic age-matched controls (recent onset: 266±11μm, n=12 versus controls: 263±7μm, n=11; insulin treated diabetics: 286±12μm n=13 versus

controls: 284±14μm, n=12). However, the media thickness of arteries from both the recent onset and insulin treated diabetic rats was significantly greater compared to their age-matched controls (recent onset diabetics: 14.82±0.64μm, n=12 versus controls:11.87±0.67μm, n=11, $p<0.01$; insulin treated:17.6±0.84μm, n=13 versus controls: 13.08±0.42μm, n=12, $p<0.001$). Likewise, the media cross-sectional area of resistance arteries from the diabetic animals were significantly greater than that of their age-matched controls (recent onset: 13131±729μm^2 ,n=12 versus controls: 10790±828μm^2 n=11, $p<0.05$; insulin treated: 16023±607μm^2 n=13, versus controls: 12500±657μm^2, n=12, $p<0.001$). A comparison between the arteries from the recent onset diabetics and their age-matched controls revealed a significant increase in the media to lumen ratio (expressed as a % as each ratio was multiplied by 100) (recent onset: 5.68±0.34% n=12 versus controls: 4.52±0.27%, n=11, $p<0.05$). A similar increase was also observed in the established insulin treated diabetic animals when compared to their age-matched non-diabetic controls (6.8±0.51% n=13 versus 4.65±0.31%, n=12, $p<0.05$).
(Table 14 & Plates 5, 6, 7, & 8: Scale shown - μm)

5.3.3 Resistance Vessel Contractility

i) High potassium

There was no significant difference in the maximum response to PSS containing high potassium in both the recent onset and the insulin treated diabetics compared with their age-matched non-diabetic controls (recent onset: 4.25±0.23mN/mm, n=13 versus controls: 4.25±0.21mN/mm, n=13; and insulin treated: 4.6±0.46mN/mm, n=8 versus controls: 4.29±0.28mN/mm, n=8).

ii) High potassium with noradrenaline

A similar pattern of results to the potassium was seen with PSS containing both potassium and noradrenaline (10^{-5}M) in that the maximum response in both the recent onset and insulin treated diabetics was not significantly different to that of their age-matched non diabetic controls (recent onset: 4.86±0.26mN/mm, n=13 versus controls: 4.97±0.31mN/mm, n=13; and insulin treated: 5.33±0.54mN/mm, n=8 versus controls: 5.14±0.33mN/mm, n=8).

iii) Noradrenaline

The results showed that there was no significant difference in the maximum contraction or the sensitivity (expressed as pD_2s), of recent onset diabetic rats compared to their age-matched non diabetic controls (4.62±0.27mN/mm, n=6 versus 4.51±0.3mN/mm, n=6 and 5.82±0.01, n=6 versus 5.8±0.01, n=6). Similarly, in the insulin treated diabetic rats there was no significant difference in the maximum contraction or sensitivity compared to their age-matched controls (5.47±0.55mN/mm, n=8 versus 4.8±0.32mN/mm, n=8 and pD_2: 5.79±0.02, n=8 versus 5.76±0.01, n=8). When the noradrenaline dose response curves were replotted for media stress, which takes into account the differences in vessel wall thickness, by expressing the active tension to NA divided by wall thickness, no significant difference in contractile response between the diabetic and control vessels was evident (Recent onset 341±28mN/mm^2 versus controls: 390±29mN/mm^2; and insulin treated: 387±21mN/mm^2 versus controls: 315±30mN/mm^2). (Table 15)

5.3.4 Endothelium-Dependent Relaxation

i) Acetylcholine

Maximum relaxation to ACh was significantly attenuated in both the recent onset diabetic rats compared to their age-matched controls (47±11% versus 92±2% $p<0.01$ n=6), and the insulin treated diabetic rats, compared to their age-matched control group (34±5%, n=7 versus 75±6%, $p<0.001$ n=8) respectively. There was a significant shift in sensitivity to ACh (pD_2) in the recent onset diabetic rats compared to their age-matched controls (7.78±0.11, n=6 versus 7.11±0.07, n=6, $p<0.001$). The sensitivity was also significantly different in the insulin treated diabetic rats when compared to their age-matched controls (7.88±0.11, n=6 versus 7.24±0.08, n=7, $p<0.001$). (Figure 11)

ii) Bradykinin

Compared to their age-matched controls, relaxation responses to bradykinin were significantly impaired in both recent onset and insulin treated diabetic rats (recent

onset:20±3%, n=5 versus control:72±7%, n=6, $p<0.001$; and insulin treated:15±4%, n=7 versus control:74±7%, n=8 respectively, $p<0.001$). The sensitivity to bradykinin could only be calculated for the age-matched control groups, as the vessels taken from the diabetic groups did not display a normal dose response curve to bradykinin (controls 11-16 weeks: 6.67±0.1, and controls 18-20 weeks: 6.85±0.25). (Figure 12)

5.3.5 Endothelium Independent Relaxation

i) Sodium Nitroprusside

The response to the endothelium independent vasodilator sodium nitroprusside was significantly impaired in the recent onset diabetics compared with their age-matched controls (74±5%, n=6 versus 94±2%, n=6, $p<0.05$). The sensitivity however, remained unchanged (6.31±0.15, n=5 versus 6.67±0.23). On the other hand, the maximum relaxation to SNP in the insulin treated diabetic rats was not significantly reduced when compared with their age-matched controls (76±6%, n=7 versus 88±4%, n=8), and similarly, the sensitivity was unaltered (6.35±0.21, n=4 versus 6.4±0.12, n=6). (Figure 13)

Table 13 Physical characteristics of recent onset and insulin treated diabetic rats compared with their non-diabetic age-matched controls.

Group	Systolic B.P. (mmHg)	Body Weight (g)	Blood Glucose (mmol/l)	Hwt./Tibia
Recent Onset	136±4 (13)	263±17 (13)	20.6±1*** (16)	1.93±0.1 (12)
Controls	128±5 (13)	241±6 (13)	3.8±0.2 (9)	2.03±0.04 (11)
Insulin Treated	119±5 (6)	333±18 (15)	14.9±1.8***(15)	2.43±0.09 (13)
Controls	125±7 (6)	304±23 (13)	3.5±0.2 (15)	2.27±0.08 (12)

Number of animals shown in brackets.

***p<0.001 Unpaired t-test diabetic versus age-matched controls.

Table 14 Morphological characteristics of mesenteric resistance arteries taken from recent onset, insulin treated diabetic rats and their age-matched non-diabetic controls.

Group	N= animals	Lumen Diameter (μm)	Media Thickness (μm)	Cross Sectional Area (μm^2)	Media : Lumen x 100 (%)
Recent Onset	12	266±11	14.82±0.64**	13131±729*	5.68±0.34*
Controls	11	263±7	11.87±0.67	10790±828	4.52±0.27
Insulin Treated	13	286±12	17.6±0.84***	16023±607***	6.8±0.51*
Controls	12	284±14	13.08±0.42	12500±657	4.65±0.31

* $p<0.05$, ** $p<0.01$, *** $p<0.001$ Unpaired t-test diabetic versus age-matched controls.

Plate 5: Light microscopy of a transverse section through a mesenteric resistance artery taken from BB recent onset diabetic rat (scale bar shown). Note thickened vessel walls.

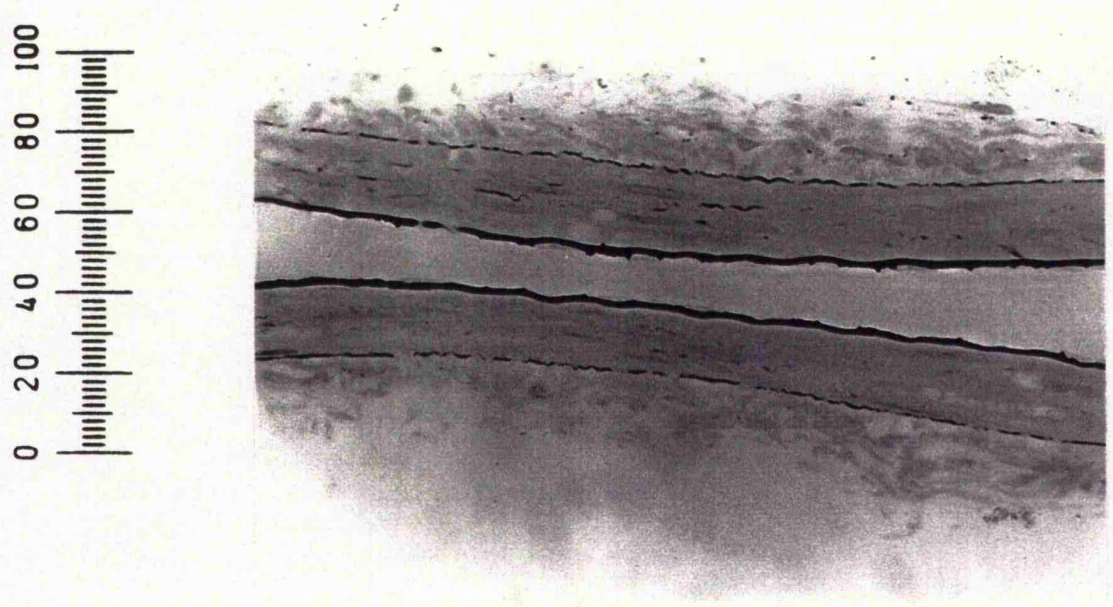

Plate 6: Light microscopy of a transverse section through a mesenteric resistance artery taken from an age-matched control (11-16 weeks) rat (scale bar shown).

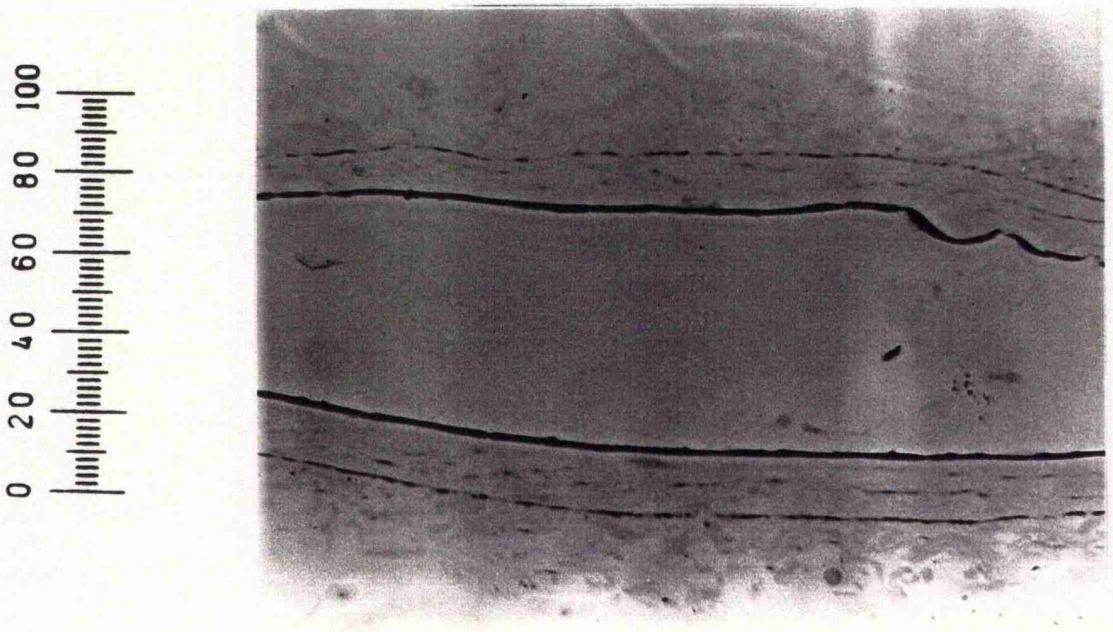

Plate 7: Light microscopy of a transverse section through a mesenteric resistance artery taken from BB insulin treated diabetic rat (scale bar shown). Note thickened vessel walls.

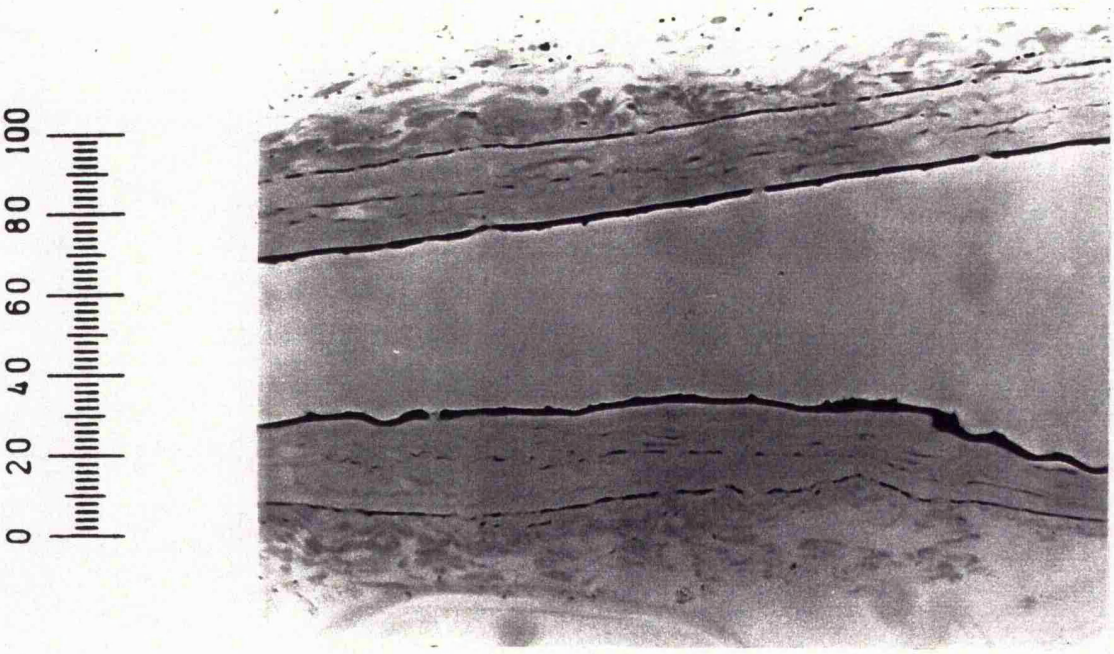

Plate 8: Light microscopy of a transverse section through a mesenteric resistance artery taken from age-matched control (18-20 weeks) rat (scale bar shown).

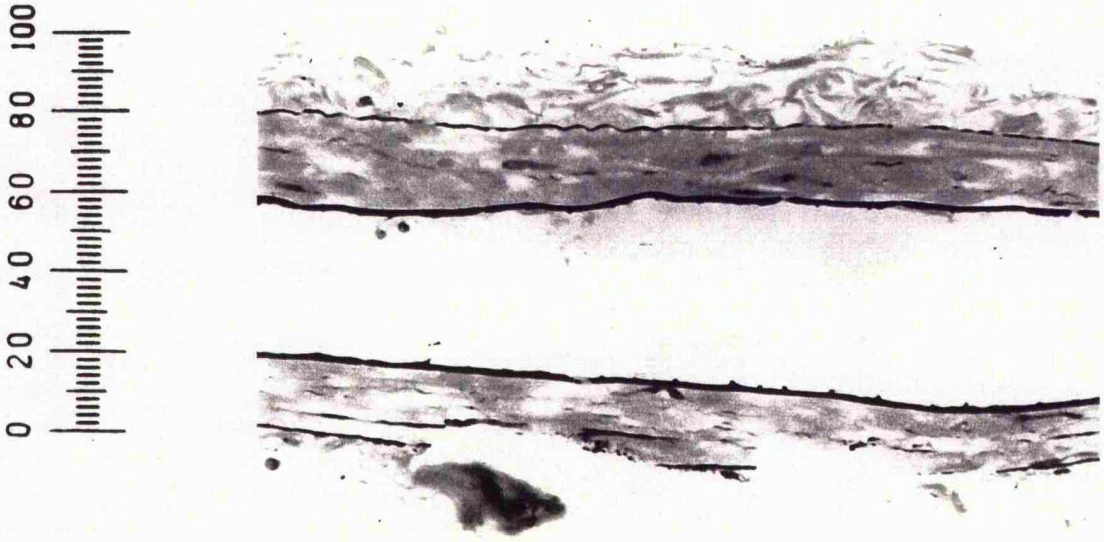

Table 15 The contractile response to PSS containing potassium (K^+), PSS + potassium and noradrenaline (NAK^+), and noradrenaline (NA) in mesenteric resistance arteries from recent onset, insulin treated diabetic rats and their age-matched controls.

Group	K^+ (mN/mm)	NAK^+ (mN/mm)	NA Max (mN/mm)	Sensitivity to NA (pD_2)	NA Media Stress (mN/mm^2)
Recent Onset	4.25±0.23 (13)	4.86±0.26 (13)	4.62±0.27 (6)	5.82±0.01 (6)	341±28 (6)
Controls	4.25±0.21 (13)	4.97±0.31 (13)	4.51±0.3 (6)	5.8±0.01 (6)	390±29 (6)
Insulin Treated	4.6±0.46 (8)	5.33±0.54 (8)	5.47±0.55 (8)	5.79±0.02 (8)	387±21 (6)
Controls	4.29±0.28 (8)	5.14±0.33 (8)	4.8±0.32 (8)	5.76±0.01 (8)	315±30 (6)

Number of animals shown in brackets.

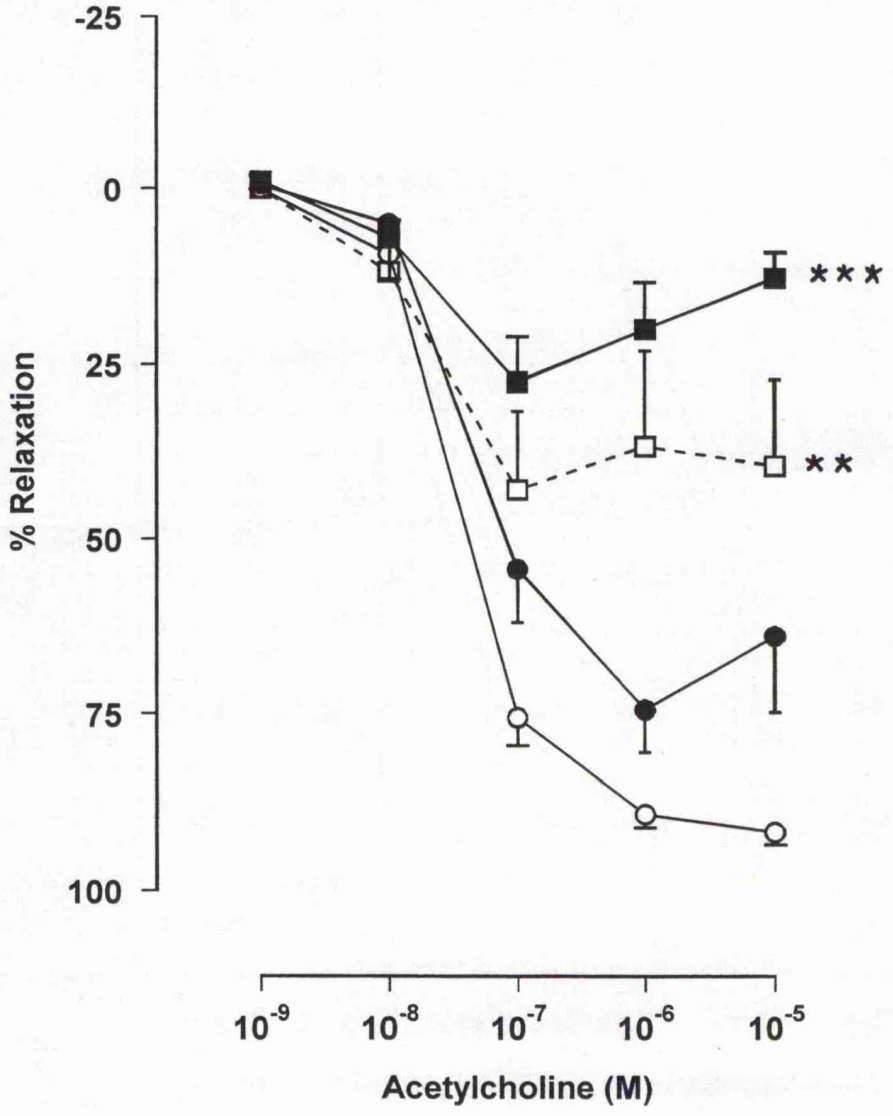

Figure 11 Acetylcholine-induced relaxation response of rat mesenteric resistance vessels from **recent onset** (❑), and **insulin treated diabetics** (■), and their age-matched **controls** (○) and (●) respectively.(**p<0.01 recent onset diabetic versus control; ***p<0.001 insulin treated diabetic versus control).

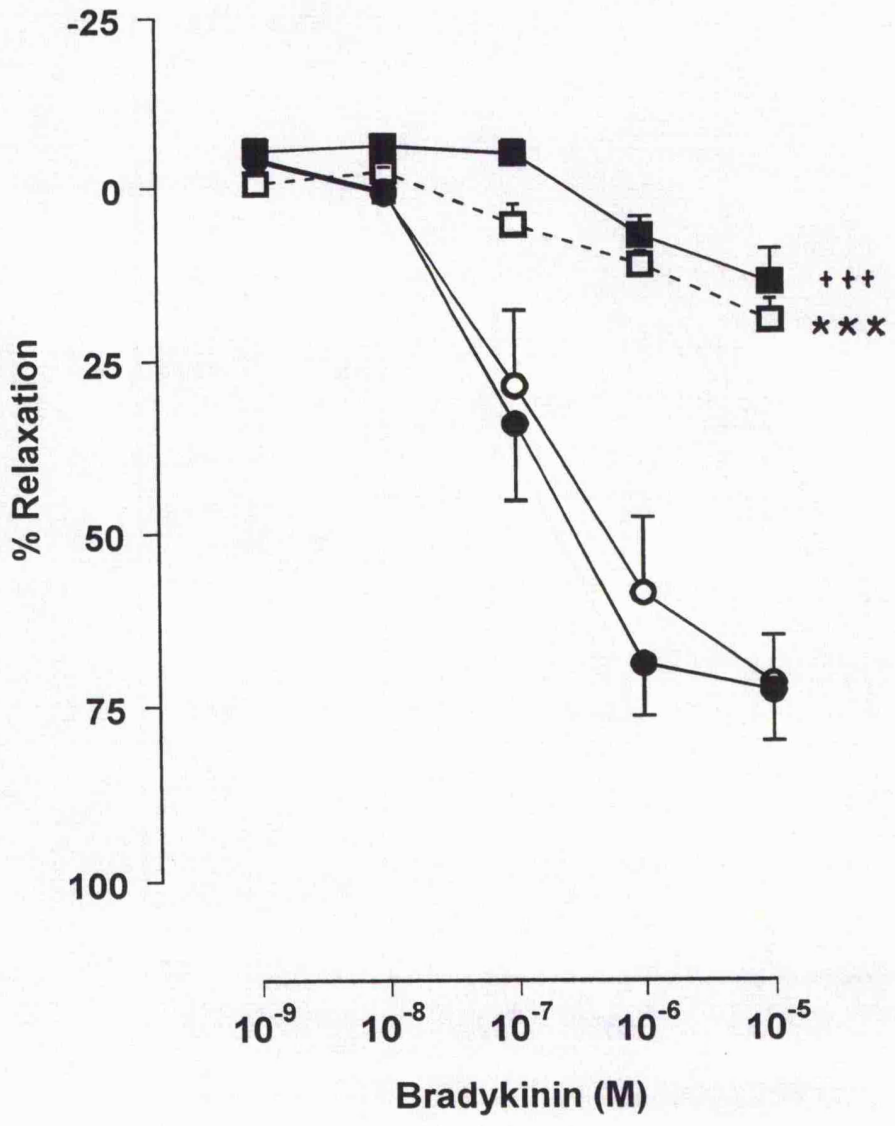

Figure 12 Bradykinin-induced relaxation response of rat mesenteric resistance vessels from **recent onset** (❑), and **insulin treated diabetics** (■), and their age-matched **controls** (○) and (●) respectively. (***$p<0.001$ recent onset diabetic versus control: $^{+++}p<0.001$ insulin treated diabetic versus control).

