DIABETES AND THE MATERNAL RESISTANCE VASCULATURE

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Diabetes and the Maternal Resistance Vasculature

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Declaration

I declare that this thesis has been composed by myself, and that I have been responsible for the patient recruitment, tissue collection and laboratory studies, unless otherwise acknowledged.

I confirm that the contents in this thesis have not been submitted elsewhere for any degree, diploma or other professional qualification.

Christine Ang

Glasgow, July 2001

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Abstract

Diabetes is the most common endocrine disorder worldwide with complications that include the development of both macro- and micro-vascular disease contributing significantly to patient morbidity and mortality. The severity of diabetic complications is thought to be amplified during pregnancy, resulting in a higher incidence of adverse pregnancy outcomes such as pre-eclampsia, placental insufficiency and stillbirth. Vascular dysfunction is thought to underlie many of these complications with the greatest effect taking place at the level of the resistance vasculature, where even small alterations in vascular reactivity can significantly modify blood flow and tissue perfusion. It is likely that problems associated with diabetic pregnancies are related, in part, to abnormal vascular function particularly dysfunction of the vascular endothelium.

Healthy pregnant women develop progressive insulin resistance and hyperinsulinaemia as a result of physiological changes in carbohydrate metabolism. Although pathological insulin resistant conditions such as type 2 diabetes and obesity are associated with abnormal vascular function, similar changes may not be demonstrable in the physiological insulin resistance of normal pregnancy. The published literature on the vascular effects in pregnancy and diabetes has been extensively reviewed, with emphasis on how modifications in the synthesis, release and action of endogenous vasoactive substances may contribute to clinically demonstrable pathology. The varying effects of diabetes on different vascular beds, in terms of both vessel size and location, reflecting the complexity of disease development has been well documented. However, the effect of pregnancy

superimposed on pre-existing type 1 diabetes has never been fully explored. Questions still persist regarding the importance of insulin itself as a direct vasodilator, its mechanism of action, and how this may alter in the insulin-resistant state of pregnancy. There has been little published literature on vascular function in pregnant women with type 1 diabetes mellitus, yet a greater understanding of the role of endothelium-dependent factors involved in the control of vascular tone is important in both healthy and diabetic pregnancies, since they are generally considered to be altered in comparison with non-pregnant subjects. The importance of endothelium-dependent hyperpolarization in human subcutaneous resistance arteries has never been investigated in either diabetes or pregnancy, while the implicit importance of endothelin-1 in these conditions requires further investigation. This thesis has examined these questions and has produced evidence that, in some cases, supports the consensus and, in others, offers new insight.

Those mechanisms most likely to be important in the control of vascular function in pregnancy have been examined here using small vessel wire myography, a laboratory-based *in vitro* technique that allows the study of isolated small resistance arteries. The direct vasodilator effect of insulin was investigated (Chapter 3) providing the first demonstration of an endothelium-independent, insulin-mediated attenuation of vascular tone in healthy pregnancy. The role of the vascular endothelium in pregnant women with and without diabetes was also examined, with particular emphasis on endothelium-dependent relaxing factors. These data provide the first confirmation that pregnant women with type 1 diabetes mellitus do not demonstrate impaired endothelial function generally considered to be a correlate of the diabetic condition, and goes on to provide some support that, in this study

sample at least, this may reflect the level of glycaemic control (Chapter 4). Furthermore, the study shows that a non-nitric oxide-dependent endothelial hyperpolarization, and not nitric oxide *per se*, contributes largely to endothelium-dependent relaxation in subcutaneous resistance arteries obtained from pregnant women whether diabetes is present or not (Chapter 4).

As well as vasodilator mechanisms, alterations in vasoconstrictor control can be implicated in a variety of vascular pathologies, including those associated with diabetes. Along with its vasodilatory action, insulin is also thought to potentiate the release and activity of the potent vasoconstrictor endothelin-1, which may be an important contributory factor in diabetic vascular disease. Endothelin-1 is a major determinant of vascular tone and has been shown to be associated with disorders of pregnancy, such as pre-eclampsia. Examination of endothelin responses in this study revealed interesting differences depending on the presence of pregnancy or diabetes. A significant increase in the maximum vasoconstriction response to endothelin-1 with no change in sensitivity was observed in pregnant compared to non-pregnant women. This contrasted with a significant reduction in sensitivity but no change in potency (maximum effect) in pregnant women with type 1 diabetes compared to healthy pregnant women. These observations clearly point to the perturbation of different aspects of endothelin-dependent vasoconstriction mechanisms being associated with pregnancy and diabetes (Chapter 5).

Another aspect of metabolic control that is sensitive to both pregnancy and diabetes is lipid metabolism. Pregnancy can potentially exacerbate the abnormal lipid patterns associated with diabetic patients. Altered lipid profiles can inactivate

endogenous vasodilatory substances, further increasing the risk of endothelial and end organ damage. Plasma lipid levels in a small number of pregnant women with and without diabetes were assessed and correlated with the *in vitro* assessments of vascular function. Plasma levels of triglycerides, total- and low-density lipoprotein (LDL)-cholesterol were increased in pregnant compared to non-pregnant women, while plasma high-density lipoprotein (HDL)-cholesterol levels remained unchanged. However, the co-existence of diabetes during pregnancy did not appear to enhance this effect (Chapter 4).

Together these data suggest that insulin, acting via an endothelium-independent mechanism, is an important vasodilator in itself. This effect of insulin is not altered in the physiological insulin resistant state of pregnancy. Although underlying diabetes does have some effect on the pregnant mother, fears of endothelial dysfunction leading to damaging vascular disorders are probably unfounded in well-controlled diabetics. Pregnancy *per se* probably does not increase this risk. The observation that endothelial hyperpolarization, rather than nitric oxide, appears more important in the endothelial control of vascular tone in small arteries whether pregnant or diabetic, supports a very recent growing body of evidence appearing in the literature. That endothelin-1 responses can be differentially affected by diabetes and pregnancy has never been demonstrated before, and requires further studies to understand the mechanisms at work.

In summary, although recent research has focused on therapeutic interventions aimed at either restoring or arresting endothelial dysfunction, a real understanding of these proposed vascular changes is required. This thesis provides important

insight into the interactions between diabetes and pregnancy and points to those areas where further insight and studies are required.

Chapter One

The Effect of Diabetes and Pregnancy on Vascular Function

1.1 Introduction

Diabetes mellitus is the most common endocrine disorder affecting approximately 30 million people worldwide. It is characterised by hyperglycaemia resulting from either a deficiency in the production or a decreased effectiveness of insulin, the main hormone involved in the regulation of plasma glucose required for cell metabolism. The three main types of diabetes mellitus seen in pregnant women are type 1 (insulin-dependent) diabetes, type 2 (non-insulin-dependent diabetes) and gestational diabetes. Type 1 or insulin-dependent diabetes mellitus is due to an inability to produce adequate amounts of insulin and occurs most often in younger adults. The majority of diabetics, however, suffer from type 2 diabetes, where there is a reduced efficacy of insulin. However, as this is more prevalent in older patients, it is seen with relatively less frequency during pregnancy. Gestational diabetes refers to women who are shown to be diabetic for the first time during pregnancy, with glucose tolerance reverting to normal post-partum. Women who suffer from gestational diabetes have a higher incidence of developing type 2 diabetes in later life.

Diabetes mellitus is associated with significant morbidity and mortality, principally due to the development of micro- and macro-angiopathy, increasing the risk of cardiovascular disease, stroke, peripheral vascular disease, retinopathy and nephropathy (Brownlee and Cerami, 1981; Merimee, 1990; Zatz and Brenner, 1986). It is estimated that around 3.5 per 1000 pregnancies are affected by diabetes, corresponding to approximately 235 women in Scotland each year (Scottish Intercollegiate Guidelines Network, 1996). Pregnant women with diabetes have an

increased risk of adverse pregnancy outcomes such as pre-eclampsia, macrosomia and stillbirth, while those with pre-existing retinopathy and nephropathy may experience rapid deterioration of these conditions (Garner *et al.*, 1990; De Swiet, 1996). Vascular changes, and in particular, endothelial dysfunction, are thought to underlie many of these complications, with the greatest effect seen at the level of the resistance vasculature where alterations in vessel reactivity can significantly alter blood flow and tissue perfusion (Roberts and Redman, 1993; McCarthy *et al.*, 1993).

1.2 Normal vascular function

Normal vascular function depends on the integrity of interactions between the endothelium and underlying smooth muscle, and is generally subdivided into those affecting the endothelium, referred to as endothelium-dependent, and those affecting the underlying smooth muscle, referred to as endothelium-independent.

The resistance to blood flow within the circulation is dependent upon the properties of the blood vessels and the blood. Although vessels of all sizes can be shown to contribute to a greater or lesser extent to resistance in the vascular bed, it is the smaller arteries (<500μm) that are most important in regulating capillary pressure and blood flow through changes in vascular tone and concomitant alterations in vessel diameter (Andersson *et al.*, 1985; Furness and Marshall, 1974). The control of peripheral vascular tone is dependent upon various vasoactive factors, some of which are synthesised and released by the endothelium.

The endothelium is a single layer of cells that lines the lumen of the entire vascular tree and expresses receptors for circulating hormones (catecholamines, angiotensin and vasopressin), and autocoids (bradykinin, serotonin and acetylcholine) (Vallance, 1992; Furchgott, 1984). Following activation by various stimuli, these receptors mediate a wide range of cellular responses based on different signal transduction mechanisms including receptors that:

a) Affect membrane electrical activity and intracellular calcium concentration by influencing transmembrane 'channels'
b) Regulate cyclic adenosine monophosphate (cAMP) formation via adenylate cyclase
c) Regulate cyclic guanosine monophosphate (cGMP) formation via guanylate cyclase
d) Affect both cytosolic calcium concentration and protein kinase C activity by influencing the metabolism of membrane phosphatidyl inositol
e) Possess protein kinase activity
f) Involve a combination of these pathways

This thesis concentrates mainly on those pathways involving receptor-operated channels and the cAMP and cGMP second messenger systems.

The endothelium also has autocrine and paracrine effects, and is able to mediate vascular responses through the synthesis and release of various vasodilators and vasoconstrictors that act on the underlying vascular smooth muscle.

1.2.1 Endothelium-dependent vasodilators

The endothelium is thought to release at least 2 vasodilator substances –nitric oxide (NO) and prostacyclin (PGI_2). A further possible 'factor' is the putative endothelium-derived hyperpolarizing factor (EDHF), which is still to be identified and is the subject of current research (Fig. 1.1).

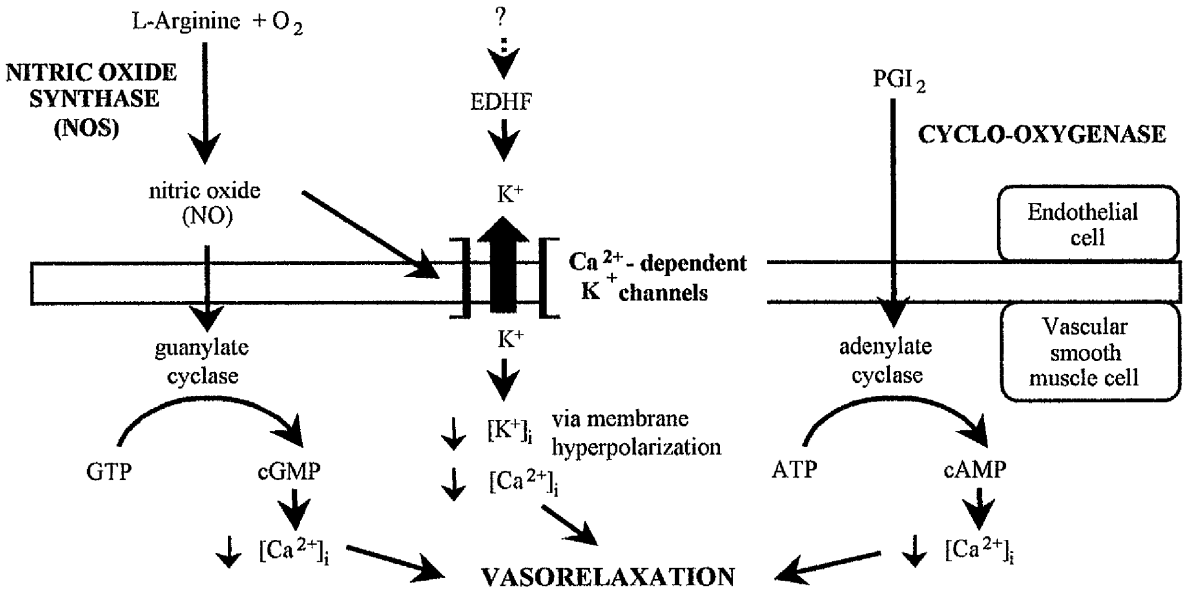


Fig. 1.1 Mechanisms of action of the endogenous endothelium-dependent vasodilators –nitric oxide (NO), prostacyclin (PGI_2) and endothelium-derived hyperpolarizing factor (EDHF). *GTP*, guanosine triphosphate; *cGMP*, guanosine 3':5' cyclic monophosphate; *ATP*, adenosine triphosphate; *cAMP*, adenosine 3':5' cyclic monophosphate; $[Ca^{2+}]_i$, intracellular calcium concentration; $[K^+]_i$, intracellular potassium concentration.

Nitric oxide (NO)

Nitric oxide is a small, labile, diffusible diatomic radical consisting of a bond order of two and a half, and a single unpaired electron. It is a reactive radical with a half-life of approximately 6-8 seconds. Nitric oxide is synthesised within the endothelial cell from the guanidine-nitrogen terminal of L-arginine in combination with molecular oxygen, via the enzyme nitric oxide synthase (NOS). Nitric oxide synthase can exist in three different isoforms –Type I, Type II and Type III. The constitutive NOS (Type III) is the isoform present in healthy endothelial cells, and is calcium-dependent, responding rapidly to changes in intracellular calcium concentration.

Nitric oxide is a potent vasodilator that causes relaxation by diffusing across the endothelial cell and stimulating soluble guanylate cyclase within vascular smooth muscle cells. This, in turn, converts guanosine triphosphate (GTP) to guanosine 3':5' cyclic monophosphate (cGMP). Relaxation results from an accumulation of cGMP, which through the cGMP-dependent modification of several intracellular processes, reduces intracellular calcium and inhibits the contractile apparatus within the vascular smooth muscle cell. However, nitric oxide has also been shown to activate potassium channels via cGMP-dependent protein kinase (Robertson *et al.*, 1993; Taniguchi *et al.*, 1993), as well as directly activating single calcium-dependent potassium channels in cell-free membrane patches without requiring cGMP (Bolotina *et al.*, 1994). Nitric oxide synthesis can be competitively inhibited by analogues of L-arginine, such as N^{ω}-nitro-L-arginine methyl ester (L-NAME) and N^{ω}-nitro-L-arginine (L-NOARG). The significant contribution of nitric oxide

to the control of vascular tone has been demonstrated by studies that have shown marked vasoconstriction following nitric oxide inhibition (Vanhoutte *et al.*, 1995; Moncada *et al.*, 1991; Rees *et al.*, 1989; Vallance *et al.*, 1989).

Prostacyclin (PGI_2)

Prostacyclin is an eicosanoid produced primarily by endothelial cells via the enzyme, cyclo-oxygenase. In contrast to nitric oxide, prostacyclin causes relaxation of vascular smooth muscle by activating adenylate cyclase and increasing the production of adenosine 3':5' cyclic monophosphate (cAMP). In many vascular beds, the contribution of prostacyclin to endothelium-dependent relaxation is negligible, contributing to as little as 2% of the total relaxation in some cases (Coats *et al.*, 2001; Kublickiene *et al.*, 1997; McCarthy *et al.*, 1994). The relative contribution of prostacyclin can be reliably assessed by inhibiting cyclo-oxygenase with indomethacin (Coats *et al.*, 2001; Gerber *et al.*, 1998).

Endothelium-derived hyperpolarizing factor (EDHF)

Before the existence of nitric oxide was recognised, several studies showed that acetylcholine could, in fact, hyperpolarize vascular smooth muscle (Kitamura and Kuriyama, 1979; Kuriyama and Suzuki, 1978). In the late 1980s, following the discovery that endothelium-derived relaxing factor was nitric oxide, electrophysiological studies suggested that this hyperpolarization was via an 'endothelium-derived hyperpolarizing factor' distinct from nitric oxide and

prostacyclin (Palmer *et al.*, 1987; Komori and Suzuki, 1987; Chen *et al.*, 1988; Félétou and Vanhoutte, 1988).

The precise chemical nature of EDHF remains unknown, and there may be different EDHFs depending on the species and vascular bed studied. Its mediation of vascular relaxation appears to be entirely dependent on smooth muscle hyperpolarization. In human subcutaneous resistance arteries, EDHF has been shown to be insensitive to the combined effects of nitric oxide synthase and cyclo-oxygenase inhibition, and is thought to be a cytochrome P450 product of arachidonic acid (Coats *et al.*, 2001). In rat hepatic and mesenteric arteries, EDHF was reported to be potassium ion released via calcium-dependent potassium channels on the vascular endothelium, which then induced membrane hyperpolarization and vasorelaxation by activating inwardly-rectifying potassium channels and the Na^+/K^+-ATPases on the smooth muscle cells (Edwards *et al.*, 1998). However, other studies have disagreed with these findings. Lacy *et al.* reported that at physiological concentrations of potassium, potassium-induced relaxation was different from acetylcholine-mediated relaxation, indicating that exogenously-added potassium did not mimic EDHF release by acetylcholine, while Doughty *et al.* concluded that although both EDHF and elevated levels of extracellular potassium were able to induce vasorelaxation, they were characteristically different (Lacy *et al.*, 2000; Doughty *et al.*, 2000).

The failure to identify a single factor as EDHF has led to very recent suggestions that endothelial-derived hyperpolarization may be, simply due to the electrical propagation across myoendothelial cells (Coleman *et al.*, 2001a; Coleman *et al.*,

2001b; Sandow and Hill, 2000). Despite its unknown chemical nature, endothelial hyperpolarization has been shown to be important in the resistance vasculature, contributing to ~80% of endothelial relaxation in some vascular beds (Shimokawa *et al.*, 1996; Coats *et al.*, 2001). Furthermore, endothelium-dependent hyperpolarization appears to be able to mediate near normal relaxation following nitric oxide inhibition, and may be important in the regulation of vascular tone in diseases where nitric oxide production or activity is impaired (Kilpatrick and Cocks, 1994).

The effects of endothelial hyperpolarization can be inhibited either by changing the cell's membrane potential using a high (25mM) potassium solution (Gerber *et al.*, 1998), or through the combined effects of charybdotoxin and apamin, which inhibit both large and small potassium conductance channels and the resulting relaxation (Zygmunt *et al.*, 1997; Petersson *et al.*, 1997).

1.2.2 Endothelium-dependent vasoconstrictors

As well as vasodilators, the endothelium has also been shown to initiate vasoconstriction through the release of various diffusible substances known as the endothelium-derived contracting factors (Rubanyi and Vanhoutte, 1985). Various endothelium-derived contracting factors have been identified, and include endothelin-1, superoxide anions (which act by scavenging nitric oxide), endoperoxides and thromboxane A_2 (Rubanyi and Vanhoutte, 1986a).

Physical factors such as pressure and stretch stimulate the release of these substances, which also provide local regulation of vascular tone. Pathological states such as pre-eclampsia, which result in increased vascular tone and systemic blood pressure, may result from an imbalance between the production of endogenous vasodilators and vasoconstrictors.

Endothelin-1 (ET-1)

Endothelin-1 (ET-1) is one of the most potent endogenous vasoconstrictor polypeptides known, primarily synthesised and released by the vascular endothelium. It is a 21 amino acid peptide formed from a larger inactive 38 amino acid precursor, bigET-1, by enzymatic cleavage via the metalloprotease endothelin-converting enzyme-1 (ECE-1) (Webb, 1997). ET-1 is preferentially and predominantly released from the vascular endothelium, and has a major role in the regulation of vascular tone. The binding of ET-1 to its receptor is facilitated by a family of guanine nucleotide regulatory proteins, or G proteins (heterotrimeric proteins comprised of distinct α, β and γ subunits) (Warner *et al.*, 1989; Douglas *et al.*, 1995). The altered G protein then reacts with either an enzyme or an ion channel evoking appropriate responses within the cell.

There are two different receptors, ET_A and ET_B, to which ET-1 has binding affinities. ET_A receptors are found on the external surface of the vascular smooth muscle cell (Lüscher *et al.*, 1993), while ET_B receptors are predominantly found on the luminal surface of the endothelial cell, but may also preside in the vascular smooth muscle cell in some vascular beds (Clozel *et al.*, 1992). Functional data

suggests that two sub-types of the ET_B receptor exist, an ET_{B1} receptor that mediates vasorelaxation via endothelium-dependent nitric oxide release, and an ET_{B2} receptor responsible for mediating peptide-induced smooth muscle vasoconstriction (Warner *et al.*, 1989; Douglas *et al.*, 1995). Upon its synthesis and release from the vascular endothelium, ET-1 exerts an autocrine effect resulting in a transient vasodilatation mediated by endothelial ET_{B1} receptors. It also exerts a paracrine effect on adjacent vascular smooth muscle cells evoking a slower but more sustained contraction via both ET_A and ET_{B2} receptors (Fig. 1.2).

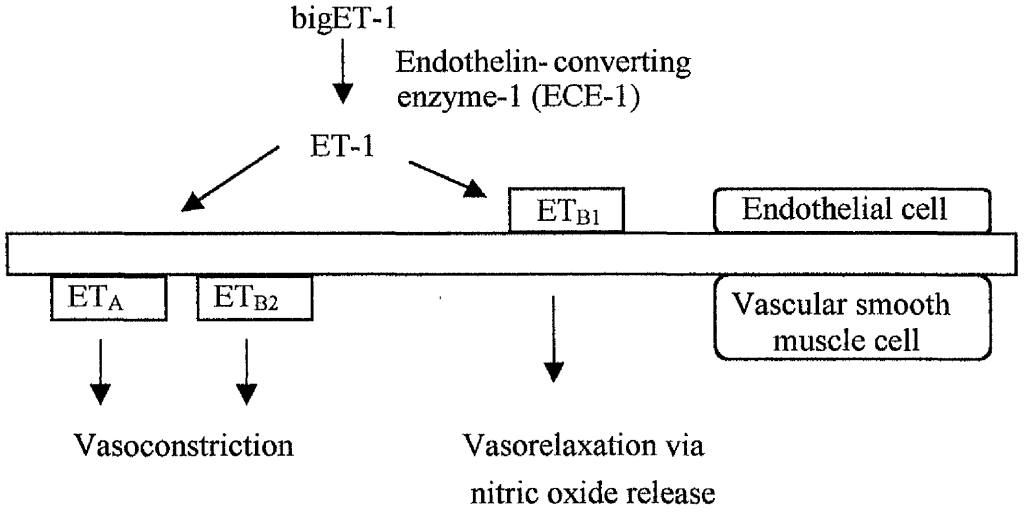


Fig. 1.2 The role of endothelin-1 (ET-1) in vascular reactivity.

Classically, the ET-1 ligand-receptor complex has been viewed as a modulator of vascular smooth muscle tone and mitogenesis, but is also known to alter smooth muscle function in non-cardiovascular tissues such as the respiratory, gastrointestinal and urogenital tracts (Bobik *et al.*, 1990; Douglas and Ohlstein, 1997). An understanding of its contribution to and importance in pregnancy

appears to be increasing, and is now thought to be involved in the modulation of placental blood flow, normal feto-placental development and in the pathogenesis of various pregnancy-related conditions, such as pre-eclampsia (Sabry *et al.*, 1995; Kohnen *et al.*, 1997; Kurihara *et al.*, 1994; Clouthier *et al.*, 1998).

In order to characterise the mechanisms at work in the regulation of vascular tone in any single experimental condition, it is often necessary to have a measure of both endothelium-dependent and endothelium-independent (and therefore smooth muscle-specific) effects. The analysis of independent smooth muscle function in conjunction with an analysis of endothelial function is necessary, so that a reliable interpretation of endothelial dysfunction will only be made if the underlying smooth muscle function is shown to be normal. Independent smooth muscle responses can be achieved by exposing the vessels to endothelium-independent agonists such as the nitric oxide donor, sodium nitroprusside.

1.3 Vascular function in diabetes

The endothelium controls vascular tone by synthesising and releasing various vasoconstrictors and vasodilators. It is likely that alterations in the synthesis of these endogenous endothelium-dependent factors may contribute to modifications in resting vascular tone and the ability of the vessels to respond rapidly to changes in metabolic requirements. This may underlie many of the complications commonly seen in conditions such as pregnancy-induced hypertension, diabetes,

cardiac and renal disease (Greer *et al.*, 1991; Greer *et al.*, 1985b; Wolff *et al.*, 1997; Maguire *et al.*, 1998; Thambyrajah *et al.*, 2000; Tomita *et al.*, 1989).

The effect of diabetes on vascular function appears to be a complex one, depending on the species and vascular bed studied. Numerous studies have provided evidence for impaired endothelium-dependent vasodilatation in patients with type 1 diabetes (Johnstone *et al.*, 1993; McNally *et al.*, 1994) and animal models with both genetic and chemically-induced type 1 diabetes mellitus (Tesfamariam and Cohen, 1992; Heygate *et al.*, 1995). However, others have also been able to demonstrate normal (McIntyre *et al.*, 2001; Smits *et al.*, 1993) and even enhanced (White and Carrier, 1986; Bhardwaj and Moore, 1988) vasodilatation. The importance of disease progression has been illustrated by one study carried out by a single investigator showing a triphasic response of increased, unaltered and impaired endothelium-dependent relaxation within the same model (Pieper, 1999). Although the interpretation of studies carried out on animal models cannot be directly transposed to humans, they provide a very important contribution to the understanding of diabetic pathophysiology.

Endothelium-independent vasorelaxation (ie. smooth muscle function) also appears to differ between different study populations, possibly either due to disease progression or to experimental methodology. Studies have shown both normal and reduced relaxation following exposure to sodium nitroprusside in patients with type 1 diabetes mellitus (McNally *et al.*, 1994; Calver *et al.*, 1992).

1.3.1 Factors influencing vascular function in diabetes

Although vascular function in diabetes can produce diverse observations, it is the impairment of endothelium-dependent vasodilatation that has been most implicated in the development of micro-angiopathy. Endothelial damage in diabetes arises from the increased likelihood of attack by the metabolic 'by-products' of diabetes (Poston, 1997). The mechanisms thought to be involved in the development of endothelial dysfunction in these patients include –a reduced synthesis or enhanced inactivation of the endothelium-derived relaxing factors; impaired diffusion of the relaxing factor to the underlying vascular smooth muscle; reduced sensitivity of the smooth muscle to the relaxing factor; or enhanced synthesis and release of endothelium-derived constricting factors (De Vriese *et al.*, 2000). However, it is more likely that a combination of these different mechanisms, rather than a single factor, accounts for the observable changes in vascular function. Numerous factors have been implicated as important determinants of vascular function, and include the age at diagnosis and duration of diabetes, the level of metabolic control, and the co-existence of lipid abnormalities.

Age of onset and disease duration

Several studies have shown a strong association between time-related risk variables, such as age at diagnosis and duration of diabetes, and the prevalence of diabetic complications (The EURODIAB IDDM Complications Study Group, 1994; Knuiman *et al.*, 1986). The onset of type 1 diabetes tends to occur at a younger age (compared to the other subtypes of diabetes), and patients therefore have a

relatively longer duration of disease, increasing the likelihood of micro-angiopathic sequelae in later life. A cross-sectional study carried out by Knuiman *et al.* on 179 insulin-dependent diabetics showed that age was the single most important time-related variable for the development of nephropathy, while both age at diagnosis and the duration of diabetes were significant risk factors for developing retinopathy and sensory neuropathy (Knuiman *et al.*, 1986). They reported that a 10-year increase in disease duration increased the prevalence of retinopathy by ~20%, while a 15-year increase in the duration of diabetes increased the prevalence to ~42%. Other significant risk factors for severe retinopathy and nephropathy include evidence of other micro-vascular damage, such as albuminuria and hypertension.

Level of glycaemic control

Hyperglycaemia, although not the only factor involved in the development of endothelial dysfunction, plays an important role in the pathogenesis of diabetic complications. Sustained episodes of hyperglycaemia resulting from chronic and poorly controlled diabetes can induce acute changes in intracellular metabolism and lead to the generation of oxygen-derived free radicals, which result in longer-term endothelial damage either by inactivating nitric oxide or by acting as vasoconstrictors (Rubanyi and Vanhoutte, 1986a; Katusic and Vanhoutte, 1989).

One of the most damaging aspects of chronic poorly controlled diabetes is the accelerated synthesis of advanced glycosylation end products (AGEs), formed by the non-enzymatic reaction between glucose and collagen (Brownlee *et al.*, 1988). AGEs reduce nitric oxide availability and impair endothelium-dependent relaxation

in experimental diabetes through the 'quenching' of nitric oxide (Bucala *et al.*, 1991). They also increase the susceptibility of low-density lipoproteins (LDLs) to free radical attack, promoting the formation of the oxidised LDLs, which are known to cause endothelial damage, inactivate nitric oxide and reduce nitric oxide synthesis (Schmidt *et al.*, 1991; Schmidt *et al.*, 1990).

Studies have examined the extent to which improved glycaemic control prevents the incidence or progression of retinopathy. One such study was a randomised controlled trial of strict versus conventional diabetic control (DCCT Research Group, 1990). This showed that strict control substantially reduced the risk of long-term diabetic complications, particularly retinopathy. The EURODIAB IDDM Complications Study also reported a decline in the frequency of background retinopathy and a coincident rise in proliferative retinopathy when glycosylated haemoglobin (HbA_{1c}) levels rose above 8%, which agreed with observations by Knuiman *et al.*, and Pirart (The EURODIAB IDDM Complications Study Group, 1994; Knuiman *et al.*, 1986; Pirart, 1978).

Plasma lipid profile

Diabetes is a risk factor for atherogenesis and vascular dysfunction related to the background population. Studies have demonstrated abnormal lipid patterns, such as elevated plasma levels of total and LDL-cholesterol in type 1 diabetics, resulting in both impaired endothelium-dependent and -independent vasodilatation (Briones *et al.*, 1984; Gibbons, 1986; Harris, 1991; Clarkson *et al.*, 1996). In fact, dyslipidaemia in diabetes has been shown to inactivate endothelial nitric oxide,

implicating it as one of the possible mechanisms by which vascular damage occurs (Bucala *et al.*, 1991; Schmidt *et al.*, 1991; Schmidt *et al.*, 1990; Jacobs *et al.*, 1990).

Hypertriglyceridaemia is often accompanied by a decrease in high-density lipoprotein (HDL)-cholesterol, and is the prominent lipoprotein defect in both treated and untreated diabetes (Abbate and Brunzell, 1990). Hypertriglyceridaemia and raised LDL-cholesterol concentrations are significant risk factors for ischaemic heart disease and directly related to serum glucose and HbA_{1c} levels (Eckel *et al.*, 1981).

1.4 Cardiovascular changes in healthy pregnancy

The changes that occur in the cardiovascular system during pregnancy are primarily to maintain adequate blood flow through the feto-placental unit, and to compensate for the inevitable maternal blood loss at delivery. Cardiac output and plasma volume increase by approximately 40% and are associated with vasodilatation in the mother to accommodate the increased circulating blood volume. These changes, apparent by the second trimester, result in a fall in mean arterial pressure, which then increases steadily to reach pre-pregnancy levels at term.