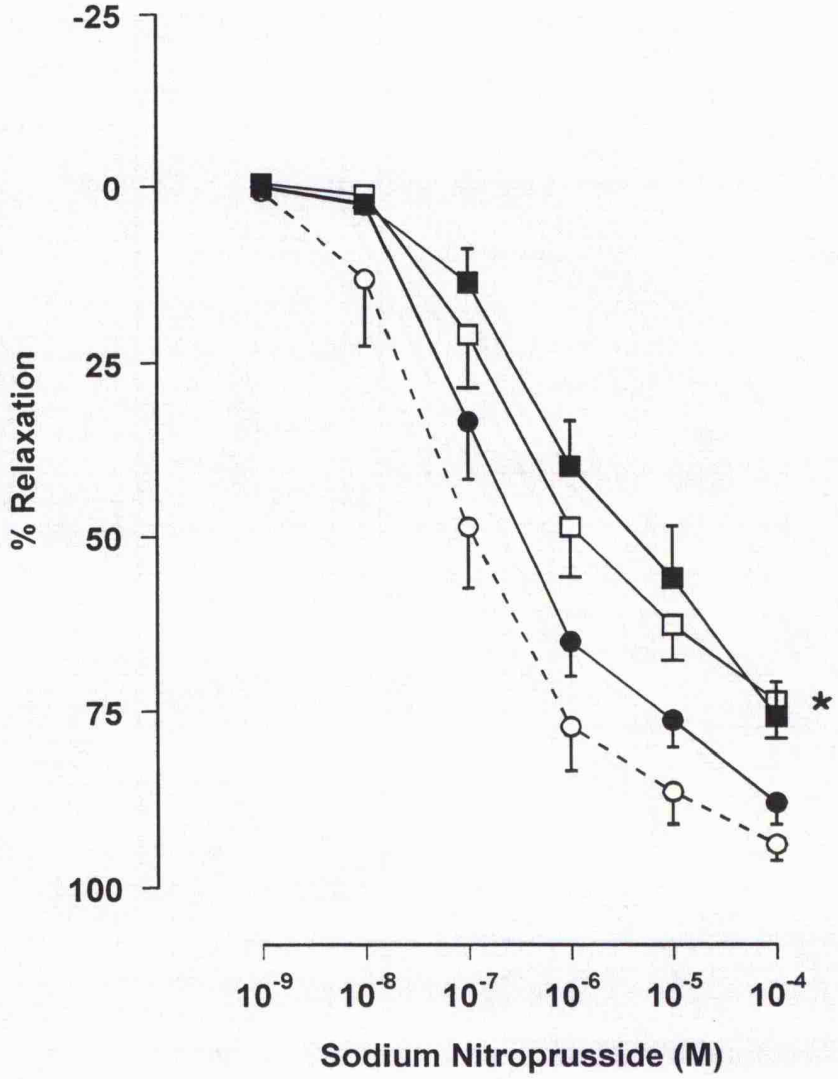

Figure 13 Sodium Nitroprusside-induced relaxation response of rat mesenteric resistance vessels from **recent onset** (❑), and **insulin treated diabetics** (■), and their age-matched **controls** (○) and (●) respectively. (*$p<0.05$ recent onset diabetic versus control).

5.4 Discussion

The present study shows no significant difference in the maximum contractile response or noradrenaline sensitivity between either the recent onset or insulin treated diabetic rats and their age-matched non diabetic controls. This is in agreement with other reports using the aortae from BB rats, where contractile responses were normal (Durante *et al.*, 1988), and in chemically induced diabetic rats where responses were impaired (Pfaffman *et al.*, 1982; Head *et al.*, 1987; and Oyama *et al.*, 1986). By contrast, the majority of studies of both large isolated arteries, and resistance arteries from rats with chemically induced diabetes have shown an enhanced sensitivity to contractile agents (Harris & MacLeod, 1988; Owen & Carrier, 1980; MacLeod, 1985; Friedman, 1989; Taylor *et al.*, 1992). Moreover, a study of insulin treated STZ-induced diabetic rats, showed that insulin did not prevent enhanced contractility to NA, although it did prevent impaired endothelium-dependent relaxation to ACh (Taylor *et al.*, 1994a). These findings contrast with human studies, which have shown resistance arteries from IDDM patients without complications other than background retinopathy, display impaired contractile responses to NA, and impaired endothelium-dependent relaxation to ACh (McNally *et al.*, 1994): Furthermore, resistance arteries from healthy volunteers exposed to increasing concentrations of insulin show decreased contractile response to NA in a dose-dependent manner (McNally *et al.*, 1995).

In this study, morphological measurements in both the recent onset and insulin treated BB rats revealed a significant increase in the media wall:lumen ratio, as well as increased cross sectional area and media thickness compared to the age-matched controls. There was not however, any associated increases in systolic blood pressure in the diabetic groups compared to their age-matched non diabetic controls. One study has shown evidence of vascular hypertrophy in the chemically induced diabetic rat, and demonstrated an increased media wall:lumen ratio, with no associated significant increase in systolic blood pressure. This study also demonstrated that *in vivo* treatment with an ACE inhibitor attenuated the development of mesenteric vascular hypertrophy in experimental diabetes (Cooper *et al.*, 1994). However, these changes were not evident until three weeks duration of diabetes (contrasting with the current study findings of changes in the recent onset BB rats). This

group went on to show in a later study that the antitrophic effect of angiotensin converting enzyme inhibitors on diabetic mesenteric arteries is mediated by inhibition of angiotensin II and by actions on the kinin-nitric oxide pathway (Rumble, Komers & Cooper, 1996). Another study of the perfused mesenteric vascular bed of STZ-induced diabetic rats reported a significant increase in the vessel wall thickness of the diabetic animals, compared to that of control animals, although again, these changes were reported after a much longer disease duration of 6 months (Olbrich *et al.*, 1996). Moreover, it was shown that pre-treatment with an ACE inhibitor partially restored impaired endothelium-dependent relaxation in diabetic animals, although the response was not fully normalised.

In this first series of experiments, there was impaired relaxation to the nitric oxide donor SNP in the diabetic vessels, suggesting a diminished vascular smooth muscle sensitivity to nitric oxide. The literature reveals a number of conflicting reports about SNP relaxation in diabetes; one *in vivo* study of anaesthetized STZ-treated rats showed a decrease in relaxation to SNP (Ha & Dunham, 1987), whereas other *in vitro* studies showed similar relaxation responses from both control and diabetic rats (Oyama *et al.*, 1986; Pieper & Gross, 1988; Taylor *et al.*, 1992). Similarly, most studies in humans with insulin-dependent diabetes have demonstrated a normal response to SNP (Elliott *et al.*, 1993; Johnstone *et al.*, 1993; McNally *et al.*, 1994), whilst others have shown the response is impaired (Calver *et al.*, 1992).

There was a significant impairment in endothelium-dependent relaxation to both acetylcholine and bradykinin in both the recent onset and the insulin treated diabetic BB rats, indicating that the attenuated endothelium-dependent relaxation responses are not dependent on the duration of diabetes. This contrasts with a study in the chemically induced diabetic rat where the impaired endothelium-dependent relaxation was related to the duration of diabetes (Otter & Chess-Williams, 1994). Other studies of chemically induced diabetic rats have shown an impaired response to acetylcholine, but bradykinin relaxation was normal (Taylor *et al.*, 1992). Impaired endothelium-dependent relaxation responses to acetylcholine with normal relaxation responses to bradykinin also have been demonstrated in human subcutaneous resistance arteries (McNally *et al.*, 1994), suggesting an abnormality at the G-protein receptor level. Therefore, a further series of studies were performed to investigate

the mechanisms underlying the attenuated endothelium-dependent relaxation responses to acetylcholine and bradykinin which was uniquely evident in both the recent onset untreated diabetic rats and the established insulin treated diabetic rats.

5.5 The Nature of the Impaired Endothelium-Dependent Relaxation Responses in the Diabetic Rats.

Resistance arteries from both the recent onset diabetic rats and the insulin treated established diabetic rats, were then arbitrarily selected for one of the five specifically designed experimental protocols which are outlined below to elucidate the mechanisms underlying the impaired endothelium dependent relaxation.

Protocol 1) The Nitric Oxide Synthetase Pathway

To ensure the impaired endothelial relaxation response of the diabetic vessels was not due to a lack of substrate for the enzyme nitric oxide synthetase, vessels were incubated with L-arginine (10^{-5}M) for 20 minutes prior to performing the ACh dose response curve. After rinsing with PSS, the vessels were then incubated with the nitric oxide synthetase *inhibitor* N^G-nitro-L-arginine (L-NOARG, 10^{-5}M) for one hour. After this, the contractile response to NA was repeated in the presence of L-NOARG to assess the role of the endothelium in modulating resistance vessel contractility. A shift factor was calculated according to the following formula to estimate the magnitude of change in noradrenaline sensitivity following incubation with L-NOARG:

$$\text{shift factor} = - \frac{(pD_2 NA) - (pD_2\ NA + \text{L-NOARG})}{pD_2\ NA} \times 100$$

ANOVA was used with the Bonferroni correction to assess statistical significance.

Dose relaxation response curves to ACh, and BK were then repeated in the presence of L-NOARG. Finally, the vessels were maximally contracted with NA 10^{-5}M and relaxed with

cumulative doses of the endothelium independent vasodilator sodium nitroprusside (SNP 10^{-9}M- 10^{-4}M).

Protocol (2) The Role of Free Radicals

The vessels were pre-treated with PSS containing the free radical scavenging enzyme superoxide dismutase (SOD 150 units ml^{-1}) and catalase (CAT 150 units ml^{-1}). After 20 minutes a dose response curve to ACh was repeated, and the vessels were brought to base line by a series of rinses with PSS.

Protocol (3) Inhibition of the Cyclo-oxygenase Pathway with Indomethacin

The vessels having been incubated with PSS containing the free radical scavenging enzymes (in protocol 2) were then subjected to protocol 3, where the ACh relaxation curve was repeated after a 30 minute incubation with a cyclo-oxygenase inhibitor indomethacin (10^{-5}M). Finally vessels were incubated in PSS containing both indomethacin and L-NOARG (10^{-5}M) for 60 minutes, after which ACh, BK, and SNP dose relaxation response curves were performed. Vessels obtained from insulin treated rats and non diabetic age-matched controls were subjected to a similar protocol except dose relaxation response curves to ACh, BK, and SNP were performed in the presence of indomethacin alone.

Protocol (4) The Importance of an Intact Endothelium

Vessels from recent onset diabetic rats and their age-matched non-diabetic controls were arbitrarily selected for either studies with an intact endothelium, or after the mechanical removal of the endothelium using a human hair. (The removal of the endothelial cells was later confirmed by scanning electron microscopy.) Following denudation, vessels were left for 45 minutes before the following protocol was performed. A cumulative dose contraction response curve to NA(10^{-8}M-3x10^{-5}M)was performed in the presence of cocaine (10^{6} M). After rinsing with PSS with a rest period of 10 minutes, the vessels were recontracted with 10^{-5}M NA and relaxation responses to ACh and BK were performed (10^{-9}M - 10^{-5}M). The

vessels were rinsed to baseline again and a dose contraction curve to increasing doses of ACh (10^{-9}M-10^{-5}M) was performed. Finally, the vessels were contracted with NA (10^{-5}M) and a dose relaxation response curve to SNP (10^{-9}M-10^{-4}M) performed.

Protocol (5) The Role of Thromboxane A_2

Vessels from recent onset diabetic rats and their age matched non-diabetic controls were incubated in PSS containing the thromboxane A_2/PGH_2 receptor antagonist SQ 29548 for 20 minutes, before dose relaxation response curves to ACh, BK and SNP were performed.

Histology and Scanning Electron Microscopy (SEM)

At the end of each study all vessels were rinsed in calcium free PSS before fixing overnight in 10% formalin for histological examination using both light microscopy and SEM.

5.6 Results

5.6.1 Resistance Vessel Contractility to NA + L-NOARG

Incubation with the nitric oxide synthetase inhibitor L-NOARG resulted in a non significant increase in maximum contractile response to noradrenaline of the recent onset diabetic control group (4.29±0.3mN/mm, n=7 versus 4.81±0.31mN/mm, n=6. p=0.058). There was also a non significant increase in NA sensitivity after the addition of L-NOARG (5.81±0.01 versus + L-NOARG: 5.78±0.02); however, analysis of the whole dose response curve (which takes into consideration each data point along the curve using one-way ANOVA for repeated measures and paired t-test), revealed a significant left-ward shift following the addition of L-NOARG ($p<0.001$). Incubation of vessels from the recent onset diabetic group with L-NOARG did not have a significant effect on either the maximum contractile response to NA (5.53±0.51mN/mm, n=7 versus 4.94±0.22mN/mm, n=6), or the sensitivity (pD_2:5.87±0.05, n=7 versus 5.84±0.02, n=6). However, similarly, a significant left-ward shift of the NA response curve was observed after the addition of L-NOARG in the recent onset diabetics, although this shift was not as marked as that found in the age-matched control vessels

($p<0.01$). (Figure 14) When the overall shift of the dose response curves produced by L-NOARG was calculated, no significant difference between the shift factor of the recent onset diabetic group compared to that of the age-matched controls was found (0.49±0.23%, n=6 versus 1.11±0.42%, n=6).

The age-matched controls of the insulin treated group (18-20 weeks) also failed to show a significant increase in maximum contractile response or sensitivity to NA after incubation with L-NOARG (4.8±0.32mN/mm, n=8 versus +L-NOARG: 4.79±0.29mN/mm, n=7 and pD_2: 5.76±0.01 versus +L-NOARG: 5.79±0.02). However, analysis of the whole dose response curve using ANOVA with paired t-test revealed a significant shift in the dose response curve following L-NOARG treatment ($p<0.05$). By contrast, there was no significant shift in dose response curve to NA in the insulin treated diabetic rats after incubation with L-NOARG. The maximum contractile response and sensitivity to NA in the insulin treated rats was also unchanged after incubation with L-NOARG (5.47mN/mm versus +L-NOARG 5.45±0.47mN/mm, n=8 and pD_2: 5.79±0.02 versus +L-NOARG:5.82±0.04, n=8) (Figure15). Calculation of the shift factor of the dose response curves produced by L-NOARG did not reveal a significant difference between the shift factor of the insulin treated diabetic group compared to that of the age-matched controls (1.52±0.58%, n=8 versus 0.91±0.26%, n=7).

+/- Endothelium

The maximum contractile and sensitivity response to NA of endothelium-denuded vessels from the recent onset diabetic rats was not significantly different from that of the endothelium-denuded vessels from their age-matched controls (recent onset: 4.46±0.54mN/mm n=8 versus controls: 3.8±0.27mN/mm n=8 and pD_2 of recent onset: 6.1±0.12 versus controls: 5.97±0.07, n=8). There was no significant difference in the NA maximum contractile response of control vessels with intact endothelium compared to control vessels denuded of endothelium (controls + endo: 3.84±0.21mN/mm versus controls -endo: 3.8±0.27mN/mm, n=8). However, there was a significant difference in the NA sensitivity (+endo: 5.76±0.07, n=7 versus -endo: 5.97±0.07, n=8, $p<0.05$). Removal of the endothelium from the recent onset diabetic vessels did not alter the maximum contractile

response or the sensitivity to NA (+endo: 5.33mN/mm, and 5.93±0.04, n=7 versus -endo: 4.46±0.54mN/mm, and 6.1±0.12, n=8, $p<0.05$).

5.6.2 Endothelium-Dependent Relaxation

i) Acetylcholine

+L-Arginine

Incubation with L-arginine failed to improve the acetylcholine relaxation response of either of the diabetic groups (recent onset:47±11%, n=6 versus +L-arginine: 44±11% n=6, and insulin treated:34±5%, n=7 versus +L-arginine: 43±9%, n=8). However, ACh sensitivity was significantly increased following incubation with L-arginine in the recent onset diabetic group, although this apparent increase in sensitivity should be interpreted with caution as the sensitivity after L-arginine could only be calculated in four of the dose response curves (recent onset pD_2: 7.77±0.11, n=6 versus +L-arginine: 7.09±0.1, n=4, $p<0.01$). The ACh sensitivity of the insulin treated diabetic group was not significantly altered by incubation with L-arginine (insulin treated pD_2: 7.88±0.11, n=6 versus +L-arginine: 7.44±0.18, n=4).

L-arginine had no significant effect on the maximum relaxation response and sensitivity to ACh in either of the age-matched control groups (recent onset controls: 92±2%, 7.11±0.07, n=6 versus +L-arginine: 91±3%, 7.1±0.07, n=6 and age-matched controls of the insulin treated group: 75±6%, n=8, 7.24±0.07, n=7 versus +L-arginine: 83±7%, n=7, 7.2±0.15, n=6).

+ L-NOARG

Inhibition of nitric oxide synthase with L-NOARG virtually abolished acetylcholine-induced relaxation in the recent onset diabetic vessels (47±11%, n=6 versus +L-NOARG: 6±4%, $p<0.01$ n=6) (Figure 16). By contrast, ACh relaxation was attenuated but not completely abolished in the age-matched controls (92±2%, n=6 versus +L-NOARG: 65±8%, n=6, $p<0.05$). Moreover, this was not accompanied by a change in ACh sensitivity (pD_2: 7.11±0.07, n=6 versus +L-NOARG: 6.96±0.17, n=5). (Figure 17)

Similar results were observed in the insulin treated diabetic vessels with a significant reduction in ACh-induced relaxation after incubation with L-NOARG (34±5%, n=7 versus

+L-NOARG: 7±5%, n=8, $p<0.05$) (Figure 18). The relaxation to ACh in this age-matched control group was significantly reduced but again not abolished by L-NOARG incubation (75±6%, n=8 versus +L-NOARG: 38±12%, n=8, $p<0.01$), which was in keeping with the response of the recent onset age-matched control group. (Figure19). The change was associated with a significant change in ACh sensitivity, although this is based on limited data (pD_2: 7.24±0.07, n=7 versus +L-NOARG: 6.74±0.08, n=4, $p<0.01$).

+ SOD & CAT

Incubation with the free radical scavenger superoxide dismutase and catalase failed to improve the acetylcholine relaxation response in either the recent onset diabetic rats (42±10%, n=7 versus +SOD: 34±10%, n=6) or the insulin treated diabetic rats (40±5%, n=7 versus +SOD: 28±7%, n=6). ACh sensitivity also was unchanged in the diabetic groups following incubation with SOD and CAT (recent onset: 7.69±0.08, n=7 versus +SOD: 7.53±0.14, n=6; and insulin treated: 7.63±0.14, n=7 versus +SOD: 7.54±0.13, n=5). Similarly, there also was no significant change in the maximum relaxation response or sensitivity to acetylcholine after the addition of SOD and CAT in the age-matched control group to the recent onset diabetics, (controls 11-16 weeks: 83 ±4% versus +SOD: 71 ±8%, n=7; and pD_2: 7.02 ±0.13, n=7 versus +SOD: 7.2±0.22, n=6). However, there was an unexpected significant reduction in the maximum relaxation response to ACh after incubation with SOD and CAT in the age-matched control group of the insulin treated rats (81 ±5%, n=7 versus +SOD: 60 ±7%, n=7, $p<0.05$). This decrease in maximum response however was not associated with a reduced sensitivity (7.2 ±0.11, n=7 versus +SOD: 7.04 ±0.15, n=7) (Table 16).

+ Indomethacin

By contrast, pretreatment with the cyclo-oxygenase inhibitor indomethacin resulted in a significant improvement in the acetylcholine induced relaxation response in both the recent onset diabetic rats (42±10%, n=7 versus +indo: 64±7%, n=7, $p<0.05$), and the insulin treated diabetic group (40±5%, n=7 versus +indo: 65±9%, n=6, $p<0.05$) (Figure 20). Moreover, after indomethacin, the ACh relaxation of the insulin treated diabetic rats was comparable to that of the age-matched control group (65 ±9% , n=6 versus 81 ±5%, n=7, $p=0.11$). The

ACh sensitivity of the recent onset diabetic group was not changed by incubation with indomethacin (7.69±0.08, n=7 versus +indo: 7.32±0.31, n=6). However, the ACh sensitivity of the insulin treated diabetic group was significantly increased after the addition of indomethacin (7.64±0.14, n=7 versus +indo: 6.9±0.16, n=6, $p<0.01$).

There was no significant change in either the maximum relaxation response or the sensitivity to acetylcholine of either age-matched control group after incubation with indomethacin (controls 11-16 weeks: 83 ±4%, n=7 versus +indo: 82±6%, n=6; and pD_2: 7.02±0.13, n=7 versus +indo: 7.22±0.07, n=6; controls 18-20 weeks: 81±5%, n=7 versus +indo: 78±6%, n=7; pD_2: 7.25±0.11, n=6 versus +indo: 6.98±0.14, n=7).

<u>± Indomethacin & L-NOARG</u>

Incubation with both indomethacin and L-NOARG caused a significant inhibition of the maximum relaxation response to ACh in the recent onset diabetic vessels (42±10 % versus 7±3%, n=7, $p<0.01$). Whereas, a comparison of the relaxation responses of the age-matched control group after the addition of indomethacin and L-NOARG showed no significant decrease in the maximum response to ACh (83±4%, n=7 versus +indo+L-NOARG: 62±12%, n=7). The sensitivity to ACh in the control group also was unchanged by incubation with indomethacin and L-NOARG (7.02±0.13, n=7 versus +indo+L-NOARG: 6.99±0.11, n=6).

<u>+/- Endothelium</u>

Mechanical removal of the endothelium resulted in a significant decrease of the acetylcholine relaxation response in both the recent onset diabetic vessels and their age-matched non diabetic controls (diabetics denuded:12±7%, n=8 versus intact:46±10%, n=8, $p<0.05$, and controls denuded:29±6%, n=8 versus intact:87±2%, n=8 respectively, $p<0.05$). Endothelial removal was confirmed by scanning electron microscopy;plate 9 shows the luminal surface of an endothelium-intact mesenteric artery from a recent onset diabetic rat, and plate 10 is a scanning electron micrograph of an endothelium-denuded mesenteric resistance artery from a recent onset diabetic rat. (Cumulative doses of acetylcholine failed to produce a contractile response in either the endothelium-denuded diabetic vessels or their endothelium-denuded age-matched controls.)

+ SQ29548

In contrast to indomethacin, pretreatment with the thromboxane A_2 receptor antagonist SQ 29548 failed to significantly improve the ACh endothelium-dependent relaxation of the endothelium-intact recent onset diabetic vessels (46±10%, n=9 versus +SQ: 64±13%, n=6, p=0.39). The ACh sensitivity also was not significantly changed by pre-treatment with SQ29548 (7.65±0.18, n=7 versus +SQ: 7.26±0.18, n=5). Similarly, there was no change in the ACh relaxation and sensitivity of the age-matched non-diabetic control group after pre-treatment with the thromboxane A_2 inhibitor (87±2% versus +SQ: 86±3%, n=8, and pD_2 : 7.53±0.14, n=8 versus SQ: 7.32±0.15, n=8) (Table 17).

ii) Bradykinin

+ L-NOARG

A one hour incubation with L-NOARG significantly inhibited the BK relaxation in the recent onset diabetic group (20±3%, n=5 versus +L-NOARG: 2±2%, n=6, $p<0.05$). On the other hand, there was no significant difference in the maximum relaxation response to bradykinin after the addition of L-NOARG in the insulin treated diabetic rats (15±4%, n=7 versus +L-NOARG: 13±8%, n=6). The maximum relaxation response of the age-matched control group of the recent onset diabetic rats was attenuated following incubation with L-NOARG, although the decrease failed to achieve significance (72±7%, n=6 versus +L-NOARG: 44±10%, n=6, p=0.06). BK sensitivity was also not significantly altered by incubation with L-NOARG (6.67±0.1, n=6 versus +L-NOARG: 6.51±0.19, n=4). However, the age-matched controls of the insulin treated group showed a significant attenuation of the maximum relaxation response to BK after incubation with L-NOARG (74±7%, n=8 versus +L-NOARG: 31±9%, n=7, $p<0.001$). The sensitivity to bradykinin in this group appeared to be unchanged following incubation with L-NOARG, although it was difficult to interpret the response to BK + L-NOARG as n=2 (6.85±0.25, n=6 versus 6.97±0.09, n=2).