The underlying mechanisms responsible for these vascular changes remain largely unclear and unidentified (Pascoal *et al.*, 1995). One possibility may be alterations in signal transduction pathways (Learmont *et al.*, 1996), which may account for the reduction of pressor responses to various vasoconstrictor agonists (Nisell *et al.*,

1985; Gant *et al.*, 1973). The increased synthesis and release of various endothelium-dependent relaxing factors, such as nitric oxide and prostacyclin (Williams *et al.*, 1997; Greer *et al.*, 1985a), a reduction in the number of adrenergic receptors or a blunting of reflex cardiac responses (Fallgren *et al.*, 1988; Hart *et al.*, 1986) have also been implicated.

Studies have demonstrated the importance of nitric oxide in pregnancy, contributing substantially to increased peripheral vasodilatation (Williams *et al.*, 1997; Anumba *et al.*, 1999). McCarthy *et al.* also observed a moderate to substantial contribution of nitric oxide (~30%) to endothelium-dependent relaxation in healthy pregnant women (McCarthy *et al.*, 1994). Along with receptor-mediated responses, flow-induced shear stress both in the resistance vasculature and feto-placental circulation has also been shown to stimulate nitric oxide release (Kublickiene *et al.*, 1997; Myatt *et al.*, 1991). Using animal models, others have also demonstrated a significantly greater contribution of endothelial hyperpolarization to vascular relaxation in pregnant compared to non-pregnant rats (Gerber *et al.*, 1998; Dalle Lucca *et al.*, 2000).

The sensitivity of the smooth muscle to nitric oxide appears to remain unaltered in pregnancy, as vascular responses to sodium nitroprusside (nitric oxide donor) have been shown to be similar in both pregnant and non-pregnant women (McCarthy *et al.*, 1994; Knock and Poston, 1996). This suggests that changes in vascular reactivity in pregnancy are more likely to be due to alterations in endothelial rather than smooth muscle function.

1.5 Type 1 diabetes superimposed on pregnancy

In 1989, the St. Vincent Declaration stated "*the outcome of diabetic pregnancy should approximate that of the non-diabetic pregnancy*" (WHO, 1990). However, despite significant advances in obstetric and midwifery care, adverse pregnancy outcomes continue to remain more common in diabetic compared to non-diabetic women, with an increase in the prevalence of pregnancy-induced hypertension, congenital anomalies, macrosomia and the increased risk of shoulder dystocia, polyhydramnios and sudden fetal death.

The 1994-1996 Report on Confidential Enquiries into Maternal Deaths in the United Kingdom reported 2 indirect maternal deaths from diabetes mellitus and 2 fortuitous late deaths from diabetic ketoacidosis (HMSO, 1998). The 6th Annual Report (1996-1997) of the Confidential Enquiry into Stillbirths and Deaths in Infancy (CESDI) recorded 16 deaths in the offspring of diabetic mothers, and 18 deaths in the offspring of women with gestational diabetes (DOH, 1998). In Scotland, a national audit was carried out in 22 consultant-led maternity units between 1998-1999 aimed at assessing maternity care for women with type 1 diabetes (Penney and Pearson, 2000). Data from 268 pregnancies were collated and of those, 209 pregnancies progressed to delivery while 59 ended in either miscarriage or abortion. The perinatal mortality rate was 4/212 (19/1000), which resulted from two stillbirths and two neonatal deaths. A total of 19 fetuses were identified as having a congenital anomaly (such as sacral agenesis, extra digit, ambiguous genitalia, ventricular septal defect) and of those, 6 were aborted.

The vascular changes associated with type 1 diabetic patients may be further amplified during pregnancy, when physiological changes within the cardiovascular system occur. It is these alterations in vessel function at the level of the resistance vasculature, which are thought to underlie some of the associated complications of diabetic pregnancies (Roberts and Redman, 1993; McCarthy *et al.*, 1993).

1.5.1 Alterations in plasma lipid levels in diabetic pregnancies

Plasma cholesterol and triglyceride levels vary with age, pregnancy and the onset of the menopause, with the increase in triglyceride concentrations being primarily due to an increase in very low-density lipoprotein (VLDL) concentrations (Stevenson *et al.*, 1993; Potter and Nestel, 1979; Sattar and Greer, 1999). The literature suggests that mean high-density lipoprotein (HDL) cholesterol levels peak at 28 weeks gestation, and are significantly higher in pregnant compared to the non-pregnant women (Sattar *et al.*, 1996). These changes are thought to be associated with elevated oestrogen levels in pregnancy, which promote the hepatic synthesis of HDL-cholesterol and reduce hepatic lipase activity (Sattar and Greer, 1999; Desoye *et al.*, 1987), and are necessary in meeting the metabolic demands of the fetus and preparing the expectant mother for lactation.

The alterations in the plasma lipid profile observed during pregnancy may be further exacerbated in the presence of diabetes, which itself exhibits lipid abnormalities (Merzouk *et al.*, 2000; Harris, 1991; Gibbons, 1986). It is possible that some of the complications associated with diabetic pregnancies may be due to endothelial damage secondary to dyslipidaemia.

1.6 The effect of insulin on vascular function

During the course of normal human pregnancy, alterations in maternal metabolism result from elevated levels of human placental lactogen (hPL), oestrogen, progesterone, cortisol and prolactin. Of these, cortisol and hPL in particular, act as physiological insulin antagonists and cause a compensatory rise in plasma insulin levels and a state of maternal 'physiological insulin resistance' (De Swiet, 1996). The consequent hyperinsulinaemia contributes to the control of maternal euglycaemia and the level of circulating glucose to the developing fetus. In normotensive women, fasting levels of insulin in the third trimester of pregnancy have been reported to reach 114pM (Martinez *et al.*, 1998), compared to non-pregnant levels of 40pM (Kazumi *et al.*, 1999).

1.6.1 Insulin-mediated vasodilatation

Insulin is one of the principle hormones involved in the regulation of plasma glucose required for cell metabolism. It is a polypeptide synthesised from an inactive precursor, proinsulin, and stored within the β-cells of the pancreas. Along with its metabolic effects, insulin is also known to be a vasodilator, and have both renal and sympathetic actions (McNally *et al.*, 1995; DeFronzo *et al.*, 1975; Rowe *et al.*, 1981).

The majority of the available literature suggests that insulin-mediated vasorelaxation is via the activation of nitric oxide synthase and the subsequent release of endothelial nitric oxide (Trovati *et al.*, 1999; Chen and Messina, 1996).

However, the vascular effects of insulin have been shown to act not only via the endothelium, but the smooth muscle as well, involving several other mechanisms such as activation of the Na^+/K^+ pump via Na^+/K^+-ATPase (Ferrannini *et al.*, 1988), a reduction in Na^+ permeability compared to K^+ permeability (Williams, 1970), and/or by a non-specific β-adrenergic mechanism (Creager *et al.*, 1985). It is also possible that this may be secondary to either a direct or indirect action on Ca^{2+}-ATPase resulting in a reduction in intracellular calcium and vasorelaxation (Zemel *et al.*, 1992). These mechanisms result in the *in vivo* modulation of vascular tone, and can be observed *in vitro* as an attenuation of vasoconstriction to a variety of vasoconstrictor agonists (McNally *et al.*, 1995; Yagi *et al.*, 1988).

The effects of insulin have been shown to be complex and variable in nature depending on the state of insulin resistance and vascular bed studied, with a reduced effect seen in obese men (Laakso *et al.*, 1990) and patients with non-insulin-dependent diabetes mellitus (Laakso *et al.*, 1992). The vascular effects of insulin may therefore play an important role in human pregnancy, and an even more critical role in diabetic pregnancies, where alterations to both the vasodilatory and vasoconstrictive capacity of the endothelium are thought to exist.

Abnormal vascular function has been observed in pathological conditions associated with insulin resistance and longer-term angiopathic sequelae (Laakso *et al.*, 1990; Laakso *et al.*, 1992; Morris *et al.*, 1995). However, similar alterations in vascular function have not been demonstrated in physiological insulin resistant states such as pregnancy, which is not known to be associated with the increased risk of small vessel disease (McCarthy *et al.*, 1994; Ashworth *et al.*, 1996).

1.6.2 Other effects of insulin on the vasculature

Along with being able to mediate vasodilatation through nitric oxide synthesis or by stimulating smooth muscle directly, recent evidence also suggests that hyperinsulinamia accompanying insulin resistance may stimulate the endothelial production and secretion of endothelin-1 (Mather *et al.*, 2001; Frank *et al.*, 1993; Hu *et al.*, 1993). The interaction between the vasodilatory effect of insulin and its ability to stimulate endothelin-1 release may be of greater importance in pregnancy, when there is an overall reduction in peripheral vascular resistance. The ultimate balance between vasodilator and vasoconstrictor production is critical in maintaining vascular function, and it may be this imbalance that contributes to the higher incidence of problems observed in pregnant women with diabetes.

1.7 Potential therapies to improve endothelial function

Over the last few years, research has begun to focus on strategies aimed at either retarding or reversing the development of small vessel disease. The aim is to reduce the incidence of morbidity and mortality associated diabetes, hypertension and hypercholesterolaemia, which have significant implications on health care.

1.7.1 Tight glycaemic control

As mentioned previously, hyperglycaemia increases the production of free radicals and advanced glycosylation end products, which either reduce the availability of

nitric oxide or inactivate it (Rubanyi and Vanhoutte, 1986a; Bucala *et al.*, 1991; Schmidt *et al.*, 1991; Schmidt *et al.*, 1990). Maintaining good glycaemic control in patients with diabetes, reduces the risk of acute and potentially life-threatening conditions such as diabetic ketoacidosis and hypoglycaemic coma, as well as longer-term micro-angiopathic sequelae (retinopathy, nephropathy and neuropathy). Achieving steady plasma glucose levels prior to conception and during the period of organogenesis (first trimester of pregnancy) reduces the production of free radicals, the subsequent risk of congenital malformations and the resulting perinatal morbidity and mortality. This can be achieved by the administration of insulin, oral hypoglycaemics or dietary modification, depending on the type of diabetes and the underlying pathology.

1.7.2 Folate

Folate has been shown by various studies to restore endothelial function in patients with familial hypercholesterolaemia, as well as healthy volunteers during a methionine load test (Usui *et al.*, 1999; Verhaar *et al.*, 1999a). Its effects may therefore also possibly extend to endothelial dysfunction in diabetes. The use of folic acid prior to conception and during early pregnancy has been shown to reduce the incidence of neural tube defects (Czeizel and Dudás, 1992), and is now a routine part of pre-natal and ante-natal care. The benefits of folate administration may therefore be two-fold in pregnant women with diabetes.

1.7.3 Antioxidants (Vitamins C and E)

The role of antioxidants in the treatment of vascular dysfunction is to decrease the production and release of oxygen-derived free radicals. Administration of Vitamin C was shown to restore impaired endothelium-dependent vasodilatation in the forearm resistance vessels of patients with type 1 diabetes mellitus (Timimi *et al.*, 1998), and reduce the incidence of congenital malformations in the offspring of streptozotocin-induced diabetic rats, supporting the theory that reactive oxygen species may be involved in embryonic dysmorphogenesis (Siman and Eriksson, 1997). Vitamin E supplementation has also been shown to partly reduce the detrimental effects of increased oxidative stress in diabetic pregnant rats and their offspring (Kinalski *et al.*, 1998). The administration of vitamin supplements is already a part of antenatal care and may also serve to improve endothelial cell function in humans.

1.7.4 Dihydropyridine calcium channel blockers

Nifedipine, commonly used to treat pregnancy-induced hypertension, has been shown to improve endothelial function in patients with hypercholesterolaemia, possibly due to a reduction in nitric oxide degradation (Verhaar *et al.*, 1999b). However, its role in normotensive pregnancies may be limited, given the associated physiological fall in blood pressure.

1.8 Summary

This chapter discusses the importance of the endothelium in resistance arteries during pregnancy. The co-existence of diabetes may further contribute to changes in vascular responses to endogenous endothelium-dependent vasodilators and vasoconstrictors. The literature suggests that endothelial function in patients with type 1 diabetes may depend on disease duration and the level of glycaemic control. The varying effects of diabetes on the different vascular beds, in terms of both vessel size and location, may reflect the complexity of disease development.

Despite previous extensive investigations of vascular function in patients with diabetes, there are few published data on the effect of type 1 diabetes on the maternal resistance vasculature in humans. A greater understanding of the role of factors involved in the control vascular tone is crucial in both healthy and diabetic pregnancies, as they are thought to be significantly altered compared to age and sex-matched non-pregnant control subjects. Although recent research has focused on therapeutic interventions aimed at either restoring or arresting endothelial dysfunction, an initial understanding of these vascular changes must be achieved if our aim is to reduce the risk of endothelial damage and the increased incidence of complications in this group of women.

1.8.1 Aims and Hypotheses

This thesis concentrates on vascular function in pregnant women with type 1 diabetes, primarily because of the significant maternal and fetal morbidity and

mortality associated with the disease, and the limited amount of published literature on the subject. The aims of this thesis were, therefore, to study the factors controlling vascular reactivity in isolated subcutaneous resistance arteries obtained from pregnant women with diabetes. The control of vascular function is multi-factorial, and the mechanisms most likely to be important were studied.

The hypotheses were:

(i) The vasodilatory effect of insulin is maintained during pregnancy through the release of endothelial nitric oxide.

(ii) Pregnant women with diabetes have abnormal vascular function, which may contribute to the increased incidence of pregnancy-related complications.

(iii) Endothelium-dependent hyperpolarization contributes significantly to the control of vascular function in diabetic pregnant women.

(iv) The vascular effects of endothelin-1 differ in pregnant women with and without diabetes.

Chapter Two

Experimental Methodology

2.1 Study Groups

Three teaching hospitals within the North Glasgow Hospitals University and Yorkhill NHS Trusts were involved in the study. Local Ethical Committee approval was granted, and the study was carried out in accordance with the Declaration of Helsinki (1996) of the World Medical Association (WMA, 1996). All patients were voluntarily recruited, provided with patient information sheets and written informed consent obtained.

Three groups of women were studied – a) type 1 diabetic pregnant women, b) non-diabetic pregnant women, and c) non-diabetic non-pregnant women. All type 1 diabetic women were recruited from joint diabetic/ante-natal clinics at the Glasgow Royal Maternity Hospital and The Queen Mother's Hospital. The non-diabetic pregnant women were recruited on admission either to the Glasgow Royal Maternity Hospital or The Queen Mother's Hospital, while the non-pregnant women were recruited from the Gynaecology ward at the Western Infirmary.

All pregnant women had caesarean sections at term, which, in the diabetic women, were carried out as either elective or emergency procedures, but were all performed electively in the non-diabetic women. The non-pregnant women were recruited from elective gynaecology operating lists for non-malignant conditions, and seen on admission to hospital. There were strict inclusion/exclusion criteria for recruitment, and this is briefly as follows:

Pregnant women

- Caesarean section at term
- Fit and well – no pregnancy related complications such as hypertension and proteinuria
- No regular medication other than insulin in the diabetic group
- Singleton fetus
- Body mass index of less than thirty

Non-pregnant women

- Elective laparotomy for non-malignant conditions
- Under 45 years of age
- Regular menstruation with no peri-menopausal symptoms
- Fit and well
- No regular medication
- Body mass index of less than thirty

2.2 Patient Characteristics

A total of 31 diabetic pregnant, 49 healthy pregnant and 23 healthy non-pregnant women were recruited over the course of 18 months. As previously mentioned, all patients were healthy and not on any regular medication, except the type 1 diabetic pregnant women who were maintained on insulin before and during the course of

their pregnancies. The smoking habits of all patients were recorded to ensure that vessel responses could be compared between women that smoked and those that did not.

Table 2.1 summarises the total number of women recruited (n), their mean ages and smoking habits. All other patient characteristics are described in the individual chapters.

Table 2.1 Basic characteristics of all women recruited into the study.

	Healthy pregnant	***Diabetic pregnant***	***Healthy non-pregnant***
n	49	31	23
Mean age (years)	32 ± 1	30 ± 1	35 ± 2
Smokers	16 (33%)	0	3 (13%)

Ages are given as mean ± standard error of the mean.

2.3 Small vessel wire myography

Numerous different techniques have been used to investigate vascular function. *In vivo* methods such as venous occlusion plethysmography, laser Doppler fluximetry and laser Doppler perfusion imaging, allow the study of arteries in their physiological environment (Johnstone *et al.*, 1993; Shore *et al.*, 1991; Jaap *et al.*, 1995). Laboratory based techniques such as small vessel (McNally *et al.*, 1994;

Knock *et al.*, 1997), large vessel (Kamata *et al.*, 1989) and perfusion (Tribe *et al.*, 1998) myography have been used to assess vascular function *in vitro*, and allow greater pharmacological manipulation that may not otherwise be possible. This thesis examines various factors controlling vascular function in pregnant women with type 1 diabetes using conventional small vessel wire myography, a laboratory-based organ bath technique.

Before the development of the small vessel myograph, it was impossible to carry out functional experimentation of small blood vessels *in vitro*. The wire myograph used in this study is a model developed by Mulvany and Halpern in 1976, based on the original 1972 suggestion by Bevan and Osher (Mulvany and Halpern, 1977; Bevan and Osher, 1972). The advantage of this technique over *in vivo* techniques is that pharmacological agents that may not be safe or possible *in vivo* can be safely studied, for example, apamin and charybdotoxin, potent toxins which in combination allow the *in vitro* study of the effects of endothelial hyperpolarization (Edwards *et al.*, 1998).

2.3.1 Tissue collection and arterial dissection

At laparotomy, the surgeons involved agreed to obtain the biopsy specimens. Strips of subcutaneous fat and skin were obtained from the incision edge unless the patient or the surgeon requested that no skin was to be taken, at which point only subcutaneous fat was excised. The biopsy was taken along the length of the incision as a wedge, measuring approximately 5cm long, 0.5cm wide and 1cm deep, allowing the cosmetic appearance of the wound to be maintained. Although the

vessels studied were subcutaneous and not cutaneous resistance arteries, many of the resistance arteries run through the subcutaneous tissue very close to the skin. Excising skin and subcutaneous tissue aids vessel identification. The biopsies were immediately transported to the laboratory in 0.9% saline in ice and placed into cold physiological saline solution (PSS) upon arrival.

The biopsies were divided into two halves to allow easier manipulation of the tissue. One half was placed in a petri dish and pinned to hold it in place, and the tissue constantly moistened with cold PSS. With the aid of a dissecting light microscope (Olympus SZ40®), small arteries (luminal diameter ~100-500μm) were isolated from the surrounding subcutaneous fat by blunt dissection with fine forceps (Agar Scientific®) and Mcpherson-Vannas dissecting scissors (World Precision Instruments®). They were identified from their venous counterparts by their physical appearances, which included wall thickness, vessel rigidity and vessel characteristics at branching points. (The vessels would be confirmed as arterial during the 'normalisation' process and 'start-up' procedure.) Isolated arteries were stored in PSS overnight at 4°C. The mean time of storage of the vessels was 15 hours (range 0-19 hours) prior to experimentation.

The storage of subcutaneous vessels overnight is not thought to affect their pH or function, and has been previously shown to be acceptable with no measurable change in function observed. McIntyre *et al.*, showed that vascular function was preserved in third order rat mesenteric arteries following cold storage for up to 4 days, when exposed to the vasoconstrictors –noradrenaline phenylephrine, potassium chloride and endothelin, and the vasodilators –acetylcholine and 3-

morpholinosydnonimine (McIntyre *et al.*, 1998). In this study, although there did not appear to be any difference between vessels experimented on immediately following dissection and those stored overnight in terms of vascular relaxation or constriction, overnight storage is an important point in the interpretation of this dataset. The relationship between storage time, maximal relaxation and vasoconstriction is shown in Table 2.2.

Table 2.2 The relationship between vessel storage time and vascular function.

Storage Time (Hours)	*Healthy pregnant (n=88)*		*Diabetic pregnant (n=32)*		*Healthy non-pregnant (n=29)*	
	Max. Relaxation (%)	*Tension (mN/mm)*	*Max. Relaxation (%)*	*Tension (mN/mm)*	*Max. Relaxation (%)*	*Tension (mN/mm)*
0	–	–	95.4 ± 1.2	2.9 ± 0.1	98.0 ± 0.8	4.2 ± 0.5
15	91.2 ± 2.2	3.8 ± 0.3	95.0 ± 0.9	3.0 ± 0.3	91.8 ± 4.3	3.7 ± 0.4
19	95.9 ± 1.4	3.2 ± 0.5	95.4 ± 1.5	2.9 ± 0.5	99.8 ± 0.2	3.0 ± 0.5

Values are expressed as mean ± standard error of the mean. *n*, number of vessels obtained.

2.3.2 Arterial mounting

Isolated arteries were mounted onto a small vessel wire myograph (Danish Myotech®, Aarhus, Denmark) to measure isometric tension (Myodaq/Myodata 2.01®) using standard methodology (Mulvany and Halpern, 1977). This involved threading two 40μm stainless steel wires through the vessel lumen under magnification at room temperature (Fig. 2.1). There was very little manipulation of

the artery with dissecting equipment during the mounting procedure, thereby minimising damage to the endothelium and underlying smooth muscle.

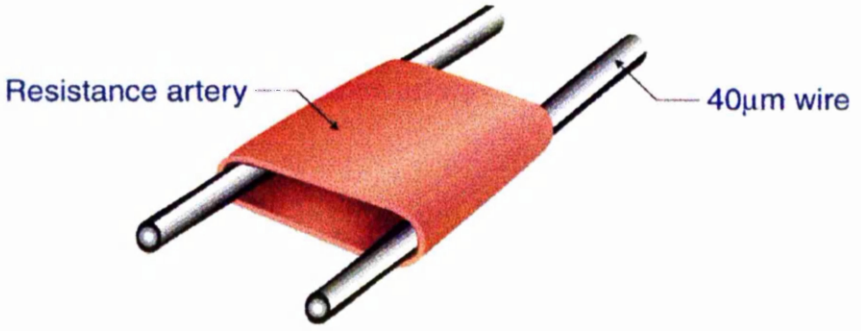


Fig. 2.1 Illustration of a resistance artery clearly showing the two wires through its lumen.

The wires were secured onto a mounting head, one end of which was attached to a micrometer and the other to a force transducer, allowing measurements of isometric tension generated by the vessel to be made (Fig. 2.2). The myograph has four sets of mounting heads, each sitting within a 5ml organ bath, allowing the responses of four vessels to be studied simultaneously under identical conditions.

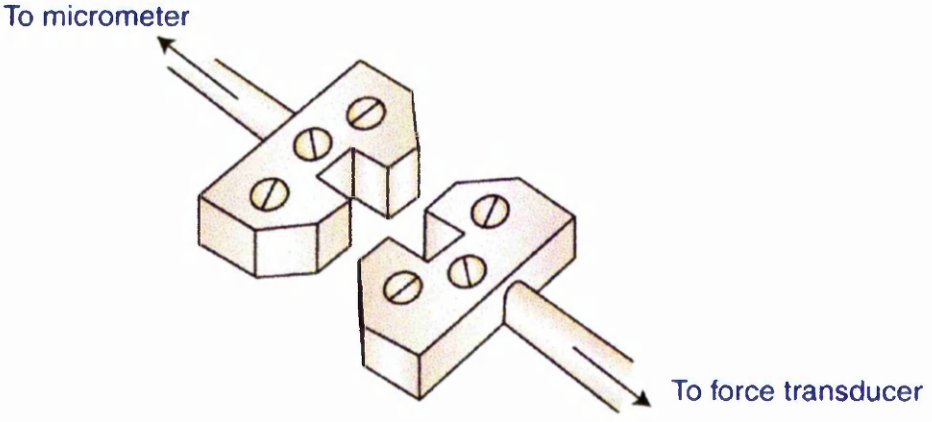


Fig. 2.2 Illustration of the mounting head of a Mulvany Halpern wire myograph. This sits within a 5ml organ bath.

2.3.3 Normalisation procedure

Once mounted, the vessels were allowed to equilibrate for one hour in PSS at 37°C and exposed to a combination of 95%O_2/5%CO_2. This 'gassing' mixture was required to keep the pH of the PSS at 7.4 when it was heated to 37°C. After this period of equilibration, the vessels were 'normalised'. This process determined each individual vessel's passive length-tension characteristics, and allowed a measure of standardisation for each individual vessel and experiment.

The normalisation procedure was performed using an iterative Myodaq/Myodata 2.01® program written by the myograph manufacturers (Danish Myotech®, Aarhus, Denmark). The procedure involved using the micrometer to stretch the vessel in a series of steps (2-3mN per stretch) at one-minute intervals. Following each stretch, the vessel relaxed to a resting tension. After one minute, the next stretch was applied. A one-minute interval per stretch allows the vessel to reach approximately 90-95% of the maximum response (increasing this to two-minute intervals or longer does not significantly affect the calculation, but merely increases the length of the protocol).

The change in tension was noted before (**tension 1**) and after (**tension 2**) each stretch to allow calculation of wall force:

Wall force (mN) = (tension 2-tension 1) x transducer sensitivity

From this and the vessel length (measured previously), the wall tension was calculated:

Wall tension (mN/mm) = Wall force/Vessel length

Internal circumference (L) at each point was calculated by:

L = (micro A-micro B) x 2 + internal circumference at B

Where micro A = micrometer reading from that stretch

micro B = micrometer reading with wires touching

Using Laplace's equation, which relates transmural tension and vessel circumference to the transmural pressure, the 'effective pressure' was calculated at each step:

Pressure = Wall tension/(internal circumference/2π)

The stretches were repeated until an effective pressure of 100mmHg (13.3kPa) was reached. The calculated effective pressure is an estimate of the transmural pressure that would be needed *in vivo* to stretch the relaxed vessel to the given internal circumference. The program then fitted an exponential curve to the results by plotting wall tension against internal circumference for each stretch. This describes increasing wall tension for each increase in circumference over the range of the normalisation procedure.

Using Laplace's law, an isobar was calculated:

Wall tension = (100mmHg) x (internal circumference/2π)

The point where the isobar and the exponential curve crossed represented the estimated internal circumference, designated L100, which this particular vessel would have under a transmural pressure of 100mmHg. The vessels were set to 90% of this value (0.9 x L100), designated L90, which has been shown to be the internal circumference at which the vessels will produce a maximum contractile response (Mulvany and Halpern, 1977). Although the L90 used in wire myography vessel normalisation was originally standardised for rat mesenteric vessels, this level of stretch in human resistance arteries is optimal for maximum active mechanical output, and has been tested in several laboratories in Glasgow, Edinburgh, Leicester and Aarhus (Hillier, personal communication). The normalised luminal diameter was calculated as:

L90/π

2.3.4 Tension measurements

Before commencing pharmacological experiments, vessel viability (contractile and endothelial function) was confirmed by carrying out a 'start-up' protocol. This involved constricting the vessels using a 123mM potassium chloride solution (KPSS), washing them out three times with PSS and allowing relaxation back to the baseline. This procedure was repeated three times (Fig 2.3).

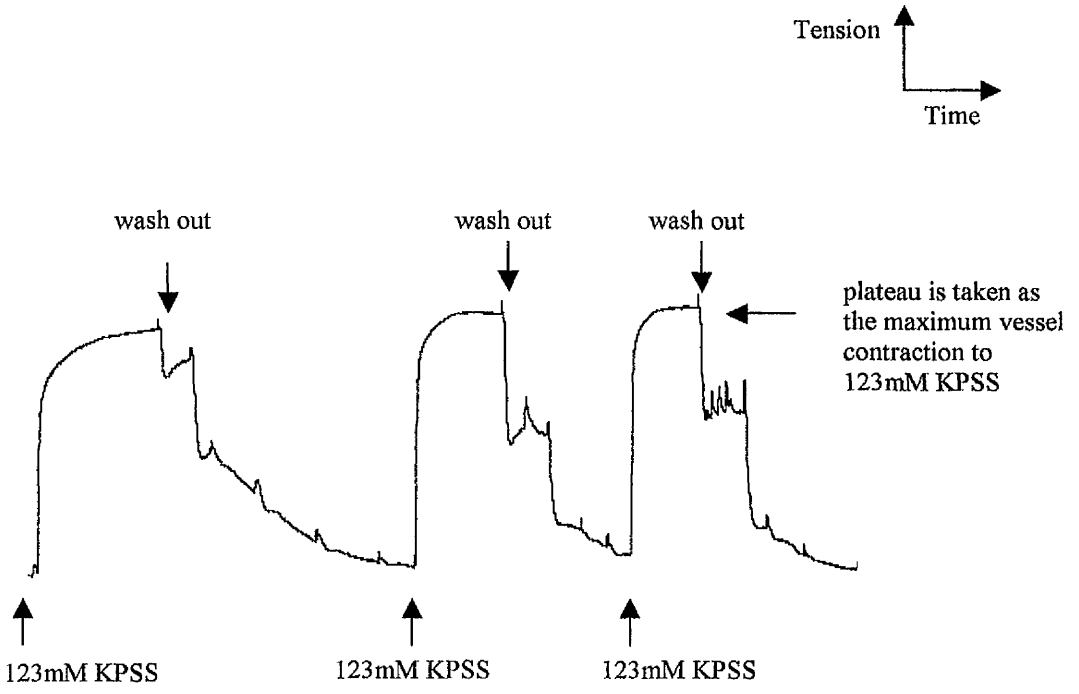


Fig 2.3 A representative trace showing constriction of a resistance artery using 123mM potassium chloride solution (KPSS).

A test of endothelial function was made by preconstricting the vessels with 10μM noradrenaline and observing relaxation to 3μM acetylcholine. In vessels requiring endothelial denudation, using a technique described by Osol et al., a human hair was washed in ethanol and rinsed in PSS prior to repeated insertion through the vessel lumen (Osol *et al.*, 1989). In these vessels, no relaxation to acetylcholine was observed (Fig. 2.4).

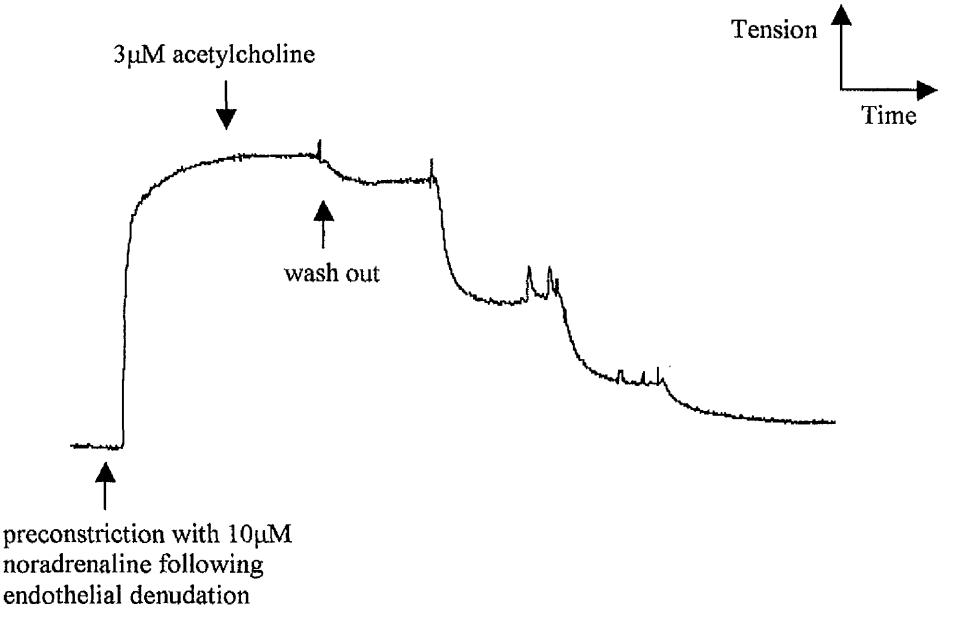


Fig. 2.4 A representative trace demonstrating a lack of relaxation to acetylcholine in a denuded resistance artery.