+ Indomethacin

Incubation with indomethacin produced a slight improvement in the relaxation response to bradykinin of the established insulin treated diabetic group, but this change did not achieve statistical significance (10±8%, n=7 versus +indo: 24±13%, n=5). Similarly, there was no

significant improvement in the relaxation response to bradykinin in the age-matched control vessels (63±9%, n=7 versus +indo: 73±6%, n=7). The addition of both indomethacin and L-NOARG to the myograph reduced the maximum relaxation to bradykinin in the recent onset diabetics (17±4%, n=6 versus +indo+L-NOARG: 8±4%, n=7) and their age-matched controls (66±8%, n=6 versus +indo+L-NOARG: 48±12%, n=7). Statistical significance was only reached in the control group when the whole dose response curve was considered using ANOVA with paired t-test , $p<0.05$. (Table 18)

+/- Endothelium

Removal of the endothelium attenuated the bradykinin relaxation response in both the recent onset diabetic vessels and their age-matched controls (diabetics denuded:1±1%, n=7 versus intact:14±10%, n=8, $p<0.05$ and controls denuded:16±5%, n=8 versus intact:75±5%, n=8, $p<0.05$). Incubation with the thromboxane A_2 inhibitor SQ29548 failed to produce a significant improvement in the bradykinin relaxation response of the endothelium intact diabetic vessels (14±10%, n=8 versus +SQ:22±14%, n=5). Similarly, SQ 29548 had no significant effect on either the maximum relaxation response or the sensitivity to BK of the endothelium intact control vessels (75±5%, n=8 versus +SQ: 76±4%, n=8 and pD_2: 6.64±0.08, n=8 versus +SQ: 6.5±0.07, n=8) (Table 19).

5.6.3 Endothelium Independent Relaxation

i) Sodium Nitroprusside

+ L-NOARG

After the addition of indomethacin and L-NOARG, maximum relaxation and sensitivity to sodium nitroprusside in the recent onset diabetic rats was very similar to their age-matched controls (recent onset: 92±2%, n=7 versus controls: 93±3%, n=7, and pD_2s recent onset: 6.45±0.08, n=7 versus controls: 6.79±0.21, n=7). This was also the case in the established insulin treated diabetic rats and their age-matched controls incubated with indomethacin alone (insulin treated: 87±3%, n=6 versus controls: 87±3%, n=7, and pD_2s insulin treated: 6.68±0.31, n=6 versus controls: 6.3±0.17, n=7) (Figure 21).

+/- Endothelium

Both the maximum relaxation response and the sensitivity to SNP of the endothelium intact recent onset diabetic vessels were similar to that before endothelium removal (+endo: 85±6%, n=5 versus -endo: 79±4%, n=8; and pD_2s +endo: 6.03±0.17, n=5 versus -endo: 6.18±0.25, n=8). Similarly, the maximum relaxation and sensitivity was not significantly different between the endothelium intact control vessels and the endothelium denuded control vessels (+endo: 91±4%, n=6 versus 86±2%, n=8; and pD_2s +endo: 6.51±0.23, n=6 versus -endo: 6.63±0.11, n=8). Likewise, there were no significant differences in SNP maximum relaxation and sensitivity of the recent onset diabetic rats denuded of endothelium compared to the endothelium-denuded age-matched control vessels. (Figure 22)

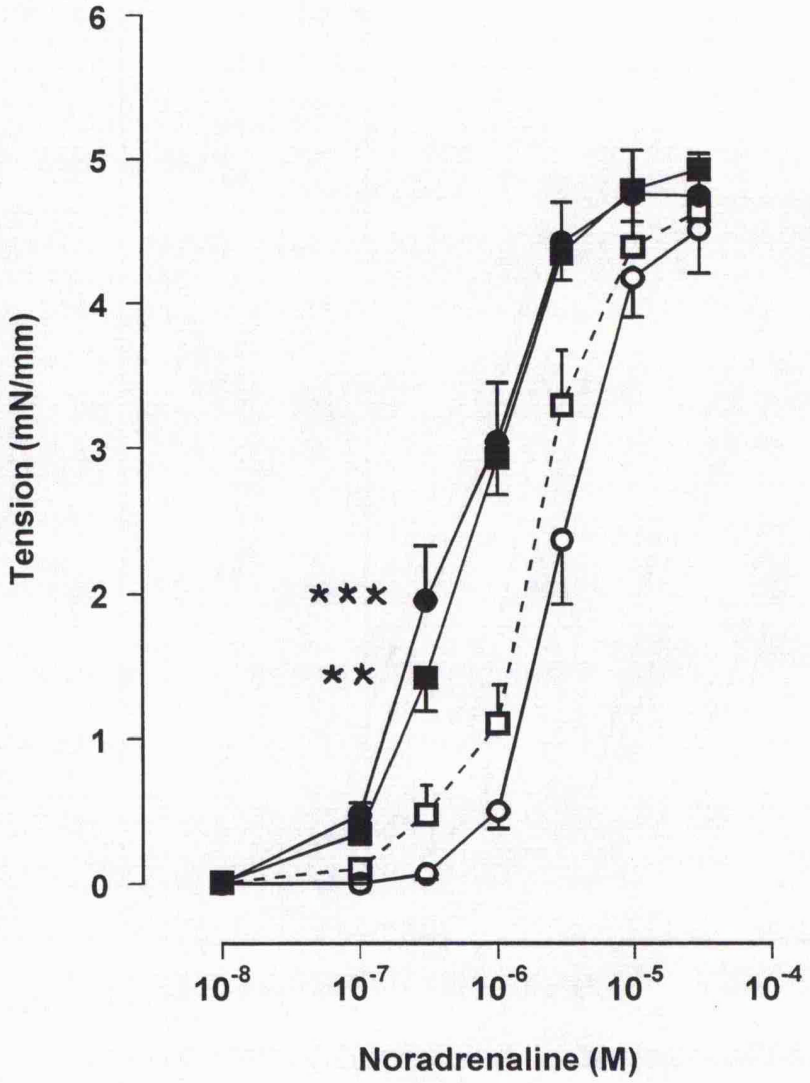

Figure 14 Noradrenaline contractile response of rat mesenteric resistance vessels from **recent onset diabetics** before (❑) and after incubation with N^G-nitro-L-arginine (L-NOARG) (■,**p<0.01), and their age-matched **controls** before (○), and after incubation with L-NOARG (●, ***p<0.001).

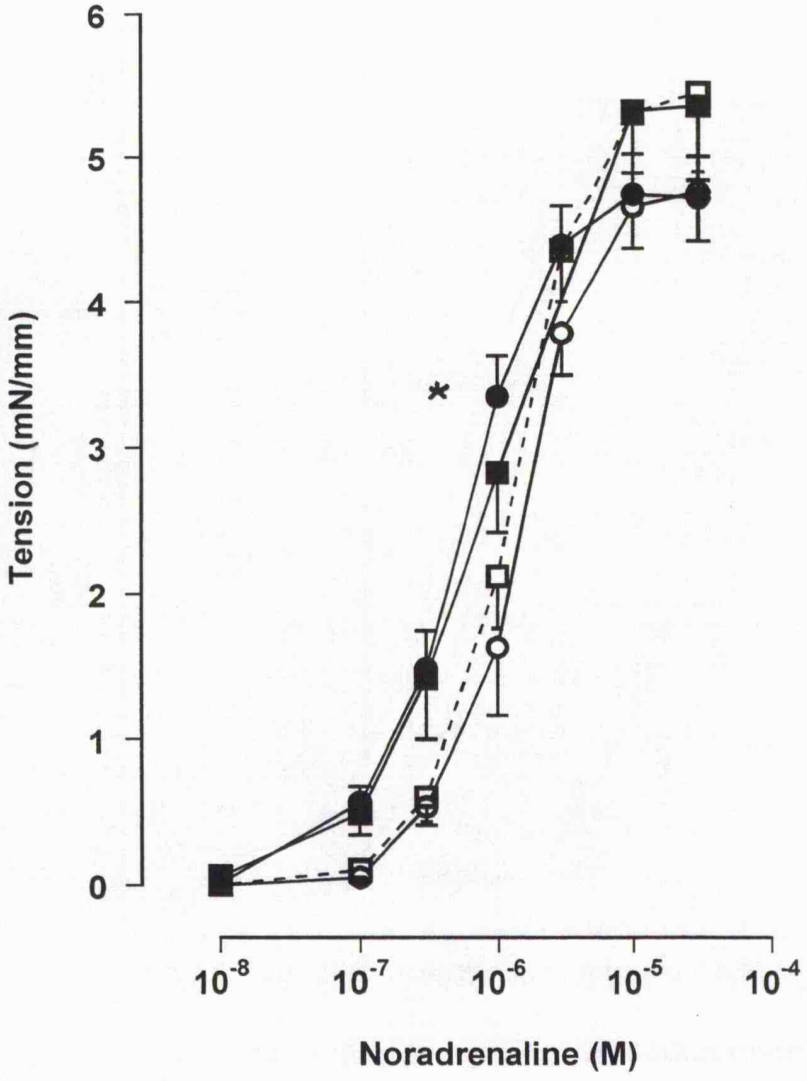

Figure 15 Noradrenaline contractile response of rat mesenteric resistance vessels from **insulin treated diabetics** before (❑) and after incubation with N^G-nitro-L-arginine (L-NOARG) (■), and their age-matched **controls** before (○), and after incubation with L-NOARG (●,*$p<0.05$).

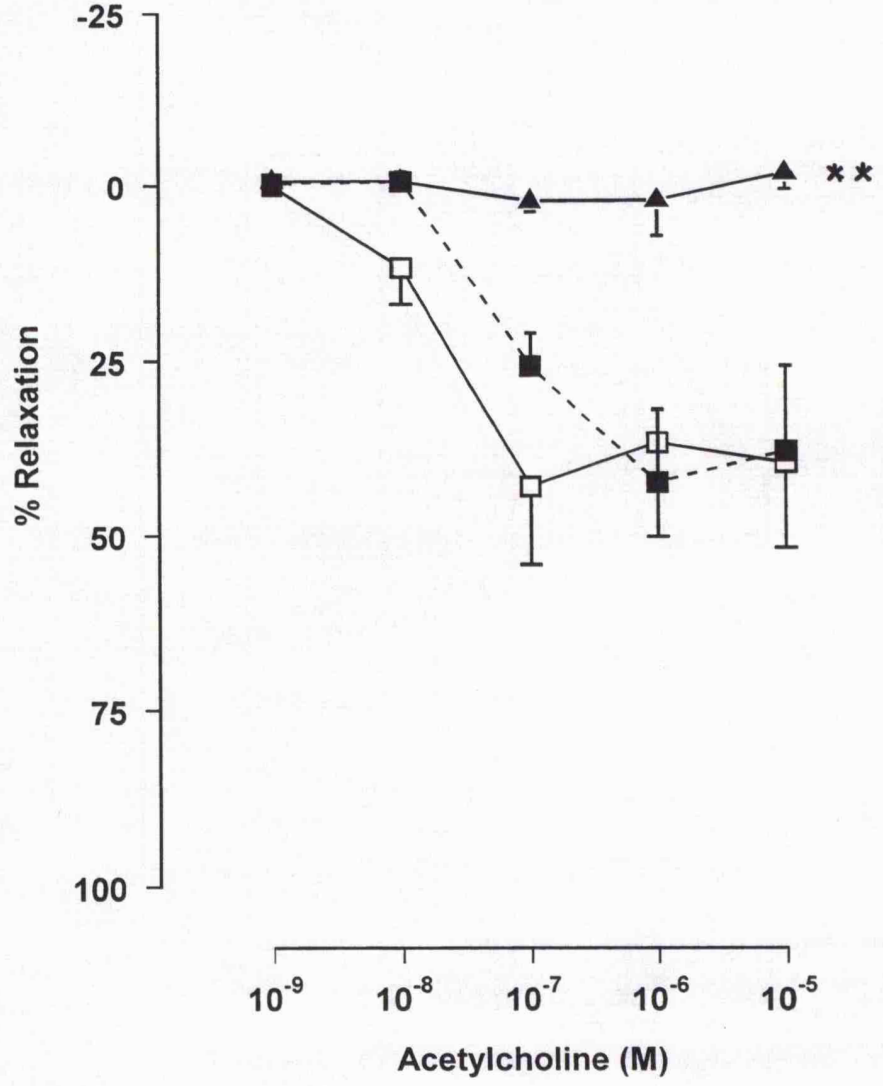

Figure 16 Acetylcholine-induced relaxation response of rat mesenteric resistance vessels from **recent onset diabetic** rats (❑), incubated with either L-arginine (■), or L-NOARG (▲, **$p<0.01$).

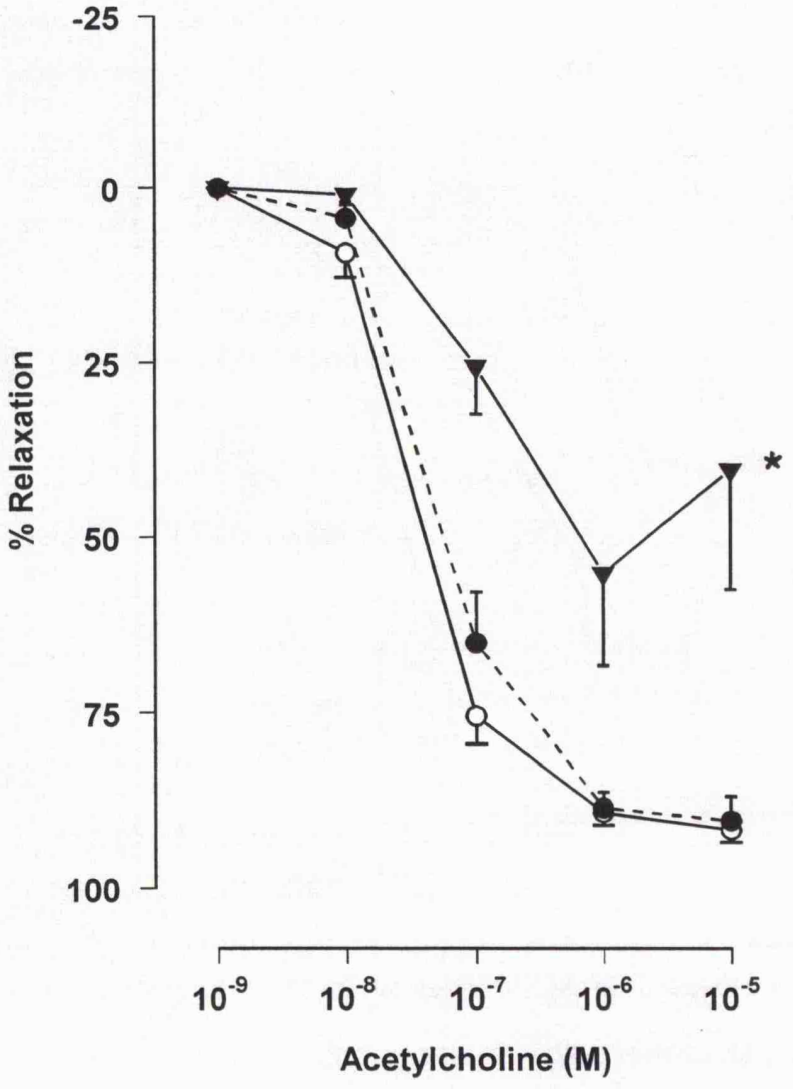

Figure 17 Acetylcholine-induced relaxation response of rat mesenteric resistance vessels from age-matched **control** rats (recent onset) (○), incubated with either L-arginine (●), or L-NOARG (▼, *$p<0.05$).

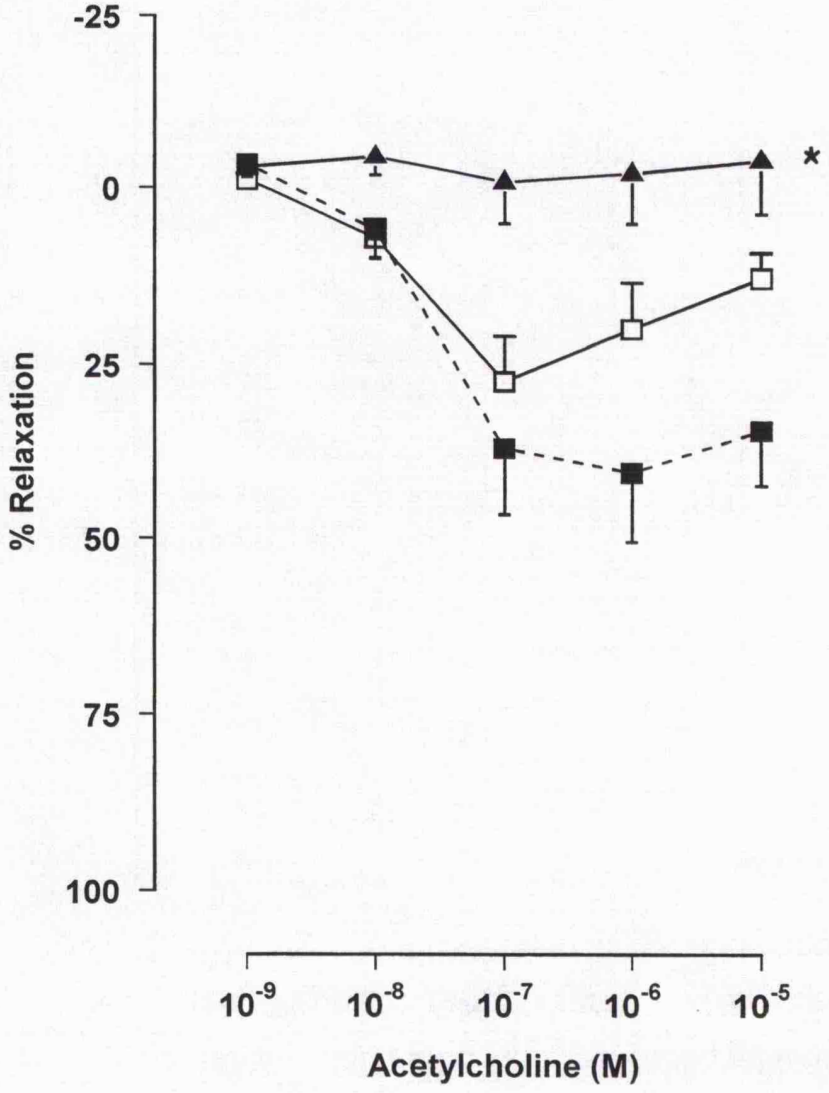

Figure 18 Acetylcholine-induced relaxation response of rat mesenteric resistance vessels from **insulin treated diabetic** rats (❑), incubated with either L-arginine (■), or L-NOARG (▲, *$p<0.05$).

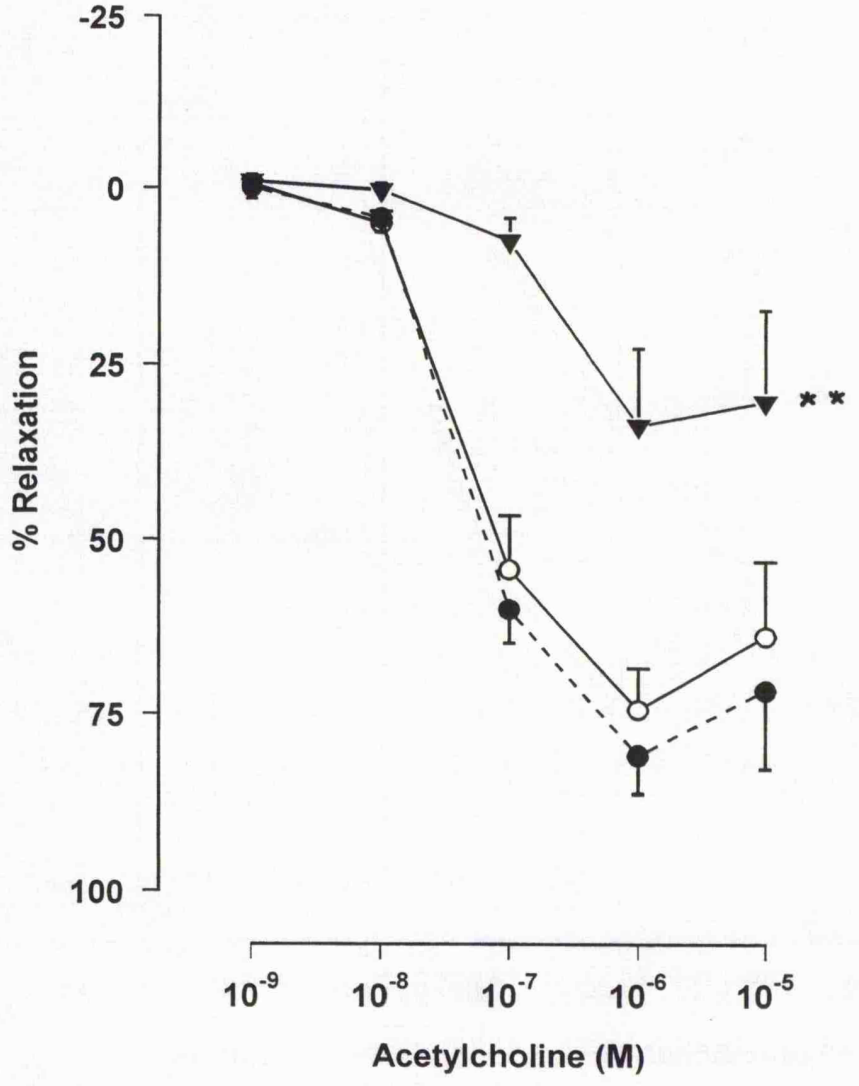

Figure 19 Acetylcholine-induced relaxation response of rat mesenteric resistance vessels from age-matched **control** rats (insulin treated) (○), incubated with either L-arginine (●), or L-NOARG (▼, **$p<0.01$).

Table 16 The maximum relaxation (%) and sensitivity (pD_2s) to ACh of resistance arteries from recent onset and insulin treated diabetic rats and their age-matched controls before and after incubation with superoxide dismutase and catalase.

Group	N = animals	ACh Max (%)	Sensitivity (pD_2)	+SOD (%)	Sensitivity (pD_2)
Recent Onset	6	42±10	7.69±0.08	34±10	7.53±0.14
Controls	7	83±4	7.02±0.13	71±8	7.2±0.22
InsulinTreated	7	40±5	7.63±0.14	28±7	7.54±0.13
Controls	7	81±5*	7.2±0.11	60±7	7.04±0.15

* $p<0.05$ paired t-test before and after incubation with SOD.