The vessels were then rinsed three times with PSS and allowed to relax to baseline. Finally, to provide a measure of maximal vasoconstriction, the vessels were constricted with 10μM noradrenaline in 123mM KPSS, rinsed three times with PSS, allowed to relax to baseline, and then equilibrated for a further hour (Fig. 2.5).

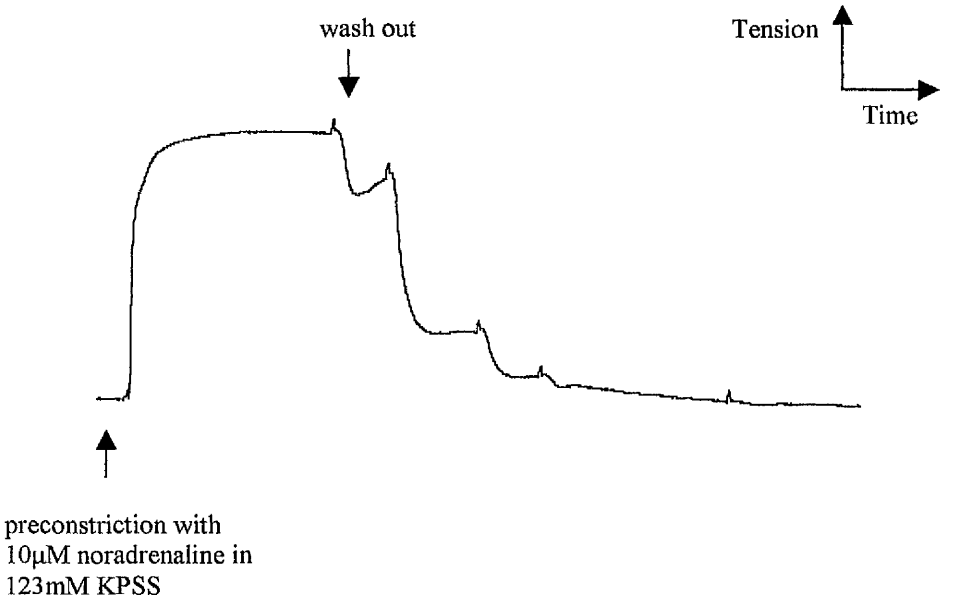


Fig. 2.5 A representative trace illustrating maximal vasoconstriction following exposure to 10μM noradrenaline in 123mM potassium chloride solution (KPSS).

2.3.5 Criteria for rejection of vessels

Endothelial function was assessed in all arteries by observing relaxation to 3μM acetylcholine following preconstriction with 10μM noradrenaline (Fig. 2.6). Arteries that did not achieve at least 60% relaxation were considered to be damaged and unsuitable for studies of endothelial function. Mechanical damage in my experience usually significantly reduces endothelial relaxation responses to below 15%. On the other hand, the literature suggests that the observed relaxation to acetylcholine in both pregnant and non-pregnant women, and patients with type 1 diabetes and gestational diabetes, will be approximately equal to or greater than

60% (McCarthy *et al.*, 1994; McNally *et al.*, 1994; Knock *et al.*, 1997). The use of a 60% cut-off therefore ensured that only mechanically damaged vessels would be excluded, and functionally impaired (by the disease process) vessels would be included. Vessels were also rejected if they did not produce sustainable contractions (greater than 3 minutes) to 123mM KPSS or 10μM noradrenaline in 123mM KPSS. Table 2.3 represents the total number of women from whom arteries were successfully dissected (No. of successful biopsies) following recruitment, the total number of isolated arteries, and the number of vessels used and rejected. There was no difference in the percentage of rejected vessels per patient group.

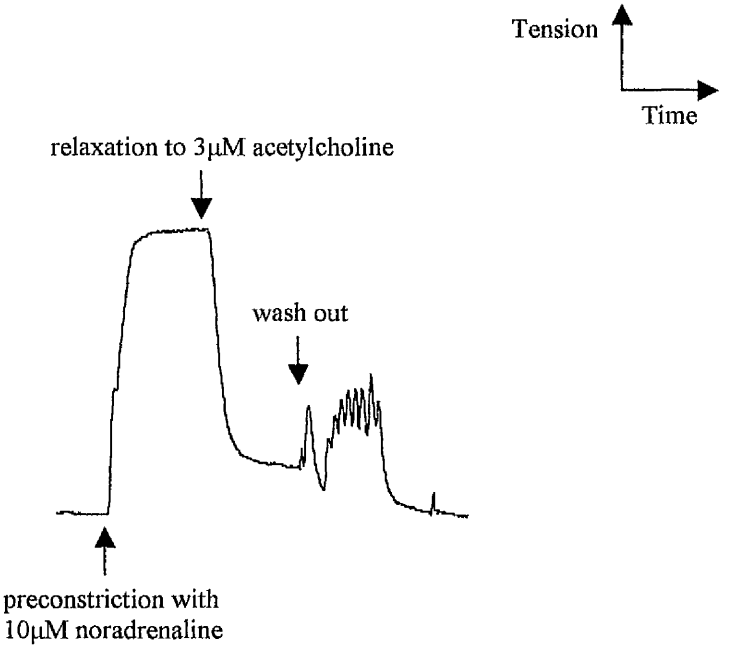


Fig. 2.6 A representative trace illustrating acetylcholine-induced relaxation in a resistance artery following preconstriction with 10μM noradrenaline.

Table 2.3 Table summarising the total number of small arteries used in and rejected in all experiments.

	Healthy pregnant	*Diabetic pregnant*	*Healthy non-pregnant*
Total no. of women recruited	49	31	23
No. of successful biopsies	34 (69%)	16 (52%)	19 (83%)
Total no. of vessels obtained	106	39	34
No. of vessels used	88 (83%)	32 (82%)	29 (85%)
No. of vessels rejected	18 (17%)	7 (18%)	5 (15%)

2.4 Pharmacological protocols

1. To investigate the effect of insulin-mediated vasodilatation in pregnant women, and the role of nitric oxide in the mediation of this response (Chapter 3).

Three study protocols were used:

Study 1.1 – Cumulative concentration response curves to noradrenaline (1nM to 30μM) were performed before and following 30 minutes incubation with insulin (1.0mU/mL). A third curve was then performed in the presence of both insulin (1.0mU/mL, 30 minutes) and N^{ω}-L-arginine methyl ester (L-NAME 0.1mM, 30 minutes), a competitive inhibitor of nitric oxide synthase.

Study 1.2 – To test for an order effect, in a different set of vessels, cumulative concentration response curves to noradrenaline (1nM to 30μM) were performed before and after incubation with L-NAME (0.1mM, 30 minutes). A third curve was then performed in the presence of both L-NAME (0.1mM, 30 minutes) and insulin (1.0mU/mL, 30 minutes).

Study 1.3 – To specifically examine the endothelium-dependent effect of insulin in pregnant women, cumulative concentration response curves to noradrenaline (1nM to 30μM) were performed, before and after 30 minutes incubation with insulin (1.0mU/mL), following endothelial denudation.

2. To investigate the contribution of each endothelium-dependent relaxing factor to vasorelaxation in pregnant women with and without type 1 diabetes (Chapter 4).

Three experimental protocols were used:

Study 2.1 – Cumulative concentration response curves were performed to carbachol (1nM to 30μM) following vessel preconstriction with 10μM noradrenaline. Three subsequent curves were then performed following a 30-minute incubation with:

a) N^{ω}-nitro-L-arginine methyl ester (nitric oxide synthase inhibitor, L-NAME 0.1mM)

b) L-NAME and indomethacin (cyclo-oxygenase inhibitor, INDO 30μM)

c) L-NAME, indomethacin and 25mM potassium chloride solution (inhibitor of endothelial hyperpolarization) (Gerber *et al.*, 1998)

Study 2.2 – To ensure that residual relaxation following nitric oxide synthase inhibition was not due to an incomplete inhibition of the pathway, the vessels were incubated for 30 minutes with L-NAME (0.1mM), indomethacin (30μM) and oxadiazoloquinoxalin (ODQ 1μM), an inhibitor of soluble guanylate cyclase.

Study 2.3 – To confirm the contribution of endothelial hyperpolarization to the residual relaxation response following inhibition of nitric oxide synthase and cyclo-oxygenase. Carbachol and sodium nitroprusside studies were repeated following 30 minutes incubation with charybdotoxin (100nM) and apamin (100nM), inhibiting both large and small conductance calcium-dependent potassium channels and the endothelium-dependent hyperpolarization (Zygmunt *et al.*, 1997; Edwards *et al.*, 1998).

To study the effectiveness of cAMP and cGMP pathways in the vascular smooth muscle, independent of endothelial modulation (Chapter 4).

Study 2.4 – Cumulative concentration response curves were performed to prostacyclin (1nM to 30μM) and sodium nitroprusside (1nM to 30μM), endothelium-independent agonists dependent on cAMP and cGMP respectively.

3. To investigate the vasoconstrictor response to noradrenaline and endothelin-1 (ET-1) in resistance arteries obtained from pregnant women with type 1 diabetes mellitus (Chapter 5).

Two study protocols were used:

Study 3.1 – Cumulative concentration response curves to noradrenaline (1nM to 30μM) were performed.

Study 3.2 – Cumulative concentration response curves were performed using ET-1 (1pM to 0.3μM).

2.5 Drugs and solutions

All drugs, obtained from Sigma, Poole, UK and CN Biosciences, Nottingham, UK, were made up with either distilled water or dimethyl sulphoxide (DMSO).

The following drugs were dissolved in distilled water:

Noradrenaline, N^{ω}-nitro-L-arginine methyl ester (L-NAME), Insulin, Carbachol, oxadiazoloquinoxalin (ODQ), Apamin, Charybdotoxin, Sodium nitroprusside, Prostacyclin, Endothelin-1.

Indomethacin was dissolved in dimethyl sulphoxide (DMSO).

PSS had the following composition: NaCl 118mM, $NaHCO_3$ 25mM, KCl 4.5mM, KH_2PO_4 1.0mM, $CaCl_2$ 2.5mM, $MgSO_4$ 1.0mM and $C_6H_{12}O_6$ (D-glucose) 6.0mM, 0.023mM EDTA and 0.03mM ascorbic acid. 25mM and 123mM KPSS had similar compositions to physiological saline, and were made by substituting NaCl for KCl on an equimolar basis.

The concentration of inhibitor drugs used are the standard concentrations known to inhibit the corresponding agonist, while the concentrations of agonists used are the ranges known to demonstrate a dose-dependent effect on vascular responses (McNally *et al.*, 1995; Gerber *et al.*, 1998; Edwards *et al.*, 1998; McIntyre *et al.*, 2001). The concentration of insulin used was chosen purely on a pharmacological basis. It has been previously shown that this particular concentration of insulin is able to demonstrate the greatest attenuation of the noradrenaline response (McNally *et al.*, 1995).

Carbachol rather that acetylcholine was used to study endothelium-dependent relaxation, as this is the standard endothelium-dependent muscarinic agonist used in our laboratory. It is a non-selective agonist that is resistant to the action of cholinesterases.

2.6 Data representation

Contractile responses are expressed as a percentage of the maximum potassium contraction for each individual vessel (%K^+), allowing for the standardisation of

responses between vessels. Relaxation responses are expressed as a percentage of the maximum tension generated following arterial pre-constriction with 10μM noradrenaline, allowing all vessel responses to be standardised.

The number of experiments is represented as n(N), where n is the number of vessels obtained and N the number of patients recruited.

2.7 Statistical analysis

Maximum contraction and relaxation were compared using either Student's *t* test, or a one-way ANOVA and Bonferroni's post hoc test for multiple comparisons. For the analysis of the cumulative concentration response curves, a one-way ANOVA for repeated measures was used, which took the 'non-independence' of the individual concentrations used into account (ie. each response is dependent on the previous response). Sensitivity is expressed as the pD_2, which is the negative log of the concentration required to produce 50% of the maximum response. The actual statistical test used for each experiment is reported in the appropriate chapter. A p value <0.05 was accepted as being statistically significant.

Chapter Three

Insulin-Mediated Vasorelaxation in Pregnancy

3.1 Introduction

Insulin is one of the few hormones that can demonstrate diverse effects, ranging from metabolic, biological and genetic influences, to mitogenesis and ion transport (Rosen, 1987; O'Brien and Granner, 1991; Moore, 1983). It can also act directly on the vasculature, and has been shown to have vasodilatory properties (Scherrer *et al.*, 1994; Steinberg *et al.*, 1994; Creager *et al.*, 1985). The mechanisms of action underlying insulin-mediated dilatation vary depending on the subject and tissue type studied, and include release of nitric oxide from the vascular endothelium, activation of the Na^+/K^+ pump on the plasma membrane, and a non-specific β-adrenergic mechanism (Scherrer *et al.*, 1994; Prakash *et al.*, 1992; Creager *et al.*, 1985). Studies have demonstrated a reduction in insulin-mediated vasorelaxation in insulin-resistant states such as obesity, type 2 diabetes and hypertension (Laakso *et al.*, 1990; Laakso *et al.*, 1992; Baron *et al.*, 1993). It has been suggested that the resulting decrease in vascular perfusion may contribute to the insulin resistance observed in these patient groups.

The changes in hormonal profile and carbohydrate metabolism during pregnancy result in physiological insulin resistance and hyperinsulinaemia, with plasma insulin levels trebling to maintain maternal and fetal euglycaemia. As the levels of insulin increase substantially during pregnancy, insulin-mediated vasodilatation may be an important factor in the control of vascular tone.

In Chapter 1, the importance of the resistance vasculature in the control of blood pressure and tissue perfusion was described. Disturbances in vascular reactivity

may result from pathologically insulin resistant states, but are unlikely to be present in physiological insulin resistance, since pregnancy *per se* is not associated with the development of micro-vascular disease. One possible explanation for this is that insulin resistance in pregnancy develops more acutely with a shorter duration of action followed by resolution post-partum.

This chapter investigates the effect of insulin-mediated vasodilatation in healthy pregnant women, and the role of nitric oxide in the mediation of this response. It was hypothesised that the vasodilatory effect of insulin is maintained in pregnancy through the release of endothelial nitric oxide.

3.2 Methods

3.2.1 Study groups

Local Ethical Committee approval was granted. Fourteen pregnant and seven non-pregnant women were recruited into the study and written informed consent obtained. All the patients were healthy, not on any regular medication and had no history of diabetes or hypertension. Blood pressure was checked prior to surgery on all women upon admission. The pregnant women underwent elective caesarean section at term (seven for breech presentation and seven for previous section), and the non-pregnant women underwent elective gynaecological surgery. Table 3.1 summarises the patient ages, blood pressures (BP) and smoking habits, and the mean luminal diameter of isolated arteries.

Table 3.1 Characteristics of patients recruited into the study investigating insulin-mediated vasodilatation in isolated small arteries.

	Healthy pregnant	*Healthy non-pregnant*	*Significance*
Age (years)	31 ± 1	33 ± 3	NS
Internal diameter (μm)	273 ± 18	274 ± 17	NS
Blood pressure (systolic) (mmHg)	114 ± 4	120 ± 5	NS
Blood pressure (diastolic) (mmHg)	70 ± 3	68 ± 3	NS
Smokers	1/14	2/7	–

Values are expressed as mean ± standard error of the mean. Significance accepted if $p<0.05$, Student's *t* test. *NS*, not significant.

3.2.2 Experimental protocols

The standard methodology of small vessel wire myography has been explained in detail in Chapter 2. Only protocols and solutions specific to this chapter have been described here.

Following the dissection of the biopsies, the vessels were tested for viability and endothelial function. The arteries obtained from pregnant women achieved a mean relaxation of 75.0 ± 2.6%, while vessels from the non-pregnant women achieved 76.0 ± 2.8% relaxation.

3.2.3 Pharmacological protocols

Three pharmacological protocols were used:

Study 1 – Cumulative response curves to noradrenaline (1nM to 30μM) were performed before and following 30 minutes incubation with insulin (1.0mU/mL) (McNally *et al.*, 1995). A third curve was then performed in the presence of both insulin (1.0mU/mL, 30 minutes) and N^{ω}-nitro-L-arginine methyl ester (L-NAME 0.1mM, 30 minutes), a competitive inhibitor of nitric oxide synthase.

Study 2 – To test for an order effect, in a different set of vessels, cumulative concentration response curves were performed to noradrenaline (1nM to 30μM) before and after incubation with L-NAME (0.1mM, 30 minutes). A third curve was then performed in the presence of both L-NAME (0.1mM, 30 minutes) and insulin (1.0mU/mL, 30 minutes).

Study 3 – To specifically examine the endothelium-dependent effect of insulin in pregnant women, cumulative concentration response curves to noradrenaline (1nM to 30μM) were performed, before and after incubation with insulin (1.0mU/mL, 30 minutes), following endothelial denudation (Chapter 2). The absence of functioning endothelium was confirmed by observing no relaxation to 3μM acetylcholine in vessels preconstricted with 10μM noradrenaline.

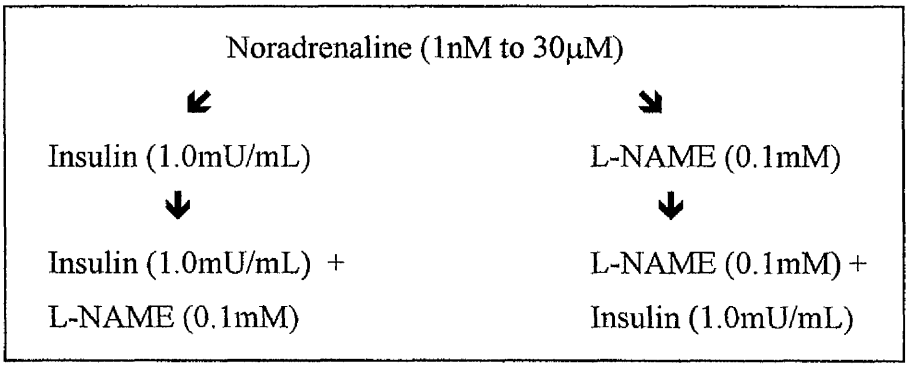


Fig. 3.1 The two pharmacological protocols used to examine the effects of noradrenaline, insulin and N^{ω}-nitro-L-arginine methyl ester (L-NAME) on isolated arteries obtained from both pregnant and non-pregnant women.

3.2.4 Drugs and solutions

All drugs were obtained from Sigma, Poole, UK, and made up in distilled H_20. PSS and KPSS were made as stated previously in Chapter 2.

3.2.5 Statistical analysis

Contractile responses are expressed as a percentage of each individual vessel's maximum potassium contraction, allowing for the standardisation of responses between vessels. Data are expressed as mean ± standard error of the mean (SEM). The number of experiments is expressed as n(N), where n is the number of vessels and N is the number of patients. Maximum contraction and sensitivity (pD_2 – negative log of the concentration required to produce 50% of the maximum response) were compared using one-way ANOVA and Bonferroni's post hoc test for multiple comparisons. A p value <0.05 was accepted as being statistically significant.

3.3 Results

Results from smokers and non-smokers were combined. Vessel responses were not noted to be obviously different (Table 3.2).

Table 3.2 Summary of maximum responses ($\%K^+$) and sensitivity (pD_2) to noradrenaline in smoking and non-smoking patients.

	Healthy pregnant		*Healthy non-pregnant*	
	Smokers	***Non-smokers***	***Smokers***	***Non-smokers***
n	1	13	2	5
NA	–			
Max. Response (%)	105.9	104.5 ± 3.4	110.6 ± 19.4	98.9 ± 0.5
pD_2	5.7	6.3 ± 0.1	6.6 ±0.3	6.7 ± 0.2
NA+INS				
Max. Response (%)	94.7	86.5 ± 7.0	99.5 ± 21.0	88.5 ± 9.1
pD_2	5.7	6.3 ± 0.3	6.5 ± 0.3	6.6 ± 0.2
NA+L-N				
Max. Response (%)	137.0	147.9 ± 15	127.6 ± 12.6	116.0 ± 13.8
pD_2	7.0	7.2 ± 0.4	6.3 ± 0.1	6.5 ± 0.2
NA+L-N+INS				
Max. Response (%)	115.6	98.5 ± 8.4	128.1 ± 4.6	101.1 ± 11.9
pD_2	7.2	7.4 ± 0.3	6.3 ± 0.1	6.6 ± 0.2
NA+INS-ENDO				
Max. Response (%)	67.5	58.3 ± 16.6	–	–
pD_2	6.4	6.6 ± 0.2	–	–

Values are mean ± SEM. *NA*, noradrenaline; *INS*, insulin; *L-N*, L-NAME; *-ENDO*, without endothelium.

No differences in the noradrenaline sensitivity (pD_2) were observed between the two groups as illustrated in Table 3.3.

Table 3.3 Table summarising the vessel sensitivity to noradrenaline (pD_2) in pregnant and non-pregnant women.

	Healthy pregnant (pD_2)	***Healthy non-pregnant (pD_2)***	***Significance***
NA	6.27 ± 0.14	6.66 ± 0.16	NS
NA+INS	6.24 ± 0.12	6.60 ± 0.17	NS
NA+L-N	7.17 ± 0.27	6.43 ± 0.15	NS
NA+L-N+INS	6.33 ± 0.15	6.77 ± 0.16	NS
NA+INS-ENDO	6.58 ± 0.17	–	–

Values are mean ± SEM. Significance assumed if $p<0.05$, Student's *t* test. *NS*, not significant; *NA*, noradrenaline; *INS*, insulin; *L-N*, L-NAME; *-ENDO*, without endothelium.

(i) Noradrenaline

There was no difference in the maximum vasoconstriction response to noradrenaline between vessels obtained from pregnant and non-pregnant women [Maximum response (%K^+) pregnant 104.6 ± 3.2%, n=17(8) *vs.* non-pregnant 100.9 ± 6.4%, n=17(8) p>0.05, Fig. 3.2].

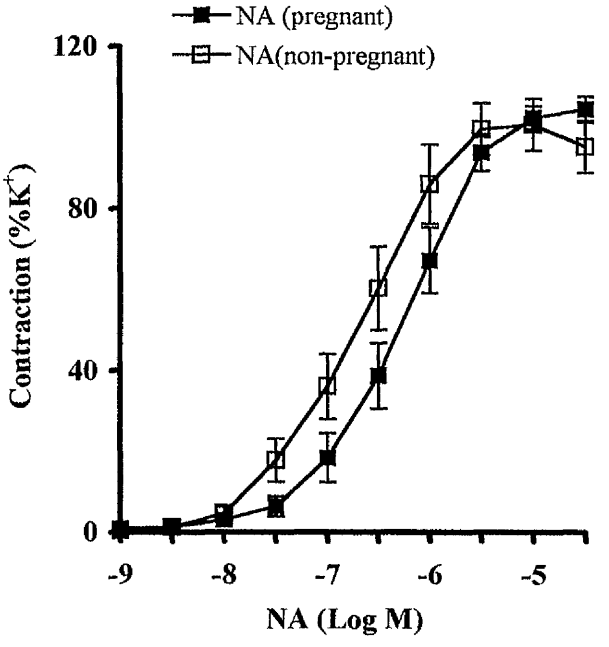


Fig. 3.2 The effect of noradrenaline (NA) on isolated resistance arteries obtained from healthy pregnant and non-pregnant women (p>0.05).

(ii) Insulin

Insulin (INS) caused a significant reduction in the maximum contractile response to noradrenaline (NA) in pregnant women. The following figure is an experimental trace of a subcutaneous resistance artery obtained from a healthy pregnant women subjected to cumulative concentrations of noradrenaline (1nM to 30μM), initially on its own, and then in the presence of insulin (1.0mU/mL) (Fig. 3.3.1).

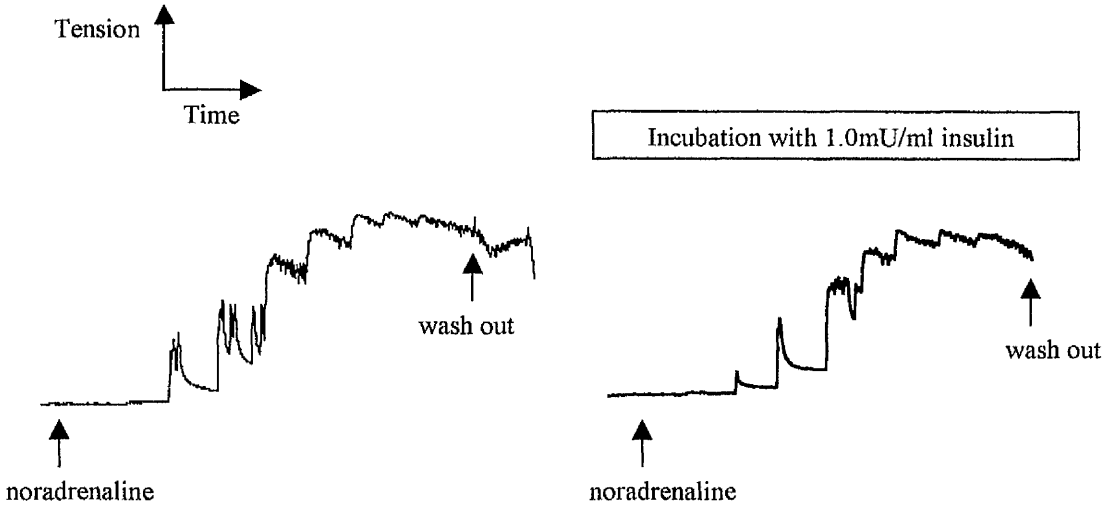


Fig. 3.3.1 A representative trace of a resistance artery exposed to cumulative concentrations of noradrenaline and then repeated following a 30 minute incubation with 1.0mU/mL insulin.

When expressed as a percentage of the maximum response to 123mM potassium chloride solution (KPSS): Maximum response (%K^+) NA 104.6 ± 3.2%, n=17(8) *vs.* NA + INS 86.7 ± 6.6%, n=17(8) *p<0.01, Fig. 3.3.2].

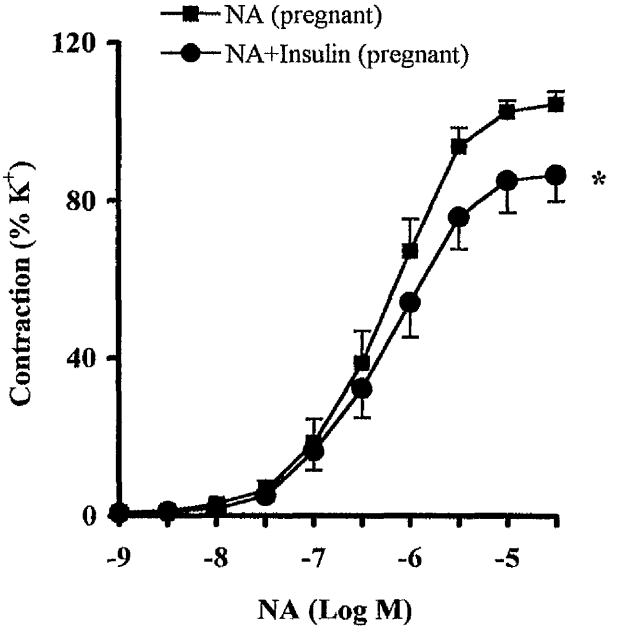


Fig. 3.3.2 The effect of insulin on vasoconstriction responses to noradrenaline (NA) in subcutaneous small arteries obtained from healthy pregnant women at term (*p<0.01).

In the non-pregnant group of women, insulin caused an attenuation of the maximum response to noradrenaline by approximately 10%, however, this was not statistically significant [Maximum response (%K^+) NA 100.9 ± 6.4%, n=11(7) *vs.* NA + INS 90.5 ± 8.5%, n=11(7) p>0.05, Fig. 3.3.3].

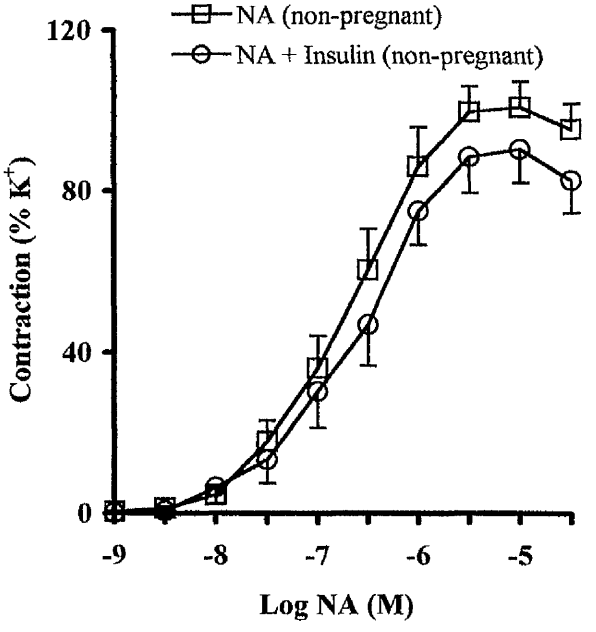


Fig. 3.3.3 The effect of insulin on vasoconstriction responses to noradrenaline (NA) in subcutaneous small arteries obtained from healthy non-pregnant women (p>0.05).

(iii) L-NAME

In a different set of vessels obtained from the same patients, incubation with L-NAME alone caused a significant potentiation of the maximum contractile response to noradrenaline in vessels obtained from pregnant women [Maximum response (%K^+) NA 118.8 ± 8.8%, n=9(5) *vs.* NA + L-NAME 145.4 ± 12.0%, n=9(5) *p<0.05, Fig. 3.4.1].

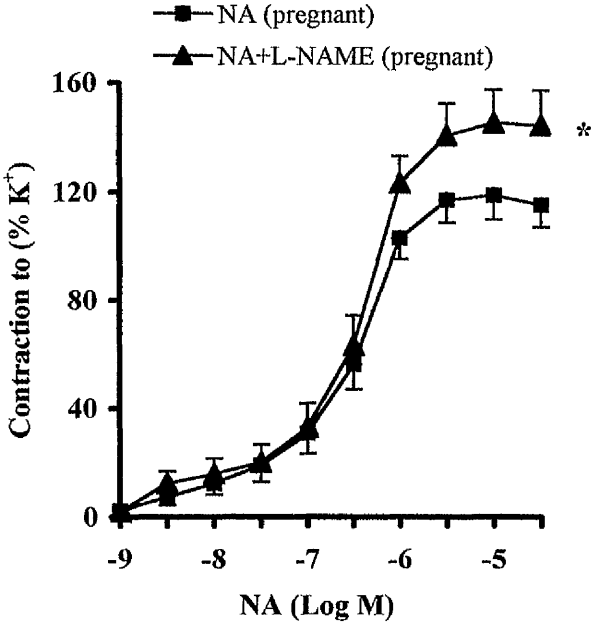


Fig. 3.4.1 The effect of incubation with L-NAME on vasoconstriction responses to noradrenaline (NA) in subcutaneous small arteries obtained from healthy pregnant women at term (*p<0.05).