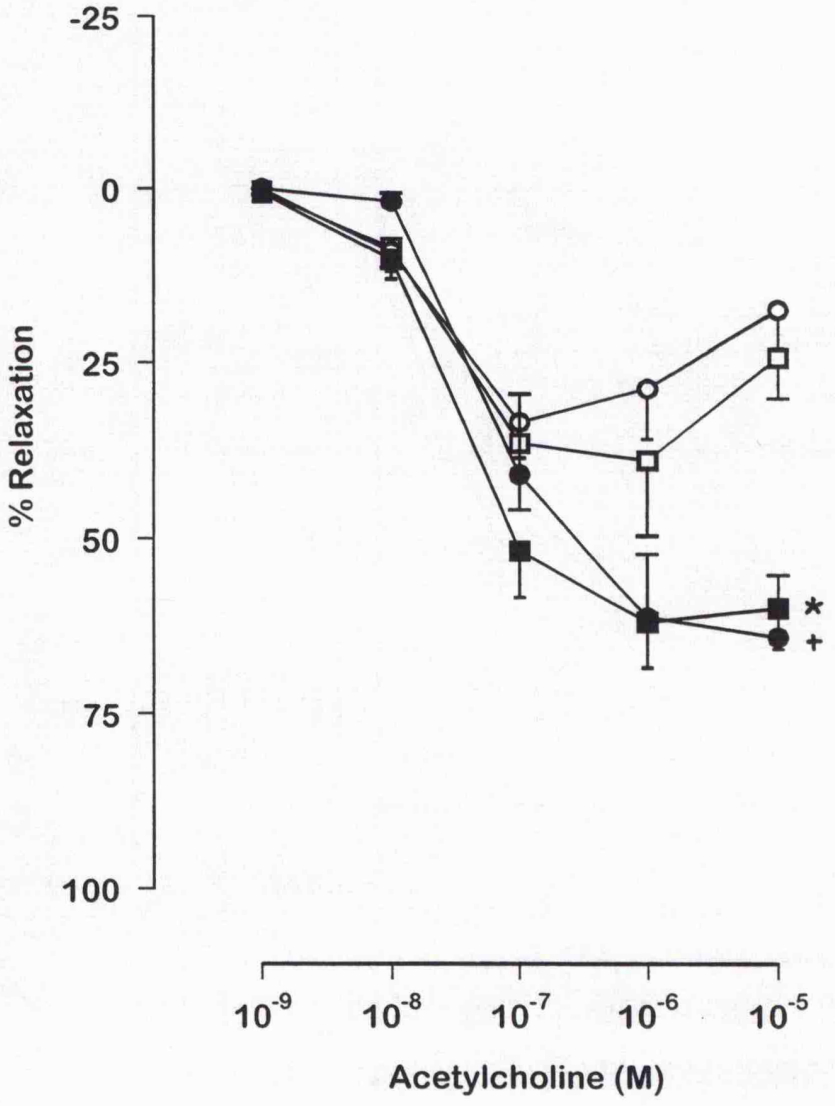

Figure 20 Acetylcholine-induced relaxation response of rat mesenteric resistance vessels from **recent onset diabetics** before (❑), and after incubation with indomethacin (■,* $p<0.05$), and **insulin treated diabetics** before (○) and after incubation with indomethacin (●,+ $p<0.05$).

Plate 9: Scanning electron micrograph of luminal surface of BB recent onset diabetic mesenteric resistance artery **with endothelium** (magnification & scale bar shown on micrograph).

Plate 10: Scanning electron micrograph of luminal surface of BB recent onset diabetic mesenteric resistance artery **denuded of endothelium** (magnification & scale bar shown on micrograph).

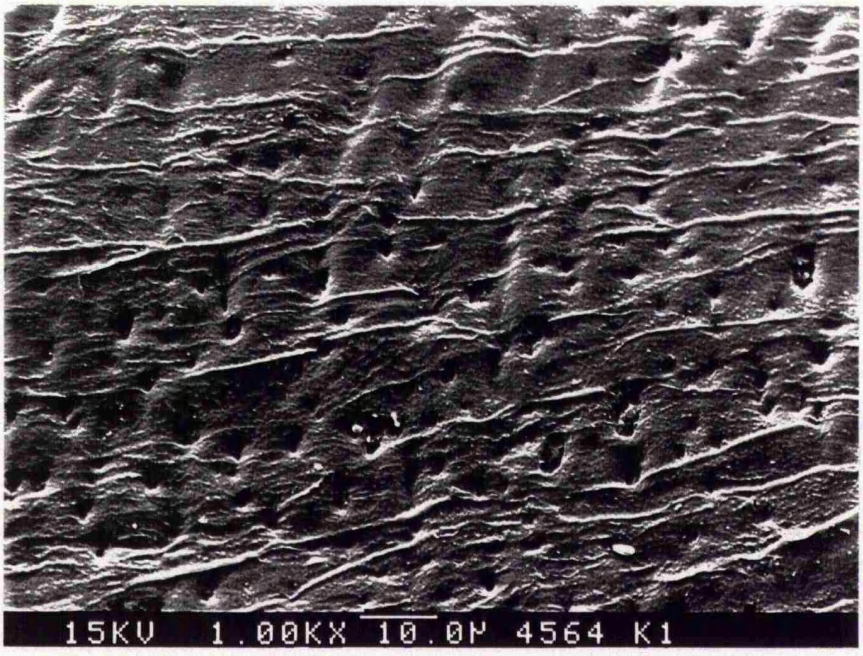

Table 17 The maximum relaxation (%) and sensitivity (pD_2s) to ACh in resistance arteries from recent onset diabetic rats and their age-matched controls, with and without endothelium; and endothelium-intact arteries incubated with SQ29548.

Group	+endo (ACh Max %)	pD_2	-endo (ACh Max %)	+ endo +SQ (ACh Max %)	pD_2
Recent Onset	46±10* (8)	7.65±0.18 (7)	12±7 (8)	64±13 (6)	7.26±0.18 (5)
Control	87±2* (8)	7.53±0.14 (8)	29±6 (8)	86±3 (8)	7.32±0.15 (8)

Number of animals shown in brackets.

* $p<0.05$ Endothelium intact versus denuded vessels (unpaired t-test).

Table 18 The maximum bradykinin relaxation response (%) of resistance arteries from diabetic rats and their age-matched controls, incubated with either indomethacin alone or indomethacin and L-NOARG.

Group	BK Max. (%)	+ Indomethacin (%)	+ Indo + L-NOARG (%)
Recent Onset	17±4 (6)		8±4 (7)
Controls	66±8 (6)		48±12 (7)
Insulin Treated	10±8 (7)	24±13 (5)	
Controls	63±9 (7)	73±6 (7)	

Number of animals shown in brackets.

Table 19 The maximum bradykinin relaxation (%) response of resistance arteries from recent onset diabetic rats and their age-matched controls, with and without endothelium; and endothelium-intact arteries incubated with SQ29548.

Group	+ Endothelium (%)	Denuded (%)	+ SQ 29548 (%)
Recent Onset	14±10*(8)	1±1 (7)	22 ±14 (5)
Controls	75±5* (8)	16±5 (8)	76±4 (8)

Number of animals shown in brackets.

* $p<0.05$, Endothelium intact versus denuded vessels (unpaired t-test).

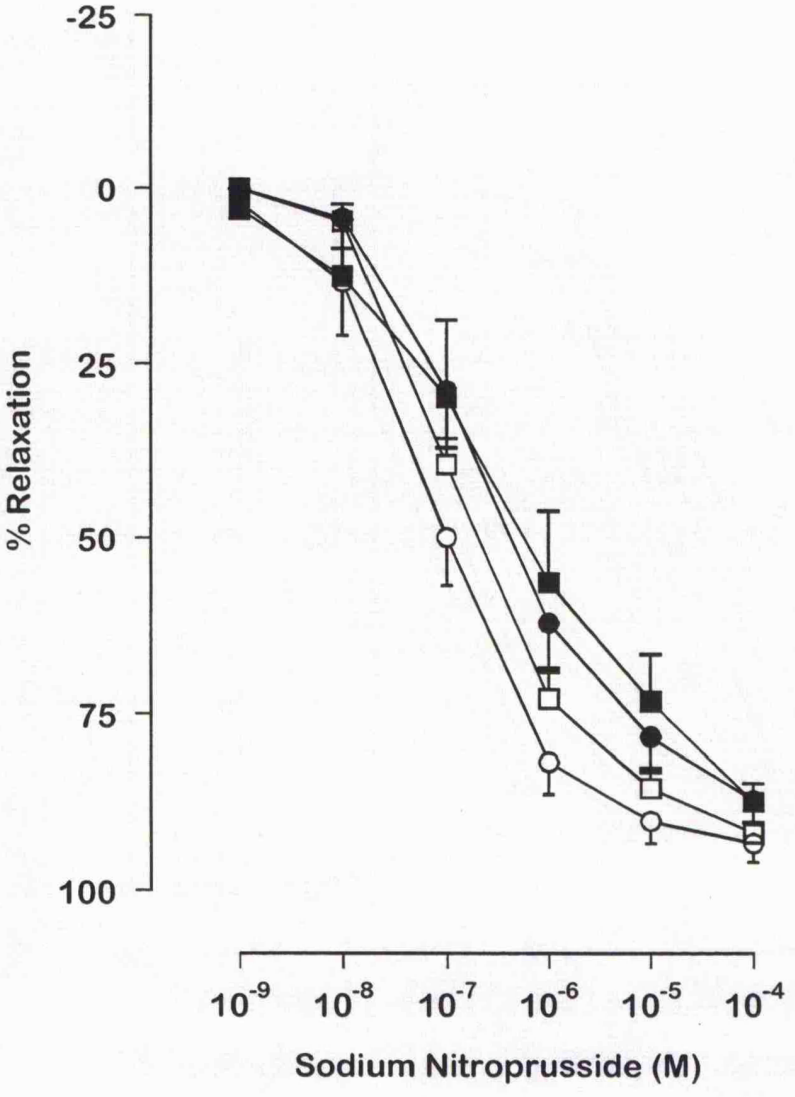

Figure 21 Endothelium-independent relaxation response to SNP of vessels from **recent onset** (❑), and their age-matched **controls** (○) incubated with indomethacin + L-NOARG, and relaxation responses of **insulin treated diabetic** (■) and their age-matched **controls** (●) incubated with indomethacin alone.

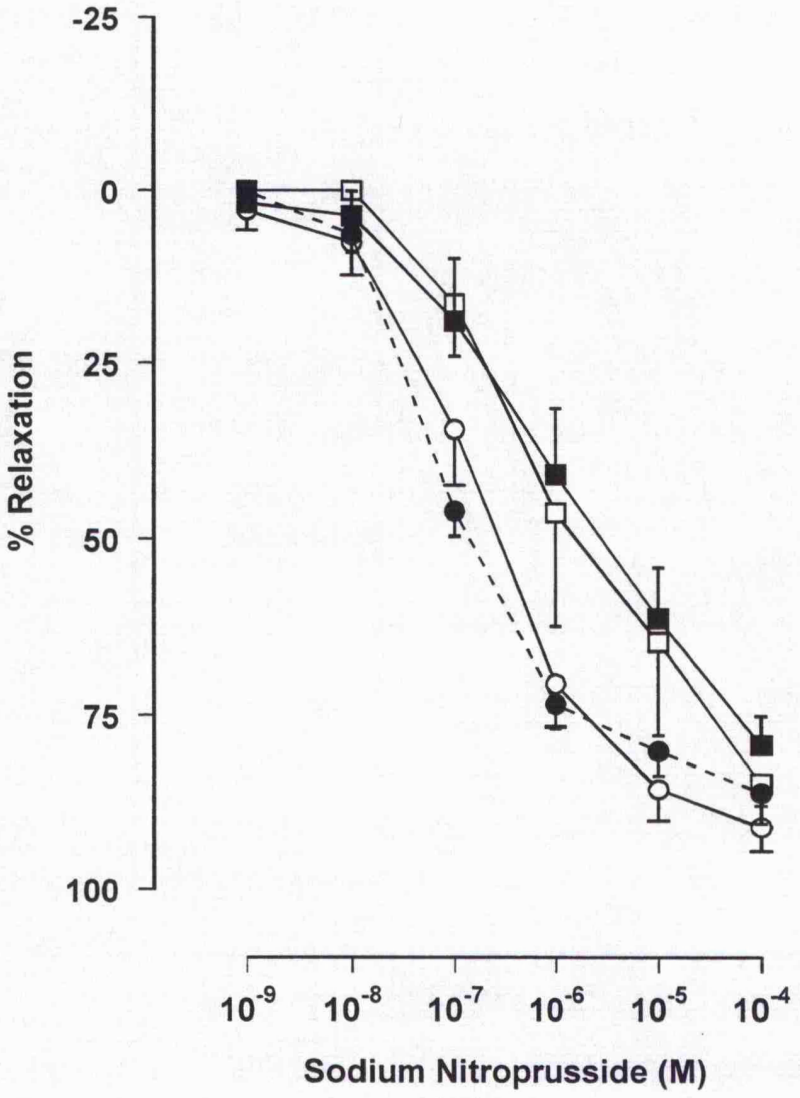

Figure 22 Endothelium-independent relaxation response to SNP of vessels from **recent onset + endothelium**(❑), **recent onset - endothelium** (■), and their age-matched **controls + endothelium** (○), **controls - endothelium** (●).

5.7 Discussion

The results from this series of studies showed that incubation with the nitric oxide synthetase inhibitor L-NOARG, resulted in an increase in maximum contractile response to noradrenaline in the controls age-matched to the recent onset diabetics. Analysis of the whole dose response curve before and after the addition of L-NOARG revealed a significant left-ward shift, which was also evident in the age-matched control group to the insulin treated diabetics, thereby indicating inhibition of basal EDRF release. However, compared to their controls, the shift in the NA dose response curve was less marked in the recent onset diabetics and no change was evident in the insulin treated diabetics. This suggests a reduction of basal EDRF release in the recent onset diabetics with a marked impairment in the insulin treated diabetic rats. These findings are in keeping with a report of reduced spontaneous release of nitric oxide in mesenteric arteries from rats with chemically induced diabetes mellitus (Taylor *et al.*,1992), although a later study showed enhanced noradrenaline sensitivity in untreated and insulin treated diabetic rats compared to controls after incubation with the nitric oxide synthetase inhibitor L-NAME (Taylor *et al.*, 1994a).

In our study, incubation with L-NOARG caused a variable decrease in maximum relaxation produced by acetylcholine and bradykinin in both diabetics and age-matched controls, suggesting that EDRF partly mediates ACh and BK relaxation in these vessels. Mechanical removal of the endothelium markedly attenuated ACh and BK relaxation responses in all vessels, confirming the pivotal role of the endothelium in mediating relaxation to these vasodilators. Although L-NOARG only partially inhibited endothelium-dependent relaxation, it is unlikely that the inhibitor concentration was insufficient to block the L-arginine pathway. Previous studies using similar concentrations have achieved comparable results (Bennett *et al.*, 1992). It is possible that the residual relaxation in the presence of L-NOARG may depend on the release of other vasodilators from the endothelium. One such vasodilator is an endothelium-derived hyperpolarising factor (EDHF), which relaxes vascular smooth muscle cells by opening potassium channels, and causing hyperpolarisation of the cell membrane (Van de Voorde *et al.*,1992).

Several mechanisms have been postulated to explain the attenuated relaxation response to acetylcholine in diabetic rats: these include a decrease in the production and release of EDRF, accelerated destruction of EDRF by free radicals, or an alteration in smooth muscle to respond to EDRF (Durante *et al.*, 1988). Incubation with the nitric oxide synthetase substrate L-arginine failed to improve the attenuated endothelium-dependent relaxation of the diabetic vessels, and it is therefore reasonable to accept that the impaired response was not due to a lack of substrate *per se*. Other studies indicate a possible role for superoxide anions interfering with EDRF activity (Katusic & Shepherd, 1991; Gryglewski *et al.*,1986a), although the literature concerning the role of free radicals in man is controversial and conflicting. However, increased levels of diene conjugation products have been demonstrated in insulin-dependent diabetic patients with microangiopathy (Jennings *et al.*, 1987b). Our studies showed no improvement of ACh relaxation after incubation with the free radical scavengers superoxide dismutase (SOD) and catalase (CAT), suggesting that excessive NO destruction by free radicals was not the cause of the impaired relaxation in the diabetic vessels. However, the present study does not exclude a role for endothelial cell membrane damage by lipid peroxidation and *in vivo* studies of the effects of treatment with free radical scavengers are needed to resolve this issue.

It is well established that the endothelium in addition to producing the vasodilators prostacyclin and EDRF, can also produce contracting factors such as thromboxane A_2, and prostaglandin H_2 via the cyclo-oxygenase pathway; and that these factors can profoundly affect vascular tone. Hypertension and atherosclerosis both have been shown to affect the endothelium so that the role of contracting factors becomes more dominant, leading to an imbalance in endothelium-dependent vascular regulation (Luscher *et al.*, 1992). Pre-treatment with the cyclo-oxygenase inhibitor indomethacin significantly improved the acetylcholine relaxation response in the diabetic vessels, suggesting increased vasoconstrictor prostanoid production may be overriding nitric oxide mediated relaxation. Similar observations have been reported using the aorta of the STZ-induced diabetic rat (Shimizu *et al.*, 1993), and rabbit aorta (Tesfamariam *et al.*, 1989), where exposure to high glucose levels caused an attenuated response to ACh-induced relaxation, which was normalized by inhibitors of cyclooxygenase and/or by PGH_2-thromboxane A_2 receptor antagonists.

Moreover, direct radio-immunoassay measurements revealed increased release of several prostanoids including thromboxane A_2 and PGF_2 (Tesfamariam *et al.*, 1989 and 1990).

In the fifth protocol of the second series of experiments, the role of thromboxane A_2 was assessed by observing the effects of the thromboxane A_2 antagonist SQ 29548 on relaxation of vessels from recent onset diabetic rats. No significant increase in ACh induced relaxation was observed, suggesting that the contracting factor was not TxA_2. Similarly, the impaired relaxation in rabbit aorta which develops with exposure to a high glucose concentration *in vitro*, can be ameliorated by indomethacin, but not with the TxA_2 antagonist (Tesfamariam *et al.*, 1990). A role for vasoconstrictor prostanoids is supported by a recent study showing treatment with aspirin restored forearm blood flow in reactive hyperaemia in diabetic patients free from complications (Steel *et al.*, 1993). On the other hand, another group observed that methacholine induced relaxation remained impaired despite aspirin therapy (Johnstone *et al.*, 1993).

The mechanism underlying the altered endothelial function has not been elucidated, but attention has focussed on glucose-induced increases of protein kinase C (PKC)(Tesfamariam *et al.*, 1991; Williams *et al.*, 1992; Johnstone *et al.*, 1993; and Cohen, 1993). These studies indicate that the abnormality of endothelium-dependent relaxation caused by elevated glucose is the result of an alteration in endothelial cell receptor-mediated signal transduction, probably mediated by protein kinase C activation (Tesfamariam *et al.*, 1991). This view is supported by the observation that the addition of protein kinase C inhibitors prevent abnormal release of prostanoids, restore endothelium-dependent relaxation, and preserves the release of PGI_2 from endothelial cells exposed to high concentrations of glucose (Tesfamariam *et al.*, 1991). Moreover, a recent *in vivo* study has demonstrated that when administered orally, a PKC inhibitor ameliorated the glomerular filtration rate, albumin excretion rate, and retinal circulation in experimental-induced diabetic rats (Ishii *et al.*, 1996).

In conclusion, this series of experiments demonstrated abnormal endothelium-dependent relaxation in resistance arteries of both recent onset and established insulin treated diabetic

BB rats. The impaired relaxation does not appear to be due to a lack of substrate for the nitric oxide synthetase enzyme, or to excessive destruction of nitric oxide by free radicals. The marked improvement of endothelial-dependent relaxation following incubation with indomethacin indicates that the abnormality results from over-production of a vasoconstricting prostanoid. However, pre-treatment with the thromboxane A_2/PGH_2 receptor antagonist SQ 29548 failed to produce a similar improvement of endothelium-dependent relaxation, suggesting that the vasoconstrictor prostanoid is not TxA_2 or PGH_2.

CHAPTER SIX

THE EFFECT OF EITHER INSULIN OR ISLET TRANSPLANTATION ON THE RESISTANCE ARTERY FUNCTION IN THE STZ-INDUCED DIABETIC RAT

6.1 Introduction

Resistance artery contractile and relaxation function was investigated in the chemically induced rat model in order to gain a different perspective regarding endothelial function in animal models of diabetes. Altered vascular reactivity and impaired endothelium-dependent relaxation has been demonstrated in conduit arteries from chemical induced animal models of diabetes, (Pieper & Gross, 1988; Cameron & Cotter, 1992). On the other hand, others have reported no change in the response to endothelium-dependent vasodilators (White & Carrier, 1986; Head *et al.*, 1987; Mulhern & Docherty, 1989). Similarly, studies of the vascular responses to noradrenaline (NA) also have revealed inconsistent results, showing both an increase in sensitivity (MacLeod & McNeill,1985; Harris & MacLeod, 1988; Pieper & Gross, 1988; Cohen *et al.*, 1990), and attenuated responses (Pfaffman *et al.*, 1982; Head *et al.*, 1987).

There have been comparatively few studies of endothelial function in resistance arteries which reflect the true state of the microvasculature and play an important role in the local control of blood flow and pressure. However, impaired endothelium-dependent relaxation has been described in resistance arteries from chemically induced diabetic animals (Takiguchi *et al.*, 1988, 1989; Taylor *et al.*, 1992, 1994a). Similarly, endothelial dysfunction has been described in studies of both human Type 1 (de Tejada *et al.*, 1989; Johnstone *et al.*, 1993; McNally *et al.*, 1994), and Type 2 (McVeigh *et al.*, 1992) diabetic subjects without complications. These observations particularly in the McNally study, support the suggestion that alterations in vascular reactivity may precede the structural changes which are the hallmark of established diabetic microangiopathy.

The introduction of insulin earlier this century removed decompensated diabetes as the major cause of death in IDDM. Subsequently, macrovascular and microvascular disease have been the main causes of morbidity and mortality. Improved glycaemic control, with intensive insulin regimes have reduced the incidence of diabetic microangiopathy (DCCT, 1993). However, optimal diabetic control remains a practical impossibility in many patients, and at present this can be achieved only by the transplantation of islets of Langerhans, or whole

pancreas (Orloff *et al.*, 1987 & 1988) to replace the function of the damaged beta cells.

In the rat, islet transplantation has been shown to reverse metabolic abnormalities, and also has beneficial effects on secondary complications associated with the diabetes (Mauer *et al.*, 1974; Gray & Watkins, 1976; Schmidt *et al.*, 1983; Pugliese *et al.*, 1990). However, to date no studies have investigated whether improved diabetic control following islet transplantation could ameliorate diabetes-induced endothelial dysfunction in the microcirculation. Accordingly, the effects of chemical induced diabetes on resistance artery contractile and relaxation function have been investigated at 6 and 12 weeks in rats after the chemical induction of diabetes. In addition, the response to *long-term* insulin treatment or intra-portal islet transplantation has been observed.

6.2 Experimental Protocol

Female WAG/Leicester rats (8-9 weeks old and weighing approximately 150g) were used for these studies. In the first study of 6 weeks of diabetes, rats were randomly divided into three experimental groups: diabetic, control, and insulin treated diabetic. Diabetes was induced by a single femoral vein injection of 55mg/kg streptozotocin (STZ) dissolved in 1ml of citrate buffer under light anaesthesia. Rats allocated to the control group received the buffer solution alone.

Blood glucose levels were measured on a daily basis using a blood glucose test strip (BM-Test 1-44) and a Reflolux meter (Boehringer Mannheim). Rats with blood glucose readings >20mmol/l were considered diabetic and were either treated with insulin, or received no insulin, and therefore remained hyperglycaemic for the duration of the study. The insulin treated group received daily subcutaneous injections of 0.8U insulin/100g body weight (Ultralente, Novo Nordisk), with the dose being regularly adjusted in order to maintain blood glucose levels within the normal range (i.e. 3-7mmol/l), according to the following dosing regime: blood glucose 3-4.9mmol/l the previous day's insulin dose was halved; blood glucose 5-9.9mmol/l, no adjustment; blood glucose >10mmol/l, the dose was increased by 0.15u insulin..

Rats were fed standard rat chow, allowed access to water *ad libitum*, and were weighed daily. Systolic blood pressure was measured by the tail cuff method one week before the animal was due for sacrifice. On the day of sacrifice, insulin treated diabetic rats did not receive their usual morning injection of insulin. Rats were culled, and mesenteric resistance arteries were dissected and mounted in a Mulvany myograph. Morphological measurements were made and the vessels normalised. Before the contraction studies were started, the vessels were incubated in PSS at 37°C for sixty minutes. Vessels then underwent the activation procedure, and following this, a cumulative concentration response curve to the contractile agent noradrenaline (NA 10^{-8}M-$3x10^{-5}$M) was carried out in the presence of cocaine (10^{-6}M). The vessels were then rinsed to baseline and allowed to recover for fifteen minutes.

The arteries were then maximally contracted with a bolus dose of NA (10^{-5}M)and once a plateau had been reached, the relaxation response to acetylcholine (ACh 10^{-9}M-10^{-5}M) was observed. Fifteen minutes after washout, arteries were maximally contracted again with NA (10^{-5}M) and were subsequently relaxed with cumulative doses of bradykinin (BK 10^{-9}M-10^{-5}M). The vessels were brought to base line with three rinses of PSS, and incubated with PSS containing N^{G}-nitro-L-arginine (L-NOARG, 10^{-5}M) for one hour. The NA, ACh, and BK dose response curves were repeated in the presence of L-NOARG. Finally, the vessels were maximally contracted with NA (10^{-5}M) and relaxed with cumulative doses of sodium nitroprusside (SNP 10^{-9}M-10^{-4}M).

6.3 Results

6.3.1 Analysis of Results

Statistical analysis of the physical characteristics of the rats and the resistance morphology was determined using the Students paired t test for comparisons within experimental groups, and the unpaired t test for comparisons between groups. Contraction and relaxation dose response curves were compared using one-way analysis of variance (ANOVA). If $p<0.05$, individual pairs of means were then compared using the Bonferroni multiple comparisons test. A shift factor was calculated to assess the magnitude of change in noradrenaline sensitivity following incubation with L-NOARG using the following formula:

$$\text{shift factor} = - \frac{(pD_2NA) - (pD_2\,NA + L\text{-}NOARG)}{pD_2\,NA} \times 100$$

ANOVA was used with the Bonferroni correction factor in order to determine statistical significance between the groups.

6.3.2 Physical Characteristics

The insulin treated rats were significantly heavier than the untreated diabetic rats (194g±6 versus 174g±4, $p<0.05$, n=8), although the weight of the insulin treated animals was not significantly different from the controls (177±8, n=5), and the body weight of the untreated diabetic rats was not significantly different from that of the controls. The heart weight/ tibia ratio was significantly greater in diabetic rats treated with insulin compared with non-diabetic controls (2±0.12, n=6 versus 1.68±0.08, n=7 $p<0.05$), but there was no significant difference in heart weight/tibia ratio between insulin treated diabetics and the untreated diabetic group (2±0.12, n=6 versus 1.73±0.03, n=8) (Table 20).

Blood glucose levels were significantly greater in the untreated diabetic group (18.4±2.2mmol/l, n=8) compared to the control group (3.1±0.2mmol/l, n=9 $p<0.001$), and those receiving insulin (9.13±2.48mmol/l, n=7 $p<0.01$). Moreover, the diabetic animals receiving insulin also had significantly higher blood glucose levels than the control animals ($p<0.01$). There was no significant difference between the systolic blood pressure measurements of the experimental groups (control: 139±4mmHg, n=8; untreated diabetic: 139±7mmHg, n=6; and insulin treated: 136±3mmHg, n=6).

i) Resistance Vessel Structure/Morphology

The lumen diameters of the resistance arteries from both the untreated and the insulin treated diabetic groups were comparable to that of the non-diabetic controls (untreated: 263±6μm, n=8; insulin treated: 249±4μm, n=8; and controls: 262±8μm, n=9) (Table 21). Likewise, there were no significant differences in media lumen, cross sectional area or media/lumen ratio between all of the experimental groups (Media thickness: untreated 10.23±0.44μm,

n=9; insulin treated 12.22 ±0.63μm, n=8; controls 11.82±0.81μm, n=9: Cross sectional area: untreated 8741±388μm^2,n=9; insulin treated 9996±532μm^2, n=8; controls 10146±847μm^2, n=9: Media:Lumen (%): untreated 3.94±0.22, n=8; insulin treated: 5±0.3, n=8; controls 4.58±0.37, n=9).

6.3.3 Resistance Vessel Contractility

i) High Potassium

There was no significant difference in the maximum response to high potassium PSS in all the three experimental groups (untreated diabetic: 3.43±0.18mN/mm, n=8; insulin treated diabetic:3.35±0.26mN/mm, n=8; control: 3.22±0.16mN/mm, n=9).

ii) High Potassium with Noradrenaline

Similarly, there was no significant difference in maximum contractile response with PSS containing both potassium and noradrenaline (NA10^{-5}M) in the three experimental groups (untreated diabetic: 4.25±0.34mN/mm, n=8; insulin treated diabetic: 4.3±0.34mN/mm, n=8; control: 4.12±0.19mN/mm, n=9).

iii) Noradrenaline

The results showed that there was no significant difference in the noradrenaline maximum contractile response or sensitivity (pD_2), of the three groups (untreated diabetic: 3.85±0.28mN/mm, 5.74±0.01, n=8; insulin treated diabetic: 3.79±0.29mN/mm, 5.8±0.01, n=8; control: 3.89±0.17mN/mm, 5.77±0.03, n=9) (Table 22).

iv) Effects of L-NOARG on the NA Contractile Response and Sensitivity

Incubation with the nitric oxide synthetase inhibitor, L-NOARG resulted in a significant increase in maximum NA contractile response in the untreated diabetic group ($p<0.05$),

along with a significant shift in the sensitivity to NA (pD_2) (Figure 23). There was no significant increase in maximum contractility or sensitivity to NA in the control group (Figure 24). The maximum contractile response was unchanged in the insulin treated diabetic group, although there was evidence of a significant shift in sensitivity ($p<0.05$) (Figure 25). The percentage shift after the addition of L-NOARG was also calculated for each group, and an analysis of variance (ANOVA) performed to compare the experimental groups. This showed that there was no significant shift evident between the groups (control: 1.35±0.41%, n=9; untreated diabetic: 0.95±0.3%, n=8; insulin treated diabetic: 1.42±0.47%, n=8) (Table 22).

6.3.4 Endothelium-Dependent Relaxation

i) Acetylcholine

Maximum relaxation to the endothelium-dependent vasodilator ACh was significantly greater in the untreated diabetic group compared to the control group (92±1%, n=7 versus 64±10%, n=8, $p<0.05$), but there was no significant difference in ACh sensitivity (control: 6.9±0.11; untreated diabetic: 6.96±0.1; insulin treated diabetic: 6.99±0.08). Relaxation in the insulin treated diabetic group was not significantly greater than that seen in the control group (79±7%, n=8 versus 64±10%, n=8) (Figure 26). A power calculation was carried out to determine the group size necessary to detect a change in sensitivity of 0.5μmol/l to ACh at the $p<0.05$ level. The calculation revealed that the number of animals in each group should be at least 4 to have an 80% power of detecting this difference in sensitivity. The group sizes used were therefore adequate.

Incubation with the nitric oxide synthetase inhibitor L-NOARG caused significant attenuation of the maximum relaxation response to ACh in all three experimental groups, with the untreated diabetic group displaying the least inhibition with L-NOARG. However, there was no change in ACh sensitivity in any of the three experimental groups. (Table 23)

ii) Bradykinin

The maximum relaxation response and sensitivity was similar in the diabetic animals compared to their age-matched controls, although there was a significant shift in sensitivity in the insulin treated diabetics compared to the age-matched controls ($p<0.05$) (control: 54±8%, 6.48±0.12, n=8; untreated diabetics: 71±6%, 6.77±0.13, n=7; insulin treated diabetics: 63±8%, 6.98±0.12, n=8) (Figure 27). A one hour incubation with L-NOARG, significantly inhibited the BK relaxation response in all three groups ($p<0.05$). Bradykinin sensitivity following incubation with L-NOARG was unchanged in both the controls and diabetic animals, however, a shift in sensitivity was evident in both the insulin treated ($p=0.059$), and the untreated diabetics ($p=0.052$), although statistical significance was not achieved. (Table24).

A power calculation was carried out to determine the group size necessary to detect a difference of 20% maximum relaxation response to BK at the $p<0.05$ level. The calculation revealed that it would be necessary to have at least 20 animals in each group to have an 80% power of detecting this difference in relaxation response. In this case the group sizes used were not sufficient to ascertain a difference in relaxation at this level. However, the group sizes were adequate for determining a difference of 0.5μmol/l in sensitivity to BK at the $p<0.05$ level. For an 80% power of detecting this difference it would be necessary to have at least 7 animals in each group.

6.3.5 Endothelium Independent Relaxation

i) Sodium Nitroprusside

There was no significant difference in the maximum relaxation response or sensitivity to the endothelium independent vasodilator sodium nitroprusside between the diabetic groups and the non-diabetic controls (untreated diabetic: 87±3%, 6.31±0.09, n=8; insulin treated diabetic: 76±5%, 6.15±0.1, n=8; control: 84±3%, 6.29±0.07, n=9), or when the untreated rats were compared directly to the insulin treated rats. (Figure 28) A power calculation revealed that it would be necessary to have at least 8 animals in each group to have an 80% chance

of detecting a difference of 20% in maximum relaxation to SNP at the $p<0.05$ level. Similarly, at least 5 animals should be in each group to ensure an 80% chance of detecting a difference of 0.5μmol/l in sensitivity to SNP at the $p<0.05$ level. The group sizes used were therefore adequate.

Table 20 Physical characteristics of STZ-induced diabetic rats, insulin treated STZ rats, and their age-matched controls (6-week study).

Group	Systolic B.P. (mmHg)	Body Weight (g)	Blood Glucose (mmol/l)	Hwt./Tibia
Control	139±4 (8)	177±8 (5)	3.1±0.2 (9)	1.68±0.08 (7)
Untreated Diabetic	139±7 (6)	174±4**(8)	18.4±2.2 (8)	1.73±0.03 (8)
Insulin Treated Diabetic	136±3 (6)	194±6 (8)	9.13±2.48 (7)	2.00±0.12* (6)

Number of animals in brackets.

* $p<0.05$ (unpaired t-test versus control)

** $p<0.01$ (unpaired t-test diabetic versus insulin treated diabetic).

Table 21 Morphological measurements of vessels of STZ-induced diabetic rats, insulin treated diabetic rats, and age-matched controls (6-week study).

Group	N = animals	Diameter (μm)	Media Thickness (μm)	Cross Sectional Area (μm^2)	Media:Lumen x 100 (%)
Control	9	262±8	11.82±0.81	10146±847	4.58±0.37
Untreated Diabetic	8	263±6	10.23±0.44	8741±388	3.94±0.22
Insulin Treated Diabetic	8	249±4	12.22±0.63	9996±532	5±0.3

Table 22 Maximum contractile response and sensitivity to noradrenaline before and after incubation with N^G-nitro-L-arginine (L-NOARG) (6-week STZ-study).

Group	N = animals	NA Max (mN/mm)	+L-NOARG (mN/mm)	pD_2	pD_2 +L-NOARG	Shift Factor (%)
Control	9	3.89±0.17	3.85±0.22	5.77±0.03	5.8±0.02	1.35±0.41
Untreated Diabetic	8	3.85±0.28*	4.32±0.33	5.74±0.01*	5.79±0.02	0.95±0.3
Insulin Treated Diabetic	8	3.79±0.29	3.95±0.35	5.8±0.01*	5.87±0.02	1.42±0.47

*$p<0.05$ (paired t test before and after L-NOARG).

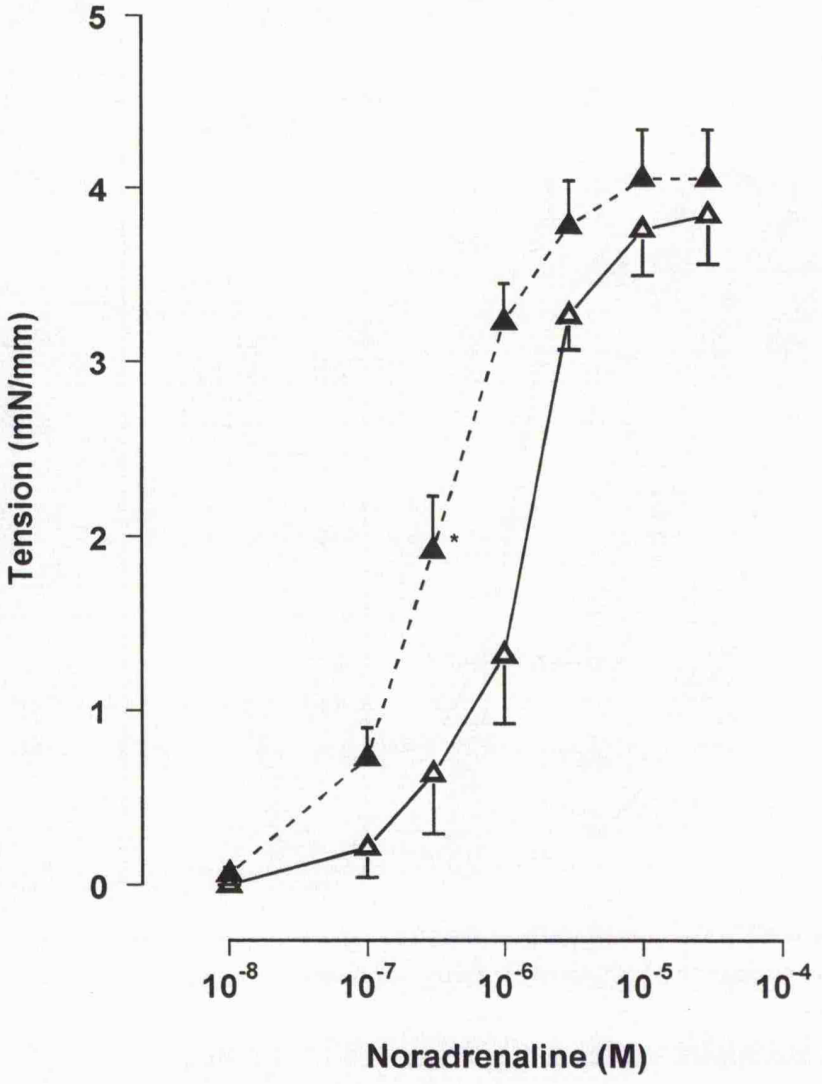

Figure 23 Noradrenaline contractile response of rat mesenteric resistance vessels (6-week study) from untreated diabetic rats before (△), and after incubation with N^G-nitro-L-arginine (L-NOARG) (▲, *$p<0.05$).

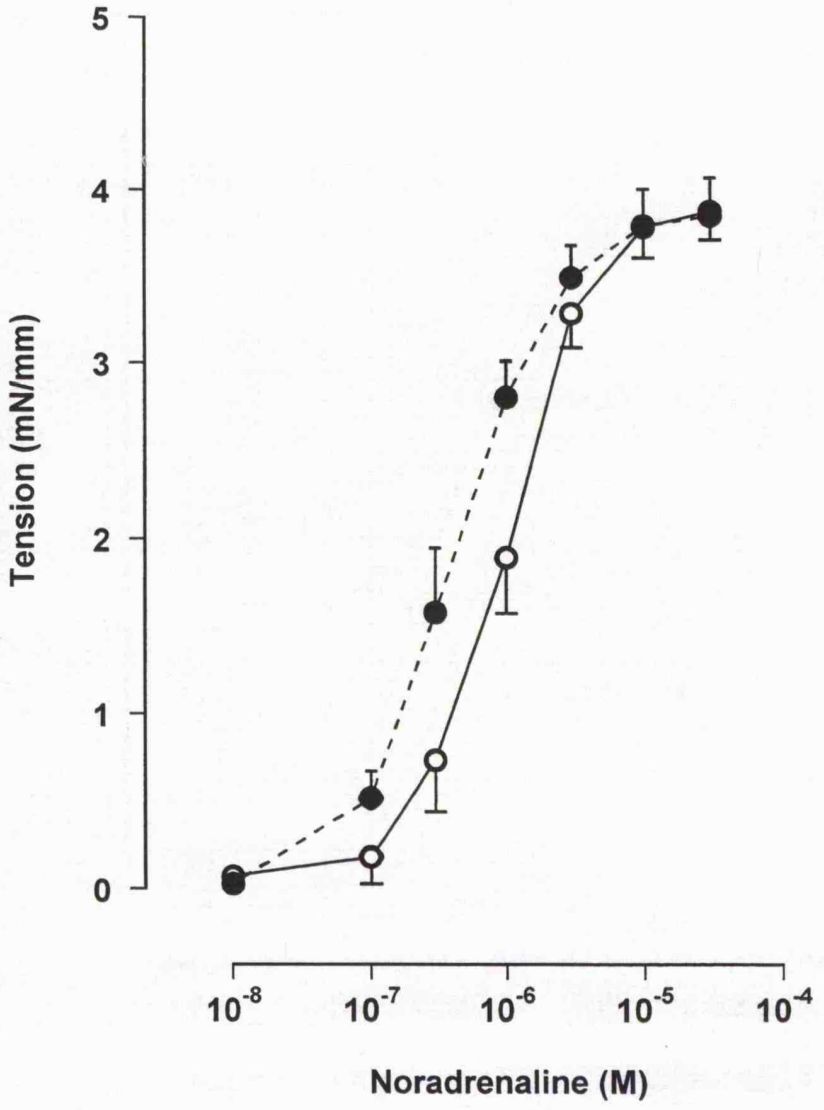

Figure 24 Noradrenaline contractile response of rat mesenteric resistance vessels (6-week study) from sham operated controls before (○) and after incubation with N^G-nitro-L-arginine (L-NOARG) (●).

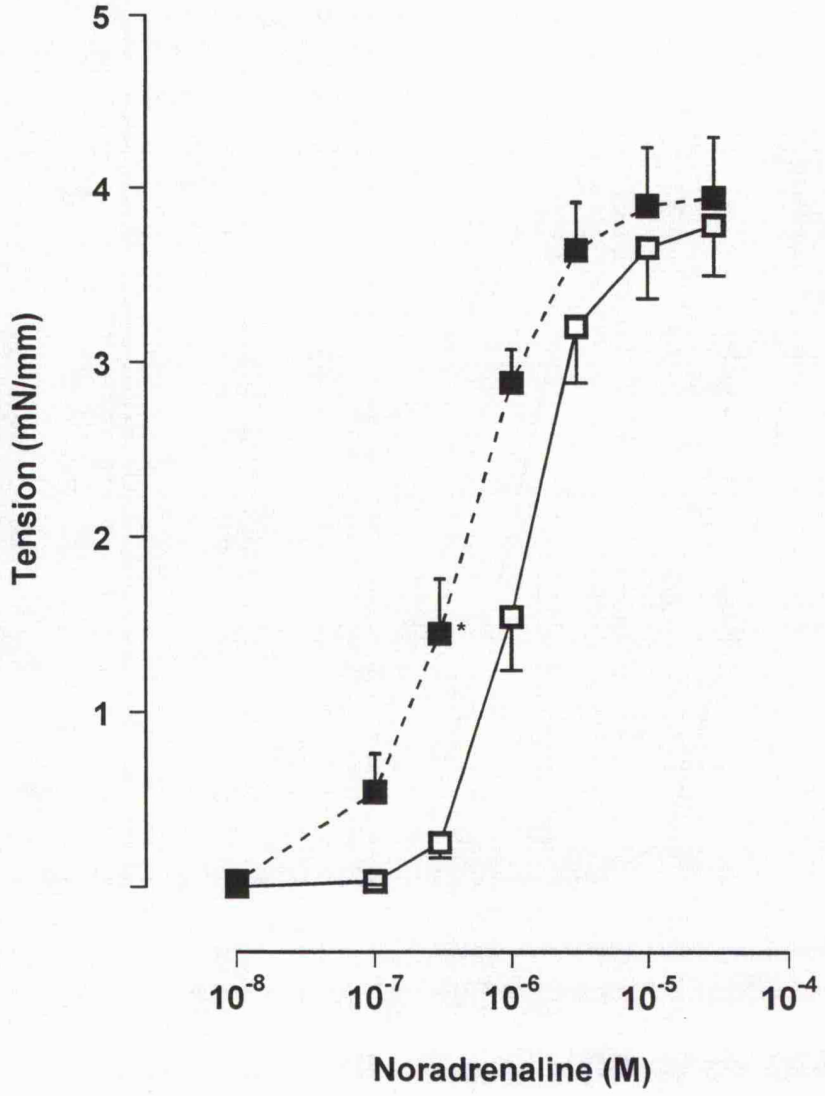

Figure 25 Noradrenaline contractile response of rat mesenteric resistance vessels (**6**-week study) from insulin treated rats before (❑) and after incubation with N^G-nitro-L-arginine (L-NOARG) (■, *$p<0.05$).

Table 23 Endothelium-dependent relaxation and sensitivity to acetylcholine before and after incubation with N^G-nitro-L-arginine (L-NOARG) (6-week STZ study).

Group	N = animals	ACh Max (%)	+L-NOARG (%)	pD_2	pD_2+L-NOARG
Control	8	64±10	30±9*	6.9±0.11	7.1±0.25
Untreated Diabetic	7	92±1$^+$	71±6*	6.96±0.1	6.66±0.11
Insulin Treated Diabetic	8	79±7	35±11*	6.99±0.08	7.01±0.07

*$p<0.05$ (paired t-test before and after L-NOARG).

$^+p<0.05$ (unpaired t-test, untreated diabetic versus control).

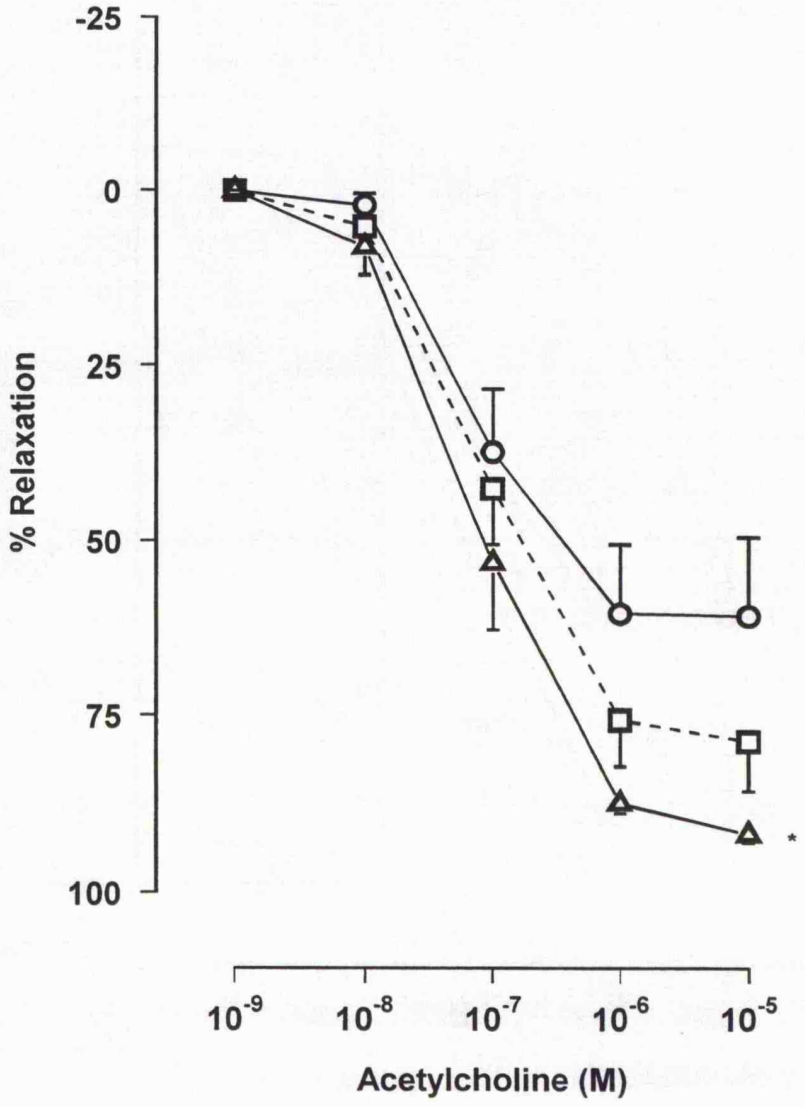

Figure 26 Acetylcholine-induced relaxation response of rat mesenteric resistance vessels (**6**-week study) from non-diabetic control rats (○), insulin treated diabetic rats (❑), and untreated diabetic rats (▵, *$p<0.05$ versus non-diabetic controls).