No significant potentiation was seen in vessels collected from non-pregnant women [Maximum response (%K^+) NA 115.8 ± 8.2%, n=10(7) *vs.* NA + L-NAME 119.7 ± 10.5%, n=10(7) p>0.05, Fig. 3.4.2].

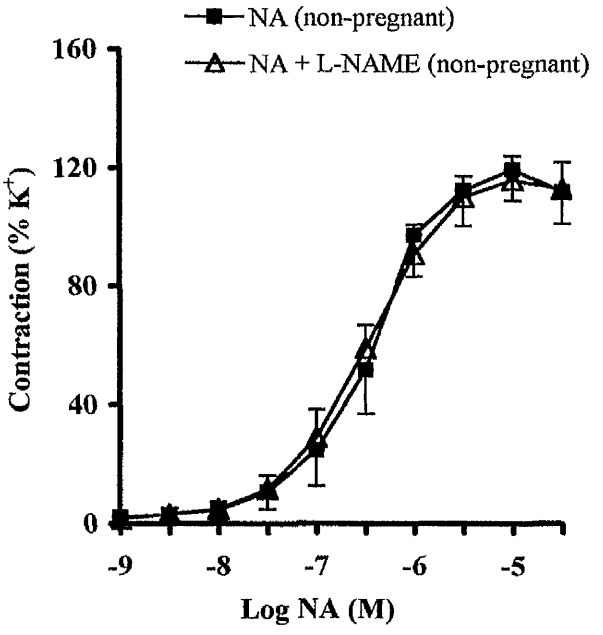


Fig. 3.4.2 The effect of incubation L-NAME on vasoconstriction responses to noradrenaline (NA) in subcutaneous small arteries obtained from healthy non-pregnant women (p>0.05).

(iv) L-NAME and Insulin

Following incubation with L-NAME, the subsequent addition of insulin resulted in significant attenuation of the maximum response in pregnant women.

Figure 3.5.1 is an experimental trace of a subcutaneous resistance artery obtained from a healthy pregnant women subjected to cumulative concentrations of noradrenaline (1nM to 30μM), following incubation with L-NAME and then with L-NAME and insulin.

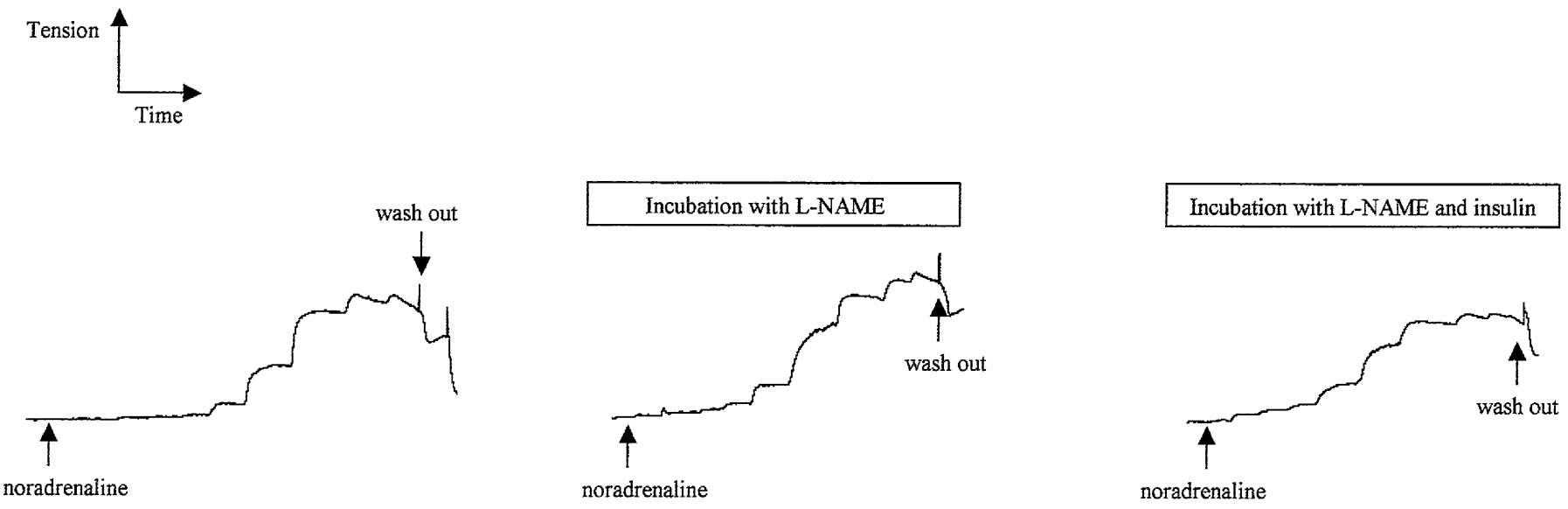


Fig. 3.5.1 A representative trace of a resistance artery exposed to cumulative concentrations of noradrenaline and then repeated following a 30 minute incubation with L-NAME and then again in the presence of L-NAME and insulin.

When expressed as a percentage of the maximum response to 123mM potassium chloride solution (KPSS): Maximum response (%K^+) NA + L-NAME 145.4 ± 12.0%, n=9(5) *vs.* NA + L-NAME + INS 97.3 ± 5.5%, n=9(5) *p<0.01, Fig. 3.5.2].

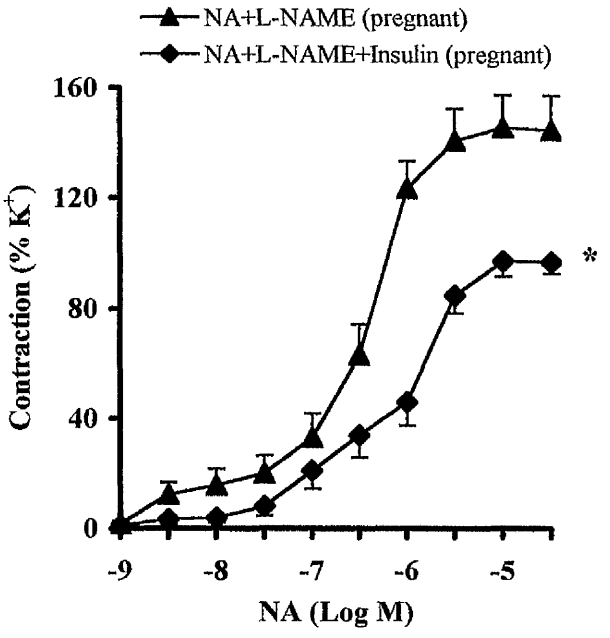


Fig. 3.5.2 The effect of the addition of L-NAME and insulin on vasoconstriction responses to noradrenaline (NA) in subcutaneous small arteries obtained from healthy pregnant women at term (*p<0.01).

Relaxation was not observed in the non-pregnant women [Maximum response (%K^+) NA + L-NAME 119.5 ± 10.5%, n=10(7) *vs.* NA + L-NAME + INS 108.5 ± 9.5%, n=10(7) p>0.05, Fig. 3.5.3].

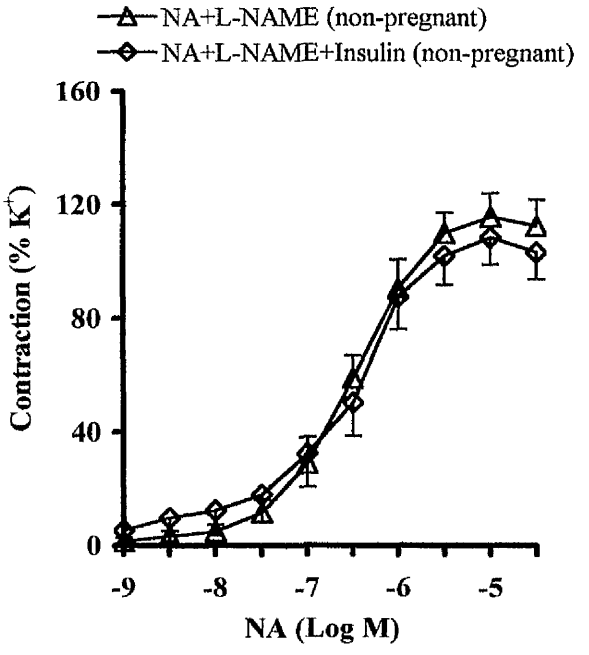


Fig. 3.5.3 The effect of the addition of L-NAME and insulin on vasoconstriction responses to noradrenaline (NA) in subcutaneous small arteries obtained from healthy non-pregnant women (p>0.05).

(v) Following endothelial denudation

Insulin caused a significant reduction in the maximum contractile response to noradrenaline in pregnant women following the removal of the endothelium [Maximum response (%K^+) NA 95.5 ± 5.5%, n=13(6) *vs.* NA + INS 64.4 ± 8.2%, n=13(6) *p<0.01, Fig. 3.6].

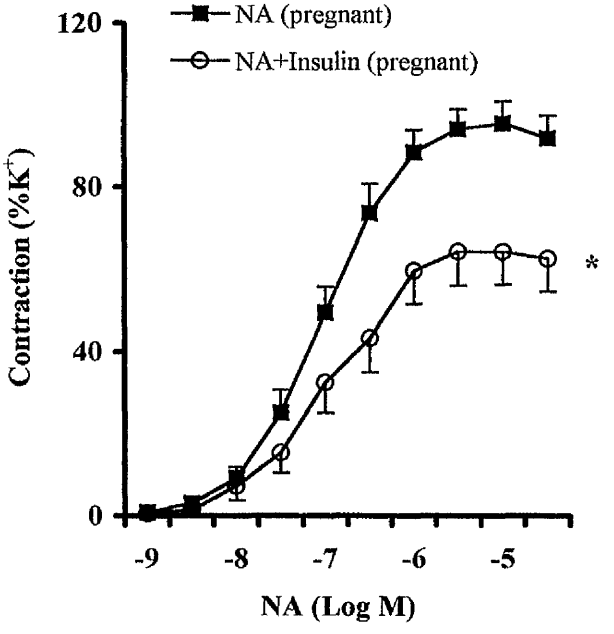


Fig. 3.6 The effect of insulin on vasoconstriction responses to noradrenaline (NA) in subcutaneous small arteries obtained from healthy pregnant women following endothelial denudation (*p<0.01).

3.4 Discussion

To my knowledge, this is the first study to demonstrate a significant attenuation of noradrenaline-induced tone, following incubation with insulin, in isolated small arteries from healthy pregnant women. Furthermore, it demonstrates that in these women, the vasodilatory effect of insulin is via an endothelium-independent mechanism.

Insulin is able to modulate the vasoconstrictor effect of noradrenaline in pregnancy, a situation in which physiological insulin resistance and hyperinsulinaemia develop. This observation is important, and along with the *in vivo* hyperinsulinaemia may account for the increased vasodilatation and reduced peripheral vascular resistance necessary to maintain blood pressure in the presence of an increased circulating blood volume. This also supports the results of previous studies investigating the effect of insulin in healthy volunteers and animal models (McNally *et al.*, 1995; Chen *et al.*, 1996).

A reduced vascular effect of insulin, seen in obese men, patients with type 2 (non-insulin dependent) diabetes mellitus, and hamsters with streptozotocin-induced diabetes, has been presumed to be a feature of the pathological insulin resistant state in these groups (Laakso *et al.*, 1990; Kinoshita *et al.*, 2000; Bouskela *et al.*, 1997). This study has demonstrated that in physiological, as opposed to, pathological insulin resistant conditions, the effects of insulin on the resistance vasculature are not diminished. This may partially explain why conditions such as type 2 diabetes are associated with the increased risk of micro-angiopathy (retinopathy,

nephropathy, peripheral vascular disease), while pregnancy *per se* does not result in long-term vascular dysfunction. Insulin-mediated attenuation of the noradrenaline response demonstrated here, was ~10% in non-pregnant women, compared to ~17% reported by McNally in a mixed group of healthy men and women (McNally *et al.*, 1995). Although the vasodilatory effect of insulin in the non-pregnant patient group was not significant (Fig. 3.3.3), there was still a trend towards blunting of noradrenaline-induced tone following incubation with insulin. The lack of statistical significance in this instance may be due to the smaller number of patients recruited in this study, but does not alter the main findings.

The present study also reports no difference in noradrenaline pressor responses between pregnant and non-pregnant women, which agrees with the published literature (Knock *et al.*, 1996; Steele *et al.*, 1993; Aalkjaer *et al.*, 1985). Although there was a trend towards a change in sensitivity, the difference was not statistically significant (Table 3.3). This finding suggests that the 'system' involved in the attenuating effect of insulin may act independently of that controlling noradrenaline pressor responses. It is possible that the endothelial modulation of noradrenaline does not play as significant a role as previously thought, as *in vitro* studies where inhibitors of nitric oxide were used showed a less significant effect in resistance arteries (Coats *et al.*, 2001; Gerber *et al.*, 1998).

In these patients, inhibition of nitric oxide synthase resulted in significant potentiation of the response to noradrenaline in the pregnant but not in the non-pregnant women. There was no alteration in the baseline response following incubation with L-NAME, prior to the commencement of the noradrenaline

concentration response curves. Within the vascular endothelium, nitric oxide release can be stimulated by a combination of biological mediators such as acetylcholine, peptides, purine metabolites and blood flow (Furchgott 1984; Rubanyi *et al.*, 1986b). In the absence of chemical stimulation from circulating neuro-hormonal factors, basal nitric oxide release is entirely dependent upon stimulation by blood flow (shear stress) on the surface of the endothelial cells. *In vitro* assessment of isolated arteries using wire myography lacks this stimulus for basal nitric oxide release. The significant potentiation in the noradrenaline response seen following nitric oxide synthase inhibition with L-NAME in pregnant women is, therefore, likely to be due to the inhibition of agonist-induced (noradrenaline) rather than basal nitric oxide. This may stimulate a concurrent increase in agonist-mediated nitric oxide release to modulate vascular tone, possibly via the activation of endothelial β_1-adrenoceptors (Graves and Poston, 1993). It may be this effect that is observed as a potentiated vasoconstrictor response following nitric oxide synthase inhibition.

Insulin-mediated vasorelaxation is thought to be due to release of endothelial nitric oxide (Williams *et al.*, 1997; Scherrer *et al.*, 1994; Steinberg *et al.*, 1994), although other mechanisms have also been implicated (Ferrannini *et al.*, 1988; Creager *et al.*, 1985). However, following incubation with L-NAME, vessels obtained from pregnant women demonstrate significant relaxation to insulin. This suggests that the control of peripheral vascular resistance in pregnancy does not depend entirely on insulin-induced nitric oxide release. Furthermore, in the presence of insulin and endothelial denudation, the continued and even exaggerated attenuation of noradrenaline-induced tone provides additional evidence that the vasodilatory effect

of insulin is likely to be endothelium-independent. As the vasodilatory effect of insulin is known to act via both the endothelium and smooth muscle, one possible explanation for this observed effect is the activation of Ca^{2+}-ATPase and Na^{+}-K^{+}-ATPase within the smooth muscle cell and subsequent smooth muscle hyperpolarization (Cleland *et al.*, 1998; Tack *et al.*, 1996; Altan *et al.*, 1989). Another possibility is that as insulin is also able to stimulate the release of the vasoconstrictor, endothelin-1, from the vascular endothelium, removing the source of endothelin-1 production could potentially result in a larger observed vasodilatory effect. However, without the benefit of further research, these mechanisms are purely speculative.

McNally *et al.* demonstrated that the vasodilatory effect of insulin on noradrenaline-induced contractility increased in a dose-dependent manner in both healthy men and women, and that using a 1.0mU/mL concentration of insulin resulted in a maximum attenuation of 17% (McNally *et al.*, 1995). In this study, a 1.0mU/mL concentration of insulin was chosen purely as a pharmacological tool to ensure that if present, the effect of insulin would be demonstrable in arteries isolated from healthy pregnant women, and was not intended to mimic *in vivo* conditions. It is possible that the use of this supraphysiological concentration of insulin (equating to approximately 6nM) may be able to overcome a loss of insulin-induced vasodilatation associated with the insulin resistant state. It would, however, be of value to investigate the effect of lower concentrations of insulin to truly reflect normal physiological conditions.