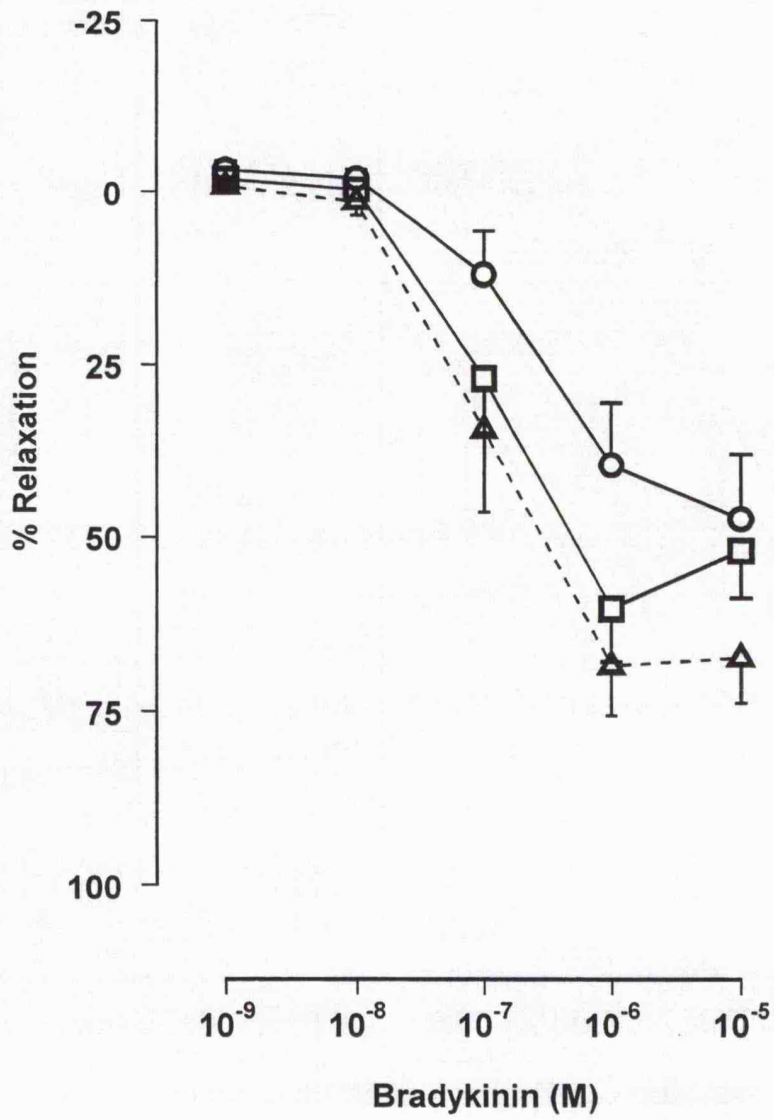

Figure 27 Bradykinin-induced relaxation response of rat mesenteric resistance vessels (**6**-week study) from non-diabetic control rats (○), insulin treated diabetic rats (□), and untreated diabetic rats (△).

Table 24 Endothelium-dependent relaxation and sensitivity to bradykinin before and after incubation with N^G-nitro-L-arginine (L-NOARG) (6-week STZ study).

Group	N = animals	BK Max (%)	+L-NOARG (%)	pD_2	pD_2+L-NOARG
Control	8	54±8	18±5**	6.48±0.12	6.42±0.14
Untreated Diabetic	7	71±6	49±4*	6.77±0.13	6.39±0.1
Insulin Treated Diabetic	8	63±8	28±11*	$6.98±0.12^+$	6.42±0.07

*p<0.05, **p<0.01 (paired t-test before and after L-NOARG).

$^+$p<0.05 (insulin treated diabetic versus control).

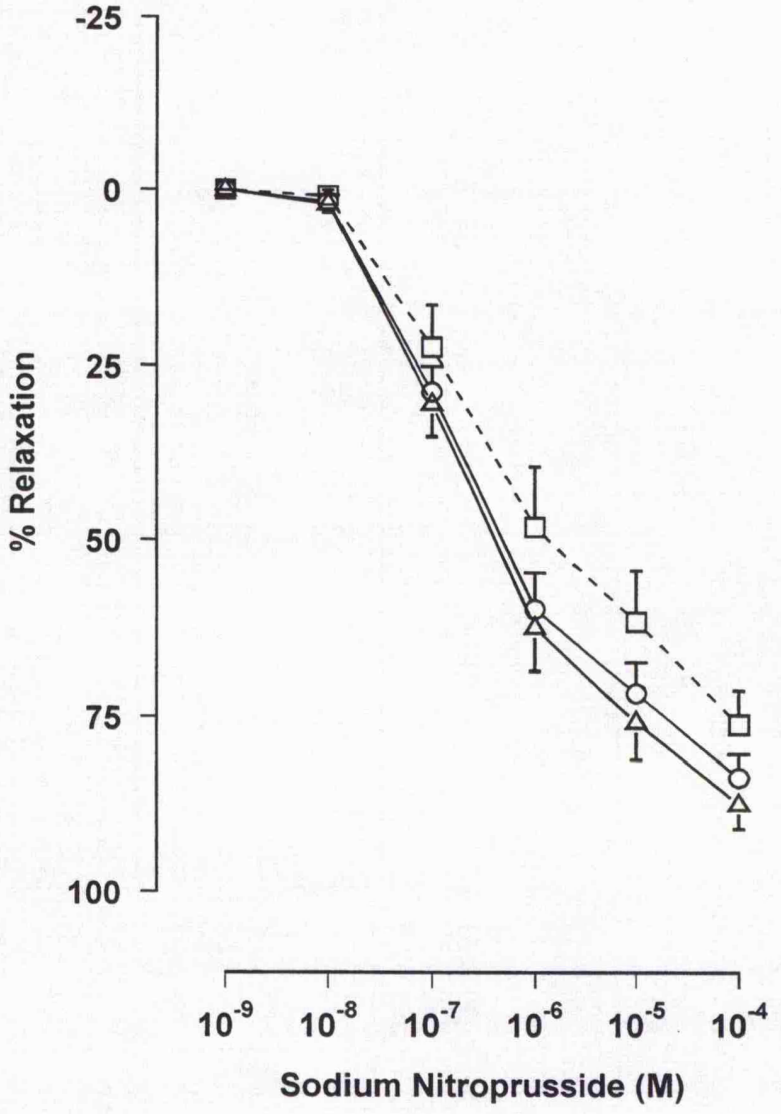

Figure 28 Endothelium-independent relaxation response to sodium nitroprusside of rat mesenteric resistance vessels (6-week study) from non-diabetic control rats (○), insulin treated diabetic rats (□), and untreated diabetic rats (△).

6.4 The Effects of Treatment with Either Insulin, or Intra-Portal Islet Cell Transplantation on the Resistance Artery Structure and Function After 12-14 Weeks of Chemical Induced Diabetes.

Further studies of longer diabetes duration were then carried out to investigate whether the alteration in basal nitric oxide release shown in the diabetic groups would still be evident after 12-14 weeks of chemically induced diabetes, and whether these changes could be ameliorated with the transplantation of islet cells. (N.B.Vessels from these animals were subjected to the same experimental protocol as the first study.)

The experimental design was similar to the first study, and consisted of four experimental groups: group 1 animals were injected with STZ and subsequently treated with insulin; group 2 were injected with STZ but did not receive insulin therapy; group 3 were injected with STZ and were treated with insulin for 14-21 days, before receiving an intraportal isograft of freshly isolated pancreatic islets (approximately 1000-1200 per recipient) from syngeneic WAG donor rats (3 donors: 1 recipient); and group 4 comprised non-diabetic control rats which underwent a sham islet transplant operation.

Reversal of diabetes in the islet transplanted group was confirmed by three consecutive blood glucose readings being consistently below 10mmol/l. Transplanted animals were culled if blood glucose readings were >20mmol/l. On sacrifice, the liver was removed from the rats that had received transplanted islets and was fixed in formalin prior to histological staining for the presence of islets. (Plates 11 & 12).

6.5 Results

6.5.1 Physical Characteristics

The body weight of the untreated diabetic animals was significantly lower than that of the other three experimental groups (152g±7 versus control 241g±4, $p<0.001$; insulin treated diabetics 210g±14, $p<0.01$; and transplanted animals 187g±7, $p<0.01$). The transplanted animals were also significantly lighter than the controls (187g±7 versus 214g±4, $p<0.01$).

Heart weight/ tibia ratio was significantly different in the untreated diabetic animals compared with the other experimental groups (1.46±0.05 versus control 1.71±0.05, $p<0.05$; insulin treated diabetics 1.83±0.08, $p<0.001$; and the transplanted group 1.71±0.06, $p<0.05$) (Table 25).

Blood glucose levels were significantly higher in the untreated diabetic group (24.2±1.1mmol/l, n=8, $p<0.001$) compared to those receiving insulin (13.4±0.7mmol/l, n=8), and the control animals (4.4±0.67mmol/l, n=6). The blood glucose was also significantly elevated in the insulin treated diabetic group compared with the non-diabetic controls ($p<0.001$). However, blood glucose levels of the transplanted animals were significantly lowered from hyperglycaemic levels to the normal range approximately ten days after islet cell transplantation (pre-transplant:16.4±1mmol/l versus post-transplant: 4.8±0.6mmol/l, n=8, $p<0.0001$). By the final week of the study (after approximately 12-14 weeks of uncontrolled diabetes and hyperglycaemia), the untreated diabetic animals had developed cataracts in both eyes. There was no significant difference in the systolic blood pressure measurements between all three of the experimental groups (untreated diabetic: 133±6mmHg, n=8; insulin treated diabetic: 125±4mmHg, n=7; islet transplanted: 132±2mmHg, n=8; control: 124±3mmHg, n=4).

i) Resistance Vessel Structure/Morphology

The lumen diameters of the resistance arteries taken from the three experimental groups were similar to those of the non-diabetic controls (untreated: 254±10μm, n=9; insulin treated: 257±7μm, n=8; islet transplanted: 261±9μm, n=8; controls: 250±6μm, n=12). Likewise, there were no significant differences in media lumen, cross sectional area or media/lumen ratio. (Media thickness: untreated 14.62±1.22μm, n=9; insulin treated 15.14 ±1.1μm, n=8; islet transplanted: 14.1 ±0.5μm, n=8; controls 12.64±0.67μm, n=12: Cross sectional area: untreated 12419±1094μm^2, n=9; insulin treated 12784±1001μm^2, n=8; islet transplanted: 12208±396μm^2, n=8; controls 11337±762μm^2, n=12: Media:Lumen (%): untreated 6.03±0.87, n=9; insulin treated: 6.15±0.55, n=8; islet transplanted: 5.48±0.33, n=8; controls 4.92±0.32, n=12) (Table 26).

6.5.2 Resistance Vessel Contractility

i) High potassium

There was a significant increase in the maximum response to PSS containing high potassium in the 12-week insulin treated diabetic group (4.43±0.28mN/mm, n=8, $p<0.05$), compared to the non-diabetic controls, the untreated diabetic and islet transplanted diabetic rats (control: 3.2±0.24mN/mm, n=12 untreated: 3.34±0.13mN/mm, n=9; islet transplanted: 3.11±0.25mN/mm, n=8).

ii) High potassium with noradrenaline

There was a significant increase in maximum contractile response to PSS containing both potassium and noradrenaline ($NA10^{-5}M$) in the insulin treated diabetic group (5.55±0.34, n=8, $p<0.05$) compared to the non-diabetic controls, the untreated diabetic and islet transplanted diabetic rats (control: 4.19±0.31mN/mm, n=12; untreated: 4.13±0.19mN/mm, n=9; islet transplanted: 4.22±0.31mN/mm, n=8).

iii) Noradrenaline

The maximum contractile response to NA was increased in the insulin treated group (4.82±0.33mN/mm, n=8, $p<0.05$) compared to the other three experimental groups (control: 3.9±0.26mN/mm, n=12; untreated diabetic: 3.96±0.2mN/mm, n=9; islet transplanted: 4.38±0.3mN/mm, n=7). However this was not associated with an increase in sensitivity (EC_{50}) to NA in the insulin treated animals (5.8±0.08, n=8) compared to the three experimental groups (control: 5.99±0.07, n=12; untreated diabetic: 6.03±0.08, n=9; islet transplanted: 6.04±0.08, n=7) (Table 27 & Figure 29).

A power calculation was carried out for the maximum contractile response to NA. This revealed that it would be necessary to have at least 12 animals in each group for an 80% chance of detecting a difference in maximum contraction of 1mN/mm at the $p<0.05$ level. In this case the group sizes used were slightly too small to ascertain a difference in maximum

contractile response at this level. However, the group sizes were adequate for determining a difference in sensitivity to NA at 80% power and $p<0.05$ level. According to the power calculation the group size should be at least 3 animals or more.

iv) Effects of L-NOARG on the NA Contractile Response and Sensitivity

In contrast to the findings of the six week study, the untreated diabetics showed no significant increase in contractility after the addition of L-NOARG, although an increase in sensitivity (pD_2) was evident ($p<0.05$) (Figure 30). Moreover, the NA contractility and sensitivity in both the control, and the islet transplanted group were not significantly changed after incubation with L-NOARG. However, incubation with L-NOARG resulted in a significant increase in both the maximum contractile response and sensitivity to NA in the insulin treated diabetics ($p<0.05$) (Figure 31). There was no significant difference in the percentage shift (after incubation with L-NOARG) evident between the experimental groups (control: 7.01±1.51%, n=10; untreated diabetic: 5.19±1.02%, n=8; insulin treated diabetic: 7.69±2.33%, n=7; islet transplanted: 4.89±1.29%, n=8) (Table 27).

6.5.3 Endothelium-Dependent Relaxation

i) Acetylcholine

There was no significant difference in ACh maximum relaxation between the experimental groups and the non-diabetic controls (untreated diabetics: 84±3%, n=8; insulin treated diabetics 75±5%, n=8; and islet transplanted: 72±7%, n=7; versus controls: 86±3%, n=10) (Figure 32). Also there was no significant difference in sensitivity to ACh between the experimental groups (control: 7.1±0.07, n=10; untreated diabetic: 7.1±0.1, n=8; insulin treated diabetic: 7.15±0.11, n=8; islet transplanted: 7.21±0.16, n=7). Incubation with L-NOARG markedly attenuated the maximum relaxation responses in all groups. However, in keeping with the six week study, there was no significant change in sensitivity following the addition of L-NOARG (Table 28).

ii) Bradykinin

There was no significant difference between maximum BK relaxation response in the experimental groups compared to the non-diabetic controls (untreated: 63±7%, n=8; insulin treated: 56±9%, n=8; and islet transplanted: 49±11, n=7 versus controls: 68±7, n=9). Also there was no change in the sensitivity between the experimental groups and the non-diabetic controls (control: 6.34±0.07, n=9; untreated diabetic: 6.85±0.18, n=8; insulin treated diabetic: 6.44±0.15, n=7; islet transplanted: 6.37±0.16, n=6) (Figure 33). Incubation with the nitric oxide synthase inhibitor, L-NOARG caused significant attenuation of the maximum relaxation response to bradykinin in all of the experimental groups, but there was no change in BK sensitivity (Table 29).

A power calculation was carried out for the maximum relaxation response to BK. This revealed that it would be necessary to have at least 15 animals in each group for an 80% chance of detecting a difference of 20% in maximum relaxation at the $p<0.05$ level. In this case the group sizes used were slightly too small to ascertain a difference in maximum relaxation at this level. Similarly, the group sizes were slightly too small to determine a change in sensitivity of 0.5μmol/l to BK at 80% power and $p<0.05$ level. According to the power calculation the group size should be at least 10 animals or more.

6.5.4 Endothelium Independent Relaxation

i) Sodium Nitroprusside

The maximum relaxation and sensitivity to sodium nitroprusside was similar in the four groups (controls: 86±3%, 6.43±0.1, n=8; untreated diabetics: 84±4%, 6.33±0.27, n=8; insulin treated diabetics: 90±4%, 6.69±0.12, n=7; islet transplanted: 87±4%, 6.5±0.18, n=8) (Figure 34). A power calculation was carried out for the maximum relaxation response to SNP and the group sizes used proved to be adequate. It would be necessary to have at least 5 animals in each group for an 80% chance of detecting a difference of 20% in maximum relaxation at the $p<0.05$ level. However, for an 80% chance of detecting a difference in sensitivity of 0.5μmol/l to SNP at the $p<0.05$ level, at least 10 animals should be in each group. Therefore the group sizes used were a little too small to ascertain a difference in sensitivity.

Plate 11: Liver tissue containing transplanted islets stained with Haematoxylin (BDH) and eosin (magnification x 20).

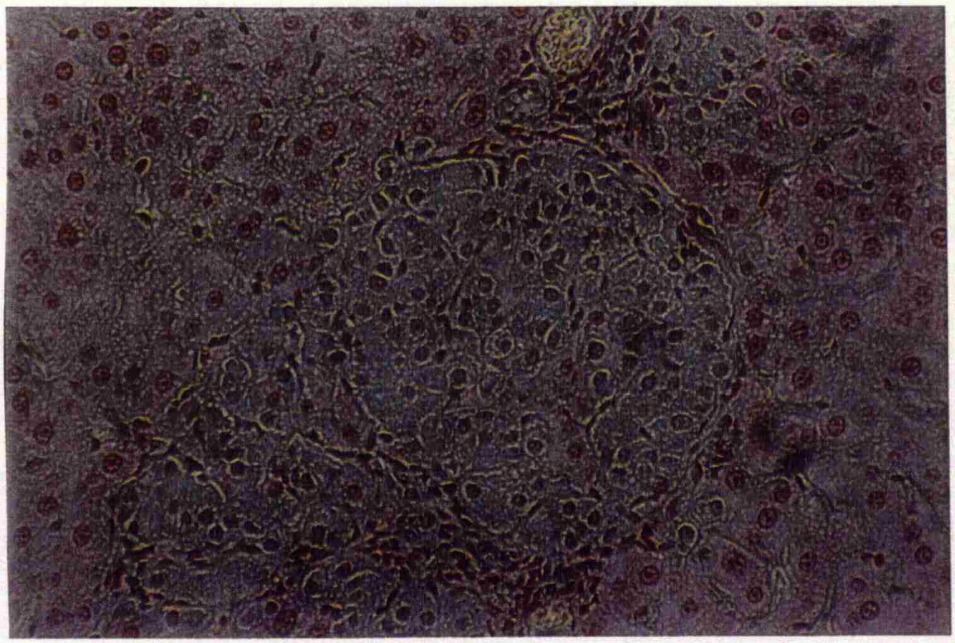

Plate 12: Transplanted islets stained for the presence of insulin using the ABC method (magnification x 20).

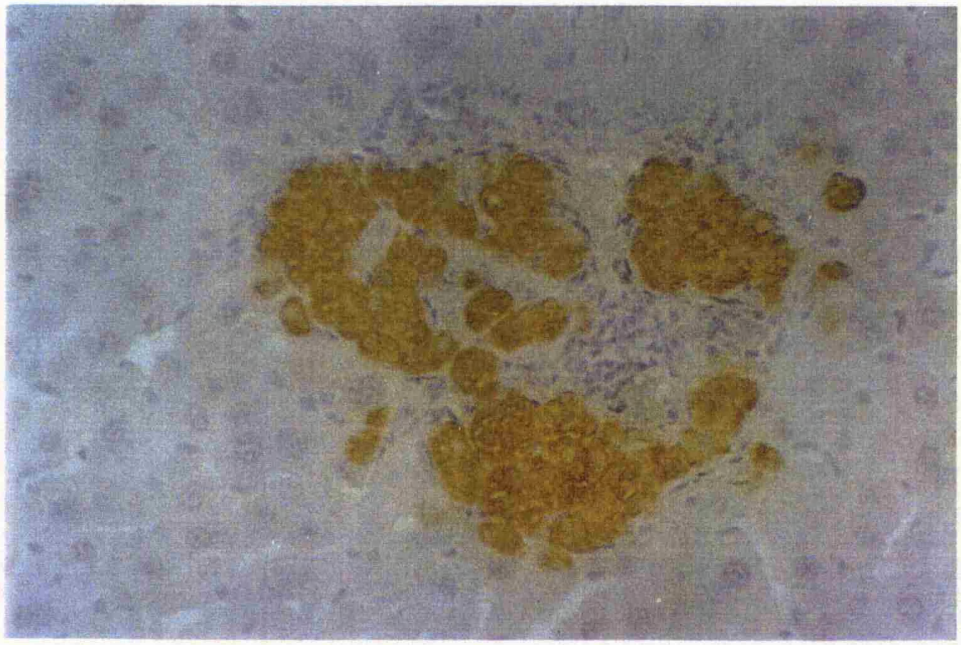

Table 25 Physical characteristics of STZ-induced diabetic rats (12-week study), insulin treated STZ rats, islet transplanted rats, and non-diabetic controls.

Group	Systolic B.P. (mmHg)	Body Weight (g)	Blood Pressure (mmHg)	Hwt./Tibia
Control	124±3 (4)	214±4 (10)	124±3 (4)	1.71±0.05 (10)
Untreated Diabetic	133±6 (8)	152±7*++ (8)	133±6 (8)	1.46±0.05+ (8)
Insulin Treated Diabetic	125±4 (7)	210±14 (8)	125±4 (7)	1.83±0.08 (8)
Islet Transplanted Diabetic	132±2 (8)	187±7 (7)	132±2 (8)	1.71±0.06 (7)

Number of animals shown in brackets

* $p<0.05$ (unpaired t-test versus control)

+ $p<0.05$ (unpaired t-test versus control, insulin treated diabetic, and islet transplanted diabetic).

++ $p<0.01$ (unpaired t-test untreated diabetic versus islet transplanted diabetic).

Table 26 Morphological characteristics of vessels STZ-induced diabetic rats (12-week study), insulin treated diabetic rats, islet transplanted rats and non-diabetic controls.

Group	N = animals	Lumen Diameter (μm)	Media Thickness (μm)	Cross Sectional Area (μm^2)	Media:Lumen x 100 (%)
Control	12	250±6	12.64±0.67	11337±762	4.92±0.32
Untreated Diabetic	9	254±10	14.62±1.22	12419±1094	6.03±0.87
Insulin Treated Diabetic	8	257±7	15.14±1.1	12784±1001	6.15±0.55
Islet Transplanted Diabetic	8	261±9	14.1±0.5	12208±396	5.48±0.33

Table 27 Maximum contractile response and sensitivity to noradrenaline before and after incubation with N^G-nitro-L-arginine (L-NOARG)(12-week STZ study).

Group	N = animals	NA Max (mN/mm)	+L-NOARG (mN/mm)	pD_2	pD_2+L-NOARG	Shift Factor(%)
Control	12	3.9±0.26	3.82±0.23	5.99±0.07	6.28±0.17	7.01±1.51 (10)
Untreated Diabetic	8	3.96±0.2	3.82±0.19	6.03±0.08*	6.27±0.07	5.19±1.02
Insulin Treated Diabetic	7	4.82±0.33*+	5.26±0.39	5.8±0.08*	6.25±0.07	7.69±2.33
Islet Transplanted Diabetic	7	4.38±0.23	4.33±0.24	6.04±0.08	6.27±0.08	4.89±1.29 (8)

*$p<0.05$ (paired t test before and after L-NOARG).

+$p<0.05$ (insulin treated diabetic versus control, untreated diabetic and islet transplanted animals)

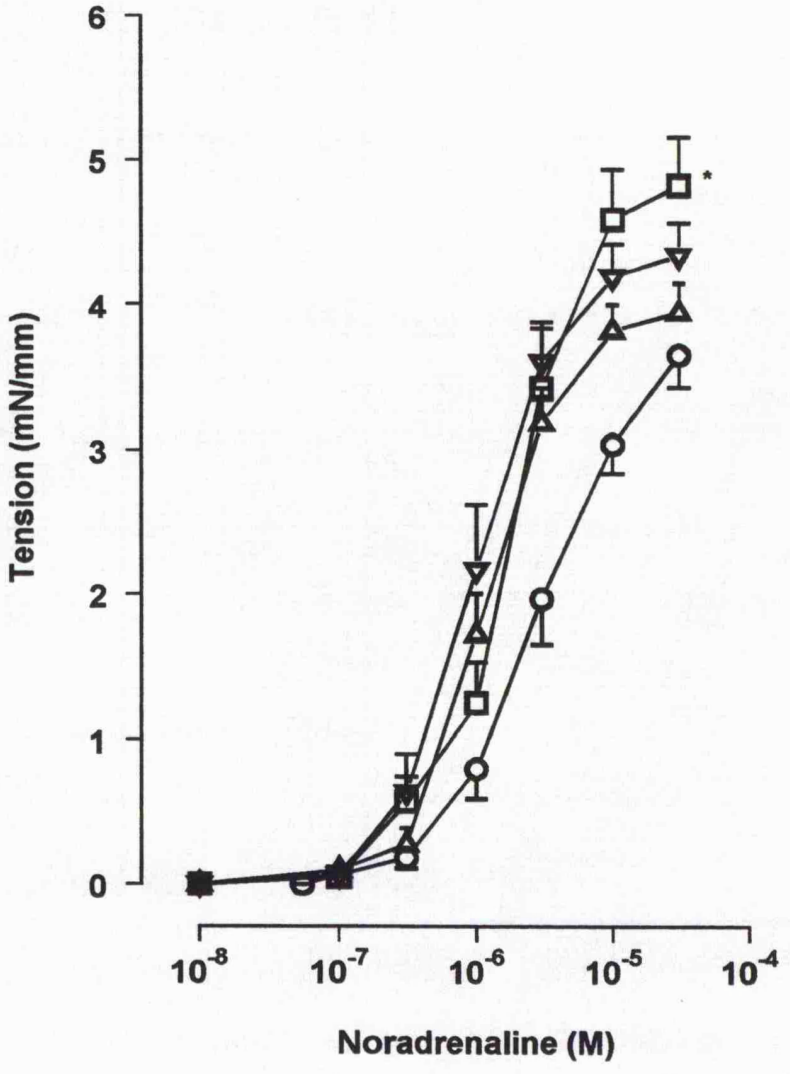

Figure 29 Noradrenaline contractile response of rat mesenteric resistance vessels (12-week study) from non-diabetic control rats (○), insulin treated diabetic rats (❑), untreated diabetic rats (▵), and islet transplanted rats (▿). (*$p<0.05$, insulin treated diabetic versus control, untreated diabetic and islet transplanted diabetic rats.)

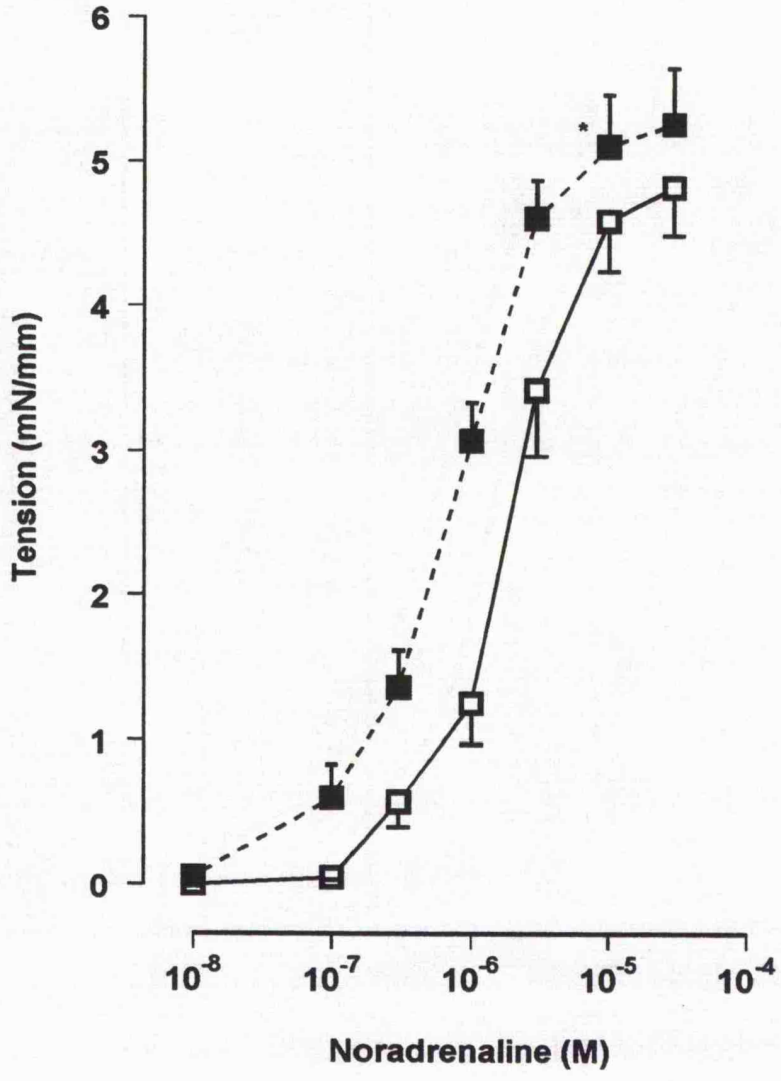

Figure 30 Noradrenaline contractile response of rat mesenteric resistance vessels (12-week study) from insulin treated rats before (❑) and after incubation with N^G-nitro-L-arginine (L-NOARG) (■) (*$p<0.05$ pD_2)..

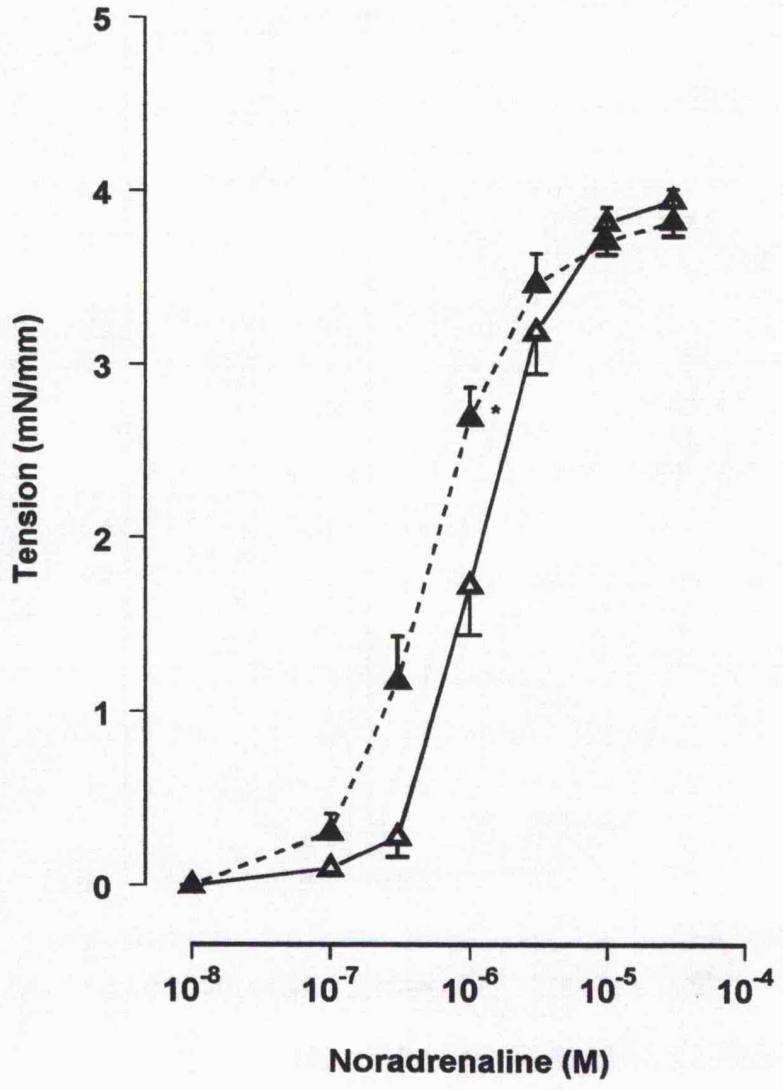

Figure 31 Noradrenaline contractile response of rat mesenteric resistance vessels (12-week study) from untreated diabetic rats before (△), and after incubation with N^G-nitro-L-arginine (L-NOARG) (▲, *$p<0.05$).

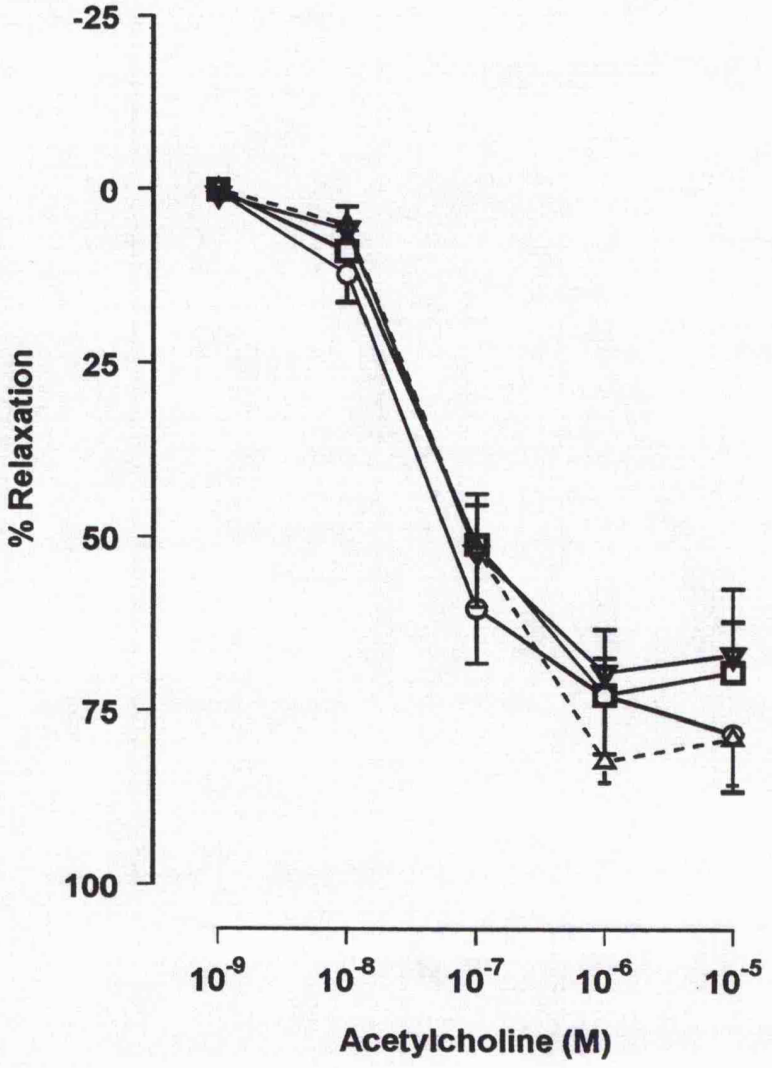

Figure 32 Acetylcholine-induced relaxation response of rat mesenteric resistance vessels (12-week study) from non-diabetic control rats (○), insulin treated diabetic rats (❑), untreated diabetic rats (▵), and islet transplanted rats (▿).

Table 28 Endothelium-dependent relaxation and sensitivity to acetylcholine before and after incubation with N^G-nitro-L-arginine (L-NOARG) (12-week STZ study).

Group	N = animals	ACh Max (%)	+L-NOARG (%)	pD_2	pD_2+L-NOARG
Control	10	86±3	34±8*	7.1±0.07	7.01±0.13
Untreated Diabetic	8	84±3	48±9*	7.1±0.1	7.03±0.2
Insulin Treated Diabetic	8	75±5	48±8*	7.15±0.11	7.09±0.1
Islet Transplanted Diabetic	7	72±7	39±11*	7.21±0.16	6.99±0.15

*$p<0.05$ (paired t-test before and after L-NOARG).

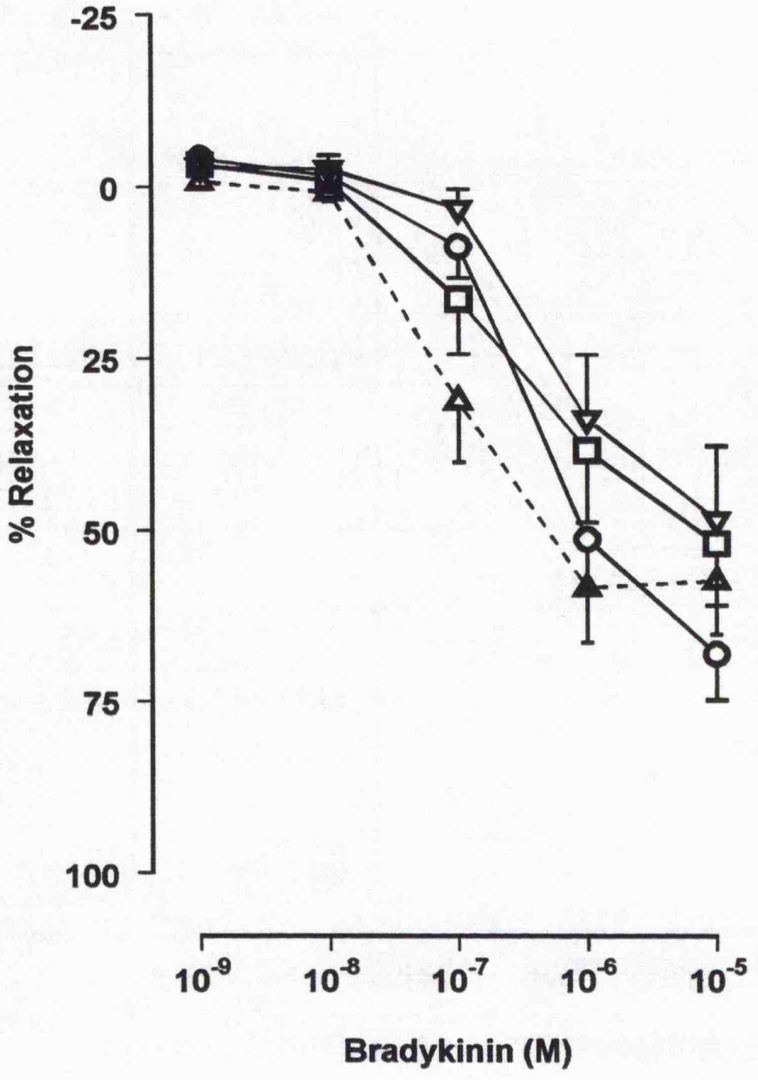

Figure 33 Bradykinin-induced relaxation response of rat mesenteric resistance vessels (12-week study) from non-diabetic control rats (○), insulin treated diabetic rats (❑), untreated diabetic rats (△), and islet transplanted rats (▽).

Table 29 Endothelium-dependent relaxation and sensitivity to bradykinin before and after incubation with N^G-nitro-L-arginine (L-NOARG) (12-week STZ study).

Group	N = animals	BK Max (%)	+L-NOARG (%)	pD_2	pD_2 +L-NOARG
Control	8	68±7	20±7**	6.34±0.07	5.92±0.11
Untreated Diabetic	8	63±7	25±7**	6.85±0.18	6.72±0.15
Insulin Treated Diabetic	8	56±9	27±10*	6.44±0.15	6.45±0.14
Islet Transplanted Diabetic	7	49±11	19±7*	6.37±0.16	6.53±0.34

*p<0.05, **p<0.01 (paired t-test before and after L-NOARG).

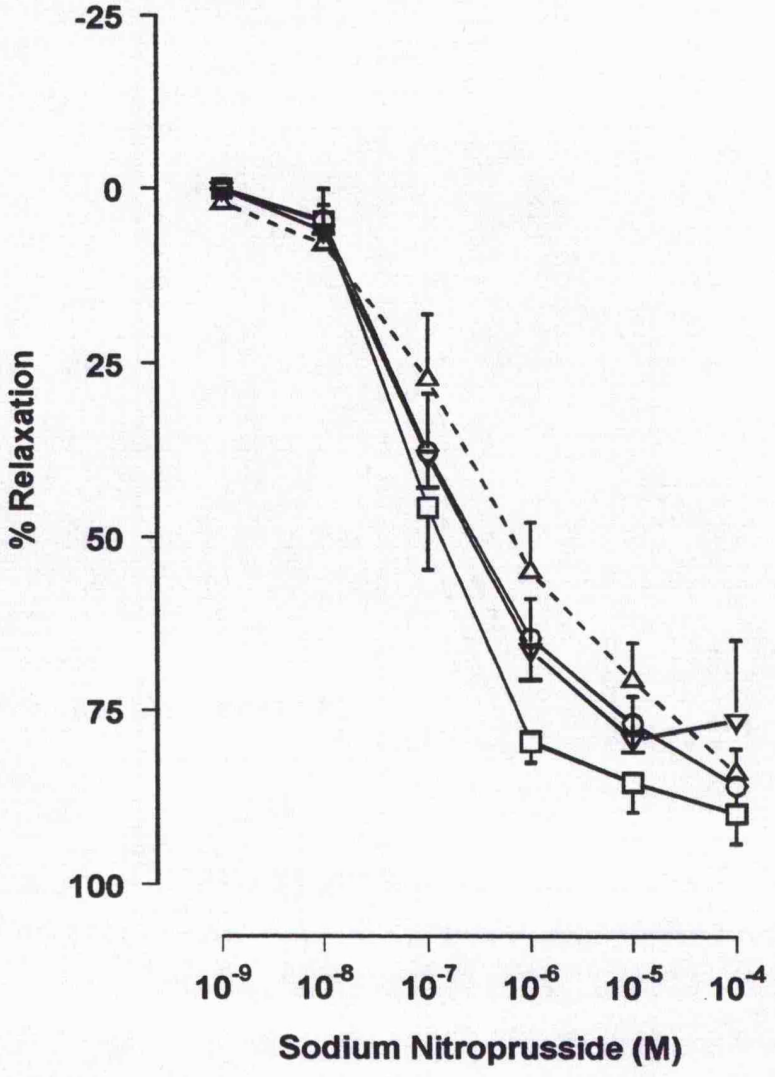

Figure 34 Endothelium-independent relaxation response to sodium nitroprusside of rat mesenteric resistance vessels (**12**-week study) from non-diabetic control rats (○), insulin treated diabetic rats (❑), untreated diabetic rats (△), and islet transplanted rats (▽).

6.6 Discussion

The results of these studies further support increased basal and stimulated release of nitric oxide in streptozotocin induced diabetic rats at both six and twelve weeks duration. Thus, incubation with the nitric oxide synthetase inhibitor N^G-nitro-L-arginine (L-NOARG) revealed a significant increase in contractility and sensitivity in the untreated diabetic vessels at 6 weeks, with increased sensitivity also displayed at 12-weeks. Insulin treated diabetic rats showed increased sensitivity at both 6 and 12 weeks with the 12-week insulin treated diabetics displaying enhanced contractility to noradrenaline. However, when the shift ratios within each of the diabetic groups were compared to the non-diabetic controls, no significant enhancement of the NA response after incubation with L-NOARG was noted in the experimental groups from either the 6-week or the 12-week study. Vascular reactivity to noradrenaline in the islet transplanted group remained unaltered, and was not significantly different to the controls. This is in agreement with the only other transplant study which has directly investigated the responses of rat blood vessels to contractile and relaxing agents (Pieper *et al.*, 1995).

In contrast to these findings, some groups who investigated aortic responses to noradrenaline reported that there was no significant difference in contractility in the diabetic vessels (Cameron & Cotter, 1992), especially when contractile force was related to tissue weight (Oyama *et al.*, 1986). However, other studies of rats with chemically induced diabetes have demonstrated an increased contractile response to noradrenaline in aortae (Mulhern & Docherty, 1989), mesenteric arteries (Agrawal & McNeill, 1987; Harris & MacLeod, 1988; White & Carrier, 1988), tail ventral arteries (Cuneo *et al.*, 1988), isolated perfused hindquarters (Friedman, 1989), and perfused kidneys (Bhardwaj & Moore, 1988). Another investigation of vascular reactivity of both aortae and mesenteric arteries in chronic experimental diabetes showed that the increased contractile response to noradrenaline was only evident in the untreated diabetics, whereas treatment with insulin completely prevented the increase (MacLeod, 1985). Similar findings were reported in a long-term study of diabetes where the aortae from untreated diabetic rats showed an increased sensitivity to the agonist phenylephrine, which was partially corrected in the insulin treated group (Chang &

Stevens, 1992).

In one study of resistance artery function, of similar experimental design to these studies, evidence of enhanced contractility to noradrenaline was revealed in both untreated and insulin treated diabetic rats, and similarly, incubation with a nitric oxide synthetase inhibitor (L-NAME) resulted in an increase in sensitivity to noradrenaline in the diabetic vessels (Taylor *et al*, 1994a). Likewise, the maximum contractile response to depolarising K^+PSS also was significantly increased in the insulin treated diabetic group.

A comparison of diabetic animal studies is difficult because of the variety of models, differing duration of diabetes and variety of vascular beds used. Studies involved are either *in vitro* or *in vivo,* and observations can be made where the same vascular bed is used (i.e. the mesenteric bed). Investigations of the mesenteric vascular bed have been carried out *in vivo* (Lash & Bohlen, 1991; Kiff *et al.*, 1991), and *in vitro* using isolated perfused mesenteric beds or isolated mesenteric resistance vessels mounted on a wire myograph (Taylor *et al.*, 1992, 1994a & b, 1995; Olbrich *et al.*, 1996).

Closer examination of these studies, (Taylor *et al.*, 1992, 1994a &b, 1995) revealed several significant discrepancies between their data and the results from my studies. There are clear differences in the strain & sex of rat used, the dose and route of administration of STZ, duration of diabetes, and degree of STZ- induced hyperglycaemia. All of these factors can influence the degree of diabetes which in turn will effect the response to vasoconstrictors and vasodilators. In addition, the region of the mesenteric bed from which the small arteries are dissected, also varied between studies, and it is possible that regional changes within the vascular bed could influence the overall vascular function. This has been demonstrated in an *in vivo* study of mesenteric vascular conductance of STZ-induced diabetic rats compared with non-diabetic control rats. These workers found that the resting mesenteric vascular conductance was elevated in the STZ group (Kiff *et al.*, 1991a). Also in another study by the same group, they reported evidence of marked hypertrophy of the mesentery in STZ-induced diabetes, and suggested that the alteration in vascularity may contribute to a change in mesenteric vascular conductances (Kiff *et al.*, 1991b).

Interestingly, in the 12-week study, contractile responses to the depolarising K^+PSS, and NAK^+ were significantly augmented in the insulin treated diabetic group alone, suggesting a chronic effect of raised exogenous insulin on the contractile mechanism of the vascular smooth muscle. In this study, the insulin treated rats received their last injection 24 hours previously, and so the plasma insulin levels in the freshly culled rat should be negligible. It is however possible that the insulin treated rats were hyperinsulinaemic and that this effect was carried over into the myograph bath. Indeed, other workers have also demonstrated a similar effect after *in vivo* treatment of diabetic rats with insulin (Taylor *et al.*, 1994a). On the other hand, one study of isolated mesenteric resistance arteries from STZ-induced diabetic rats have demonstrated a decrease in the vascular sensitivity to noradrenaline in vessels from rats treated *in vivo* with insulin, and that vessels taken from untreated diabetic rats tended to be more reactive than either the non-diabetic control vessels of those taken from the insulin treated rats (Savage *et al.*, 1995).

Moreover, an *in vivo* study showed that hyperinsulinaemia attenuated the vascular reactivity to phenylephrine and angiotensin II in the forearm resistance vessels of healthy humans (Sakai *et al.*, 1993); although in this particular study, only short-term effects of insulin were investigated in healthy, young subjects. Similarly, another study in healthy volunteers demonstrated that infusion of insulin into the femoral artery resulted in a significant increase in leg blood flow, and that this insulin-mediated vasodilation of skeletal muscle vasculature was endothelium-derived nitric oxide dependent (Steinberg *et al.*, 1994). As for the mechanism of the vasodilator action of insulin, a recent *in vitro* study of isolated rat aorta from normal rats demonstrated that insulin acts both on the vascular endothelium by increasing endothelial [Ca^{2+}] and releasing NO, which decreases smooth muscle [Ca^{2+}] as well as acting directly on the smooth muscle and causes a decrease in smooth muscle Ca^{2+} concentration (Han *et al.*, 1995).

However, because of the evident non-selectivity in the increased vascular reactivity of the insulin treated diabetics in the 12-week study to both K^+PSS and NA, it is possible that the responsiveness of mesenteric arteries from diabetic rats may be enhanced by two different

mechanisms: Noradrenaline is a non-selective α-adrenoceptor agonist, which activates both α_1, and α_2 receptors. In rat aortae as well as mesenteric arteries, noradrenaline is believed to cause an increase in cytoplasmic Ca^{2+} levels by increasing the influx of extracellular Ca^{2+} through receptor-operated channels, and by causing the release of intracellular Ca^{2+} (Scarborough & Carrier, 1984). As an increased reactivity to noradrenaline was seen in this study, it is possible that there could be a subsequent change in either the density or the affinity of the α-receptors in the blood vessels. The insulin treated diabetics in the twelve week study also showed enhanced vascular reactivity to the depolarising K^+PSS, as well as to noradrenaline, and so it is possible that the increase in Ca^{2+} influx could be due to the involvement of both potential-sensitive, and receptor-operated Ca^{2+} channels.

There was no evidence of impaired endothelium-dependent relaxation to acetylcholine or bradykinin in the untreated or the insulin treated diabetics of the 6 or 12 week study. This is in agreement with other studies which investigated the vasodilator response of the mesenteric vascular bed (Kiff *et al.*, 1991a), and the aortae (Head *et al.*, 1987; Taylor *et al.*, 1994b), of chemical induced diabetic rats. In our 6-week study, the maximum relaxation response to ACh was significantly *augmented* in the untreated diabetic group. This increased vasodilator response again suggests an increased release of nitric oxide, which appears to be absent in more established diabetes of longer duration (i.e. 12-14 weeks). Indeed, further evidence to support this is again provided by the incubation of the vessels with L-NOARG. In the presence of the nitric oxide synthetase inhibitor, the untreated diabetic vessels from the 6-week study failed to display such a marked reduction in endothelium-dependent relaxation as that of the insulin treated diabetic and control vessels. Another study of STZ induced diabetic rats (of 3 weeks duration) also has demonstrated an increased release of NO in the early stages of insulin-dependent diabetes. In this study the glomerular filtration rate was increased, as well as renal plasma flow, polyuria, and basal urinary NO_2/NO_3 excretion (Komers *et al.*, 1994) . These results along with findings from similar studies, therefore suggest that increased renal production and/or sensitivity to EDRF/NO may play a role in the genesis of diabetic hyperfiltration of early diabetes, and perhaps the increase in regional blood flow to other organs (Bhardwaj & Moore, 1988; Bank & Aynedjian, 1993; Komers *et al.*, 1994).

An alternative explanation for the increased relaxation response to ACh is the release of an additional relaxing factor, endothelium-derived hyperpolarising factor (EDHF). This has been shown to relax vascular smooth muscle cells by opening potassium channels and hyperpolarising the cell membrane (Van der Voorde *et al.*, 1992). It is possible that the simultaneous release of vasodilator eicosanoids could also be responsible for altering endothelium-mediated relaxation to acetylcholine in the diabetic blood vessels. Several studies have demonstrated an augmented release of prostacyclin from diabetic arteries, and this increased production, coupled with its synergistic action on EDRF may play a role in mediating endothelium-dependent relaxation in certain diabetic vascular beds (White & Carrier, 1986).

In keeping with contractile agonists, the literature on the responses to endothelium-dependent vasodilators is inconsistent; with increased relaxation being reported in one study (White & Carrier, 1986), and a decrease in sensitivity, but not maximum relaxation being reported in another (Kamata *et al.*, 1989). In contrast, other investigations of endothelial-dependent responses in chemical induced diabetes, have demonstrated *attenuation* of endothelium-dependent relaxation to ACh in both mesenteric arteries (Taylor *et al.*, 1994b) and aortae (Oyama *et al.*, 1986; Cameron & Cotter, 1992) from diabetic rats.

However, acetylcholine is not an important physiological mediator of endothelium-dependent relaxation *in vivo*, but the influence of shear stress on the subsequent release of NO has been directly linked to the same G-protein as ACh (Ohno *et al.*, 1993). It is therefore possible to extrapolate from these findings, and suggest that flow-induced NO release could be abnormal in diabetes, and may have important implications in the determination of peripheral tone. Indeed, increased blood flow has been shown in the early stages of insulin-dependent diabetes mellitus in man, resulting in precapillary vasodilatation (Jaap & Tooke, 1995). One *in vivo* animal study which compared regional blood flow in different vascular beds in rats, which had been diabetic for four weeks, reported a significant increase in blood flow to the mesenteric bed, whereas blood flow to other abdominal and thoracic organs was unchanged, and those to skin and muscle was reduced (Hill & Larkins, 1989). Moreover, increased renal and mesenteric blood flow has been demonstrated in conscious, chronically

instrumented rats following streptozotocin treatment (Kiff *et al.*, 1991a, b).

The relaxation response to the endothelium-independent vasodilator sodium nitroprusside, was similar in all the experimental groups irrespective of the duration of diabetes. The literature reveals conflicting reports about alterations in the SNP response in diabetes. The majority of studies show that the SNP response is not impaired in diabetics versus controls (Oyama *et al.*, 1986; Mulhern & Docherty, 1989; Pieper *et al.*, 1995; Taylor *et al.*, 1995a,b), but one study showed a decrease in relaxation to SNP in diabetic arteries, (Kiff *et al.*, 1991a). Similarly, in man some forearm studies in insulin-dependent diabetic subjects, have shown normal responses to SNP (Elliott *et al.*, 1993; Johnstone *et al.*, 1993; McNally *et al.*, 1994), whilst others have demonstrated an impaired response (Calver *et al.*, 1992).

The transient changes in reactivity observed in these diabetic animal and human studies, suggest that complex alterations in vascular function develop in the early stages of diabetes, and that these changes continue to be modulated by other intrinsic or extrinsic factors that come into play as the disease progresses (Kobbah *et al.*, 1988; Katz & McNeill, 1987). Since the arterioles are the major site of vascular resistance, small changes in arteriolar sensitivity could have a significant effect on blood pressure, and the regulation of tissue blood flow (Morff, 1990).

Several studies of islet cell transplantation in diabetic rats have been performed demonstrating the potential to reverse the diabetic state and associated microvascular complications (Mauer *et al.*, 1975; Finch *et al.*, 1977; Trimble *et al.*, 1980; Schmidt *et al.*, 1983; Orloff *et al.*, 1987; Fan *et al.*, 1990). One such study reported that the development of nerve fibre abnormalities could be prevented if islet cells were transplanted soon after the onset of diabetes. However, where islet transplantation was delayed, abnormal fibre morphology occurred, and complete reversal of the diabetic neuropathy was not achieved despite consistent euglycaemia (Britland *et al.*, 1991).

More recently, a study has shown that transplantation of an adequate mass of pancreatic islet tissue to the liver or beneath the kidney capsule, and the subsequent long-term reversal of

hyperglycaemia, can prevent the development of glomerular basement membrane thickening found in untreated diabetic animals (Leow *et al.*,1995). Interestingly, another recent study demonstrated that pancreatic islet transplantation not only restored euglycaemia, but also reversed the endothelial dysfunction in rat aortae. Moreover, this group showed islet transplantation completely normalised indices of oxidative stress in various blood vessels even after eight weeks of diabetes; and since oxidising free radicals are known to have a detrimental effect on endothelial function, this improvement must be considered to be an additional benefit of islet transplantation (Pieper *et al.*, 1995).

In conclusion, it can be suggested that in the early stages of chemical-induced diabetes there was an enhanced release of nitric oxide, which was more evident in the untreated diabetic rats, than the insulin treated, and appeared to normalise as the duration of diabetes progressed. This study also uniquely showed that the alteration in vascular reactivity of the resistance arteries was restored within normal limits by the transplantation of islets of Langerhans. Islet transplantation also successfully corrected the metabolic abnormalities of the insulin dependent diabetic state, and did not appear to have any detrimental effects on the contractile and relaxation responses of the mesenteric resistance vasculature. Further studies of islet transplantation in diabetic rats with different lengths of disease duration are necessary to determine whether an end-point for the reversibility of the diabetic state, the amelioration of altered vascular reactivity or indeed, endothelial dysfunction exists.

CHAPTER 7

SUMMARY & CONCLUSIONS

7.1 The Effect of Hyper-and Hypoglycaemia on the Endothelium-Dependent Relaxation of Resistance Arteries from Normal Rats

i) In the first experimental chapter, acute exposure of normal rat mesenteric resistance arteries to hyperglycaemia caused an impairment of endothelium-dependent relaxation. This was marked in arteries incubated in PSS containing the highest concentration of glucose (40mM) where the maximum acetylcholine relaxation response was significantly impaired at 4 & 6 hours. No impairment to ACh relaxation was observed in vessels incubated in PSS containing 20mM glucose. The effect of 40mM glucose was not a result of the increase in osmolarity since no change in the acetylcholine response was observed in arteries incubated in PSS containing 4mM glucose + 36mM mannose.

ii) The relaxation response to bradykinin was not impaired by incubation in PSS containing 20mM glucose. Unfortunately, a dose response curve to bradykinin was not performed after incubation in PSS containing 40mM glucose. It is therefore not possible to predict whether the response would be impaired after exposure to a more hyperglycaemic medium.

iii) Incubation with the nitric oxide synthetase inhibitor L-NOARG attenuated acetylcholine-induced relaxation in vessels incubated in PSS containing 4mM glucose, 4mM glucose + 16mM mannose, and 20mM glucose. However, a complete blockade of endothelium-dependent relaxation did not occur. This may be the result of incomplete inhibition of nitric oxide synthetase, or implicate other mechanisms for ACh-mediated relaxation, i.e. the release of prostacyclin or the endothelium-derived hyperpolarising factor (EDHF). The maximum relaxation response to bradykinin was not significantly changed by incubation with L-NOARG, suggesting that the relaxation response involves the release of other vasodilator substances.

iv) Incubating vessels in a hypoglycaemic medium (PSS containing 2mM glucose) had no deleterious effects on the endothelium-dependent relaxation responses to acetylcholine, bradykinin, or the endothelium-independent relaxation to sodium nitroprusside. This suggests that acute hypoglycaemia *per se* has no significant effect on the

endothelium function or the vascular smooth muscle second messenger system in resistance arteries.

7.2 The Effect of Diabetes Duration and Insulin Treatment on Resistance Artery Structure & Function in the BioBred Rat

i) Studies of resistance arteries from the spontaneously diabetic Bio Bred rat at the onset of diabetes and after insulin treatment of 6-8 weeks revealed a significant increase in the media wall:lumen ratio, as well as increased cross sectional area and media thickness compared to their age-matched non-diabetic controls. The blood pressure of the diabetic rats was not significantly different from the non-diabetic controls. The presence of significant morphological change in the recent onset diabetic BB rats indicates that vascular alterations were taking place before the clinical onset of insulin-dependent diabetes.

ii) Inhibition of nitric oxide synthetase with L-NOARG caused a significant left-ward shift in the dose response curve to noradrenaline in vessels from the recent onset diabetic vessels, but not the insulin treated. However, the shift demonstrated in the recent onset diabetic vessels was not as marked as that observed in the age-matched control vessels. This suggests there was a reduction of basal EDRF release in the recent onset diabetics, with a marked impairment of basal EDRF release in the long term insulin treated diabetics.

iii) The endothelium-dependent relaxation response to acetylcholine and bradykinin was significantly impaired in both the recent onset and the insulin treated diabetic BB rats. Incubation with L-NOARG caused a partial reduction in the maximum endothelium-dependent relaxation response to acetylcholine and bradykinin in both the diabetic groups and their age-matched controls, suggesting that EDRF only partly mediates the relaxation response to ACh and BK in these vessels.

iv) Incubation with the nitric oxide synthetase substrate, L-arginine failed to restore the impaired ACh relaxation response in the diabetic vessels, indicating that the impaired response was not due to lack of substrate *per se*. Incubation with the free radical scavengers

superoxide dismutase and catalase also failed to improve the relaxation response, suggesting that excessive destruction of nitric oxide by free radicals was not the explanation of impaired relaxation. However, these studies do not exclude a role for endothelial cell membrane damage by lipid peroxidation, and *in vivo* studies of the effects of treatment with free radical scavengers are required to resolve this issue.

v) Pretreatment with a cyclo-oxygenase inhibitor, indomethacin produced a significant improvement of acetylcholine-induced relaxation in both the recent onset and insulin treated diabetic vessels. This suggests that the endothelial abnormality may involve an increase in vasoconstrictor prostanoid production which over-rides nitric oxide-mediated relaxation. However, incubation with the thromboxane A_2 and prostaglandin H_2 receptor antagonist SQ 29548 failed to improve the impaired ACh relaxation response, indicating that the contracting factor is not TxA_2 or PGH_2 and the exact identity of the vasoconstrictor remains undetermined.

7.3 The Effect of Either Insulin Treatment or Islet Transplantation on the Resistance Artery Function in the Streptozotocin-Induced Diabetic Rat.

i) The third experimental chapter describes investigations of resistance artery structure and function in a different animal model of experimentally induced diabetes. Resistance arteries were examined after 6 and 12 weeks of streptozotocin-induced diabetes, and in 12-week STZ rats treated with insulin or intra-portal transplantation of islets of Langerhans. Resistance artery structure was similar in the diabetic groups compared to the non-diabetic age-matched controls in both the 6 week or the 12 week study. It appears therefore that up to 12 weeks of untreated chemical-induced diabetes causes no significant changes in the structure of the resistance arteries. This contrasts with the morphological findings of the spontaneously diabetic BB rat study, where there was evidence of vascular changes at the clinical onset of diabetes and after 6-8 weeks of insulin treated diabetes.

ii) Incubation with L-NOARG caused a significant increase in both the contractile response

and sensitivity to noradrenaline in the untreated diabetic vessels at 6 weeks, with only an increase in sensitivity at 12 weeks. The insulin treated diabetic rats showed an increase in sensitivity at both 6 and 12 weeks with an enhanced NA contractile response also demonstrated in the 12-week insulin treated diabetics. Vascular reactivity in the age-matched non-diabetic groups and the islet-transplanted group was unchanged. These results support the increased basal release of nitric oxide in streptozotocin-induced diabetic rats.

iii) In contrast to the findings of the spontaneously diabetic Bio Bred rat study, there was no evidence of impaired endothelium-dependent relaxation to acetylcholine or bradykinin in the untreated or the insulin treated diabetic rats of the 6 or 12-week study. Indeed, the maximum relaxation response to ACh was significantly augmented in the 6-week untreated diabetic rats. This increased vasodilator response again suggests an increased release of nitric oxide, which was absent in diabetes of 12-14 weeks. Indeed, further evidence to support this is provided by incubation of the vessels with L-NOARG. Inhibition of nitric oxide synthetase caused a significant inhibition of both ACh and BK maximum relaxation response in all of the experimental groups, although the untreated diabetic group (6-week study) failed to display such a marked reduction in endothelium-dependent relaxation as that in the insulin treated diabetic and control vessels. The augmented relaxation response may depend on an increased release of nitric oxide, but also there may be the release of an additional relaxing factor such as endothelium-derived hyperpolarising factor, or possibly due to the release of prostacyclin.

iv) The endothelium independent relaxation response to sodium nitroprusside was similar in both the untreated diabetics, and the insulin treated diabetics compared to the age-matched non-diabetic controls. Also, the relaxation response to SNP in the islet transplanted group was not different from that in the control vessels. Thus it appears that the smooth muscle second messengers systems are not affected by chemical-induced diabetes regardless of whether the diabetic animal is untreated, insulin treated or receives a transplantation of islets.

v) This study showed that resistance artery vascular reactivity of the 12-week diabetic rats

could be restored to normal by the islet transplantation; and moreover islet transplantation is an effective strategy for the correction of the metabolic abnormalities associated with experimental-induced insulin-dependent diabetes. However, islet transplantation to reverse the diabetic state was performed after only three weeks of diabetes. Further studies of islet transplantation after longer periods of diabetes are required to establish whether there is an end-point for the reversibility of the diabetic state and the abnormal vascular reactivity.

Conclusions

The experimental findings described in this thesis highlight endothelial dysfunction as a feature of diabetes. In the spontaneously diabetic BioBred rat this leads to an impaired response to acetylcholine which depends on an alteration in the balance between the release of EDRF and vasoconstrictor prostanoids. The endothelial abnormalities take on a different form in the experimentally induced STZ diabetic rat with enhanced EDRF release in the early phase of diabetes, and curiously, this was associated with an increased contractile response to noradrenaline. There were differences between the two diabetic models with evidence of vascular hypertrophy in the BioBred rat, but no such changes were evident at either 6 or 12 weeks after the experimental induction of diabetes. The use of two different animal models of insulin-dependent diabetes has both highlighted the advantages and limitations of animal experimentation. None the less, findings described in this thesis support the view that vascular endothelial dysfunction may be an early and relevant feature in the pathogenesis of diabetic microangiopathy.

APPENDICES

APPENDIX A Equipment & Supplies

Unless otherwise stated all equipment was obtained from suppliers within the United Kingdom.

Myograph (model 400A)	JP Trading, Aarhus, Denmark
Kistler Morse transducers	JP Trading, Aarhus, Denmark
Stainless steel wire (40μm diameter)	JP Trading, Aarhus, Denmark
Technical 2 Stereomicroscope	Carl Zeiss Jena Ltd.
Olympus stage light microscope	Olympus Ltd.
Filar micrometer eyepiece (x8)	Carl Zeiss Ltd.
Water immersion objective lens (x25)	Leitz Ltd.
Flat bed 2 channel recorder	Fisons Ltd.
Heidolph T50 water heater & circulator	Scientific Industries Ltd.
Large water bath	Grant Instruments Ltd
Suction Pump	BDH Ltd.
5%CO_2/ 95% O_2	BOC Ltd.
Sutures	Ethicon Ltd.
Blood pressure equipment	IITC INC/Life Science Instruments, Woodland Hills, California.
Sphygmomanometer	Richardons Ltd.
3" opthalmic scissors (Hans Gueder)	Altomed Ltd.
Watchmaker forceps size 5	Richardsons Ltd.
Assorted dissecting instruments	Richardsons Ltd.
Araldite resin/ Coffin moulds	Agar Aids.

Chemicals

Acetylcholine chloride, bradykinin, cocaine hydrochloride, indomethacin, noradrenaline, sodium nitroprusside, L-arginine, superoxide dismutase, catalase, and L-NOARG were obtained from Sigma Chemical Company, Poole, Dorset. The thromboxane A_2 receptor

antagonist SQ 29548 was a generous gift from Bristol-Myers Squibb Pharmaceuticals.

APPENDIX B Solutions & Drugs

Physiological Salt Solution (PSS)

Compound	Molarity (mM)	g/L
NaCl	118	6.90
KCl	4.5	0.34
$CaCl_2$	2.5	0.37
$MgSO_4.7H_2O$	1.0	0.25
KH_2PO_4	1.0	0.14
$NaHCO_3$	25	2.10
Glucose	6.0	1.08

High Potassium Physiological Salt Solution

A solution of high potassium PSS was made by substituting the sodium chloride with an equimolar concentration of potassium chloride.

Compound	Molarity (mM)	g/L
KCl	122.5	9.26
CaCl2	2.5	0.37
MgSO4.7H2O	1.0	0.25
KH2PO4	1.0	0.14
NaHCO3	25	2.10
Glucose	6.0	1.08

10% Formalin

A solution prepared by diluting Formalin 40 (Formalin 100%) with distilled water.

RPMI 1640 medium without L-glutamine

Supplier: GIBCO BRL.

Stock solution in 500ml bottle.

Catalogue Number: 31870-025.

Manufactured by Life technologies Ltd. Paisley, Scotland.

To make 5x 100mls of RPMI for incubating islets

Add the following to 500ml bottle of RPMI medium without L-glutamine, (in a sterile hood).

Foetal Calf Serum	50mls
Penicillin/Strep	10mls
Pyruvate	1ml
2-mercaptoethanol	0.5ml
Hydrocortizone	0.5ml
Herpes buffer	10ml

Aliquot into 5x 100ml sterile bottles, & store in the fridge (4°C). Just before adding to islets as incubating medium, add 1ml L-glutamine to 100mls of RPMI medium. (The breakdown products of L-glutamine can affect the viability of the islets, and so this is why the L-glutamine is added to the media immediately prior to use.)

Bovine Serum Albumin Solution

Supplier: Advanced Protein Products Ltd., Brockmoor, West Midlands.

Osmolarity regulated: 300mMols/kg (Nominal).

Density 1.1g/ml (Nominal).

Catalogue Number: PS-280-50.

500ml stock solution. Store at 4°C.

To make up density gradients for preparation of islets

Using the stock bottle of 300mMols/kg, the following amounts of BSA and MEM were weighed out into sterile Duron bottles.

1.085g/cm3	1.069g/cm3	1.056g/cm3
21.585g MEM	47.385g MEM	68.355g MEM
128.415g BSA	102.61g BSA	81.645g BSA

The density of each solution was checked by squirting 2mls into a densitometer, and adjusted accordingly. (If the density reading was too high, more MEM would be added; too low, more BSA would be added.)

Collagenase

Supplied by: Serva

Lot Number: 03092C, or 13073C.

Stored at -20°C.

To make up collagenase for pancreas distension

Add to 100 mls of MEM in sterile hood:

1.5ml $Ca\ Cl_2$ (15mM)

10ml DNase (Serva DN25 0.2mg/ml).

Add 1ml MEM /1mg collagenase. (Approximately 20mg of collagenase is needed to distend three pancreata.)

Minimum Essential Medium-Eagle 500ml (Stock Solution 10x)

With Hanks' salts, without L-Glutamine and $NaHCO_3$.

Supplier: GIBCO BRL

Manufactured by: Life Technologies Ltd., Paisley, Scotland.

Catalogue Number: 21581-020.

To make up MEM + BSA

In a sterile hood, add to a new 500ml bottle of MEM 5mls of 35% BSA (300mmols/kg)

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