3.5 Conclusions

This study suggests that physiological insulin resistance and hyperinsulinaemia in pregnancy are not associated with the diminished vascular response to insulin observed in pathological states. Furthermore, insulin mediates vasorelaxation via an endothelium-independent mechanism. Conditions in which insulin resistance develops as part of the pathological process, such as type 2 diabetes, have been shown to be associated with a reduced vascular effect of insulin. This is not observed with the physiological insulin resistance associated with healthy pregnancies.

Chapter Four

Endothelium –Dependent and –Independent Relaxation in Pregnant Diabetic Women

4.1 Introduction

Numerous studies have demonstrated impaired endothelial function in diabetes, implicating it as one of the mechanisms underlying the complications associated with the disorder (Johnstone *et al.*, 1993; McNally *et al.*, 1994). Abnormal vascular responsiveness in diabetics, due particularly to endothelial dysfunction, can deteriorate rapidly during pregnancy, when the ability of the maternal vasculature to respond rapidly to changes in metabolic requirements may be further compromised. The increased incidence of complications such as pre-eclampsia and placental insufficiency, associated with increased maternal and fetal vascular resistance, contributes greatly to both maternal and fetal morbidity and mortality (Garner *et al.*, 1990; Redman, 1991). However, vascular dysfunction can also reflect changes in smooth muscle function, which may result in altered responses of the vascular smooth muscle to stimuli.

There is a considerable heterogeneity in the pattern of vascular dysfunction observed in different disease states, reflecting complex and varied underlying mechanisms. In general, the description of vascular dysfunction can be conveniently separated into two relatively simple states, endothelium-dependent or endothelium-independent. A state of endothelium-independent dysfunction is almost exclusively accepted to be due to abnormally functioning vascular smooth muscle. Following these straightforward criteria, an interpretation of endothelial dysfunction can therefore normally only be suggested if some measure of 'normal' underlying smooth muscle functionality has been made. Without this comparison, there would be difficulty in differentiating between endothelial and smooth muscle

effects. A 'misdiagnosis' of endothelial dysfunction may therefore, in fact, be due to alterations in smooth muscle responses. In diabetes, where vascular dysfunction, particularly micro-vascular in origin, is a major contributor to morbidity and mortality, the specific underlying mechanisms responsible appear to differ according to the diabetic model and vascular bed studied. In diabetic research, a complex picture has emerged whereby different clinical and experimental studies have demonstrated both preservation and impairment of endothelium-dependent and -independent vasodilatation (Smits *et al.*, 1993; Johnstone *et al.*, 1993; Heygate *et al.*, 1995; Calver *et al.*, 1992).

Nitric oxide (NO) has been one of the most widely studied endothelium-dependent relaxing factors, inducing vasodilatation via the guanosine 3':5' cyclic monophosphate (cGMP) pathway. It has been shown to contribute significantly to the control of vascular tone by studies that have shown marked vasoconstriction following nitric oxide synthase inhibition with analogues of L-arginine (Vanhoutte *et al.*, 1995; Bennett *et al.*, 1992). In most human vascular beds, the contribution of prostacyclin (PGI_2) to endothelium-dependent relaxation is much less significant (Kublickiene *et al.*, 1997; McCarthy *et al.*, 1994), but along with nitric oxide, is thought to be associated with the physiological vasodilatation seen in pregnancy (Greer *et al.*, 1985a). Prostacyclin differs from nitric oxide in that it acts via the adenosine 3':5' cyclic monophosphate (cAMP) pathway, and can be assessed by inhibiting the prostacyclin-converting enzyme, cyclo-oxygenase (COX).

Until very recently, there has been little emphasis on the contribution of endothelial hyperpolarization to vasorelaxation. This hyperpolarization, which was initially

thought to be due to a putative endothelium-derived hyperpolarizing factor (EDHF) whose precise chemical identity remains uncertain, has been shown to be insensitive to both nitric oxide synthase and cyclo-oxygenase inhibition (Coats *et al.*, 2001). However, current literature suggests that endothelium-dependent hyperpolarization is more likely due to electrical coupling through myoendothelial gap junctions, rather than a single chemical factor (Coleman *et al.*, 2001a; Coleman *et al.*, 2001b; Sandow and Hill, 2000). Furthermore, its importance appears to increase as the size of the vessel decreases, contributing significantly to the maintenance of tone within the resistance vasculature (Shimokawa *et al.*, 1996; Coats *et al.*, 2001). The effects of endothelial hyperpolarization can be inhibited by the use of 25mM potassium chloride, or by the combined effects of apamin and charybdotoxin (Gerber *et al.*, 1998; Edwards *et al.*, 1998). Endothelial vasodilatory function can, therefore, be relatively comprehensively characterised by manipulation of nitric oxide, prostacyclin and endothelial hyperpolarization by inhibitors and blockers.

On the other hand, the characterisation of smooth muscle vasodilator function generally focuses on investigating the two most important second messenger systems, which transduce stimulation by either agonists or endothelial factors. As described above, nitric oxide and prostacyclin are the guanylate cyclase and adenylate cyclase systems that result in increases in cGMP and cAMP respectively. These pathways can be studied independently from the endothelium by exposing the vessels to the nitric oxide donor, sodium nitroprusside (which acts via guanylate cyclase), and to exogenously-applied prostacyclin (acting via adenylate cyclase).

Cholesterol is an important lipid involved in the biosynthesis of plasma membranes, bile salts and steroid hormones. The tight control of plasma cholesterol is important, because high levels have been shown to increase the risk of ischaemic heart disease and stroke. Diabetes is, itself, a risk factor for atherogenesis and vascular dysfunction compared with the general population. Cholesterol and triglyceride circulate in plasma as part of various lipoprotein complexes, which include high-density lipoproteins (HDLs), low-density lipoproteins (LDLs), and very low-density lipoproteins (VLDLs). LDLs are the main transporters that carry cholesterol from the liver to most cells for metabolic and structural requirements, at the same time as enhancing arterial wall deposition and intimal thickening. HDL, on the other hand, promotes the removal of cellular cholesterol and its secretion into bile by the liver. The ratio of plasma LDL- to HDL-cholesterol, and not the actual plasma cholesterol level, reflects the risk of developing atherosclerotic heart disease.

Plasma cholesterol levels have been shown to vary with age, pregnancy and the onset of the menopause, and are thought to be associated with alterations in plasma oestrogen levels (Stevenson *et al.*, 1993). During normal human pregnancy, the maternal plasma lipid profile undergoes a series of changes necessary for maintaining pregnancy, normal fetal development and preparing the mother for lactation. Plasma cholesterol typically increases by around 50-70% between 12 and 36 weeks gestation. HDL-cholesterol rises to a maximum at around 28 weeks gestation, and then declines towards term. Triglyceride concentrations increase markedly by around three-fold most dramatically in the second half of pregnancy (Sattar *et al.*, 1996).

Few studies have been published investigating the influence of pre-existing type 1 diabetes mellitus on maternal vascular responses, particularly since the important contribution of endothelial hyperpolarization has become clear. The aim of this study, therefore, was to determine the contribution of endothelium-derived hyperpolarization to endothelium-dependent vascular relaxation in pregnant women with and without type 1 diabetes mellitus. This would be achieved by measuring agonist-induced vasorelaxation responses in subcutaneous resistance arteries following incubation with inhibitors of endothelial mechanisms. Secondly, smooth muscle function, distinct from endothelial effects, would be assessed by observing cGMP- and cAMP-dependent vasorelaxation in the same patients using specific endothelium-independent agonists. It is also reasonable to suggest that some variability in total-, HDL- and LDL-cholesterol may correlate with functional measures of endothelial function, more specifically, between vascular dysfunction and lipid derangement in pregnant women with type 1 diabetes, since there are abundant data from the atherogenesis field demonstrating associations between lipids and vascular function (Sattar *et al.*, 1998; Sattar and Greer, 1999).

4.2 Methods

4.2.1 Study groups

Local Ethical Committee approval was granted. Written informed consent was obtained prior to caesarean section in eleven healthy pregnant and nine diabetic pregnant women at term, and prior to elective laparotomy in seven healthy non-pregnant women. All the women were normotensive and matched for maternal and gestational age. The diabetic women were well controlled on insulin, and did not require significant manipulation of their regimes with advancing gestation. The non-pregnant women were age-matched, fit and well, and not on any regular medication. Further patient characteristics including blood pressure, birthweight, glycosylated haemoglobin (HbA_{1c}) levels and disease duration are summarised in Table 4.1.

Standardising recruitment to include only women having abdominal surgery enabled the same vascular bed (abdominal subcutaneous resistance arteries) to be studied. The indications for caesarean section in the healthy pregnant women were previous caesarean section (seven), previous traumatic delivery (two), fetal distress (one) and breech presentation (one). The indications for caesarean section in the diabetic pregnant women were previous caesarean section (three), fetal distress (three) and failed induction of labour (three). The non-pregnant women were recruited from those admitted for elective gynaecological surgery, the indications of which were ovarian cystectomy (three), total abdominal hysterectomy (two), colposuspension (one) and myomectomy (one).

Table 4.1 Characteristics of patients recruited into the study investigating endothelium-dependent and -independent vasodilatation in isolated small arteries.

	Healthy pregnant	*Diabetic pregnant*	*Healthy non-pregnant*	*Significance*
Age (years)	30 ± 2 (n=11)	28 ± 2 (n=8)	35 ± 3 (n=7)	NS
Gestational age (weeks)	39 ± 0.2	37.5 ± 0.4	–	S
Birthweight (g)	3542 ± 151	3991 ± 242	–	NS
Maternal weight (kg)	63 ± 3	72 ± 4	69 ± 6	NS
Maternal BMI (kg/m^2)	25 ± 1	27 ± 1	26 ± 2	NS
Blood pressure (systolic) (mmHg)	114 ± 3	127 ± 4	123 ± 7	NS
Blood pressure (diastolic) (mmHg)	70 ± 3	79 ± 2	80 ± 4	NS
Smokers	1/11	0/11	3/7	–
HbA_{1c} (%)	–	6.3 ± 0.3	–	–
Duration of diabetes	–	15.1 ± 2.1	–	–

Values are expressed as mean ± standard error of the mean. n represents the number of women recruited. Significance assumed if $p<0.05$, one-way ANOVA and Bonferroni's post hoc test. *S*, significant; *NS*, not significant; *BMI*, body mass index; *HbA_{1c}*, glycosylated haemoglobin.

4.2.2 Experimental protocols

The standard methodology of small vessel wire myography has been explained in detail in Chapter 2. Only protocols and solutions specific to this chapter have been described.

A total of 34 small arteries with a mean luminal diameter of ~295μm were dissected free from the biopsies obtained. The vessels isolated achieved a mean relaxation of 84.4 ± 2.5% in healthy pregnant women, 81.5 ± 3.1% in diabetic pregnant women, and 82.1 ± 3.3% in non-pregnant women. Arteries that did not achieve a minimum relaxation of at least 60% were deemed to be unsuitable for studies of endothelial function and were excluded from the experiment. The rationale behind this has been previously explained in Chapter 2.

4.2.3 Pharmacological protocols

Four pharmacological protocols were used. All experiments were carried out in arteries pre-constricted with 10μM noradrenaline, which produces a sustained maximal contraction during the duration of the response curve. However, in studies where vessels were incubated with 25mM potassium chloride, the concentration of noradrenaline was titrated to achieve a similar pre-constriction tension to previous cumulative concentration response curves.

1. To quantify the contribution of hyperpolarization to endothelium-dependent relaxation, both nitric oxide and prostacyclin were inhibited. Cumulative

concentration response curves were performed to carbachol (1nM to 30μM), before and after 30 minutes incubation with:

a) N^{ω}-nitro-L-arginine methyl ester (nitric oxide synthase inhibitor, L-NAME 0.1mM)
b) L-NAME and indomethacin (cyclo-oxygenase inhibitor, INDO 30μM)
c) L-NAME, indomethacin and 25mM potassium chloride (inhibitor of endothelial hyperpolarization) (Gerber *et al.*, 1998).

2. To study the effectiveness of cAMP and cGMP pathways in the vascular smooth muscle, independent of endothelial modulation. Cumulative concentration response curves were performed to prostacyclin (1nM to 30μM) and sodium nitroprusside (1nM to 30μM), endothelium-independent vasodilatory agonists dependent on cAMP and cGMP respectively.

3. To ensure that any residual relaxation following nitric oxide synthase inhibition was not due to an incomplete inhibition of the pathway, the vessels were incubated for 30 minutes with L-NAME (0.1mM), indomethacin (30μM) and oxadiazoloquinoxalin, an inhibitor of soluble guanylate cyclase (ODQ 1μM), before studies were performed with carbachol (1nM to 30μM) and sodium nitroprusside (1nM to 30μM).

4. To confirm the contribution of endothelial hyperpolarization to the residual relaxation response following inhibition of nitric oxide synthase and cyclo-oxygenase. Carbachol and sodium nitroprusside studies were repeated following 30 minutes incubation with charybdotoxin (100nM) and apamin

(100nM), inhibiting both large and small conductance calcium-dependent potassium channels and endothelium-dependent hyperpolarization (Edwards *et al.*, 1998; Zygmunt *et al.*, 1997).

4.2.4 Measurement of plasma lipids

In a small number of women –eight healthy pregnant, three diabetic pregnant and three healthy non-pregnant, 5ml of venous blood was obtained from the ante-cubital fossa for measurements of plasma triglyceride, total-, HDL- and LDL-cholesterol levels after an overnight fast, and collected in bottles containing lithium heparin. The blood samples were centrifuged immediately upon collection for 10 minutes. The supernatant plasma was then removed and stored at -70°C, until serum analysis was performed.

4.2.5 Drugs and solutions

The drugs used were obtained from Sigma, Poole, UK, and CN Biosciences, Nottingham, UK, and made up in distilled H_2O, except for indomethacin which had to be dissolved in dimethyl sulphoxide (DMSO). The composition of the physiological saline solution (PSS) used has been described in Chapter 2. 25mM and 123mM potassium chloride solutions (KPSS) were made by substituting NaCl for KCl on an equimolar basis.

4.2.6 Statistical analysis

Relaxation is expressed as a percentage relative to the maximum tension generated following arterial pre-constriction with noradrenaline, mean ± standard error of the mean (SEM). The maximum response for each protocol was the greatest mean relaxation achieved and not the percentage relaxation attained at the highest concentration of carbachol. The number of experiments is represented as n(N), where n is the number of vessels and N the number of patients.

Maximum relaxation and sensitivity (pD_2 – negative log of the concentration required to produce 50% of the maximum response) were compared using one-way ANOVA and Bonferroni's post hoc test for multiple comparisons. A p value <0.05 was accepted as being statistically significant.

4.3 Results

4.3.1 Myography

1. Endothelium-dependent relaxation. As there were no differences in vessel responses between smokers and non-smokers, all results were combined (Table 4.2).

Table 4.2 Summary of maximum responses (%K^+) and sensitivity (pD_2) to carbachol in smoking and non-smoking patients.

	Healthy pregnant		*Healthy non-pregnant*	
	Smokers	***Non-smokers***	***Smokers***	***Non-smokers***
n	1	10	3	4
Carbachol				
Max. Relaxation (%)	94.4	92.5 ± 2.3	94.6	88.3 ± 6.8
pD_2	6.7	6.9 ± 0.1	6.6 ± 0.1	7.2 ± 0.3
+L-NAME				
Max. Relaxation (%)	72.3	74.4 ± 4.2	81.8 ± 1.5	79.9 ± 7.2
pD_2	6.2	6.7 ± 0.1	6.2	6.7 ± 0.3
+L-NAME+INDO				
Max. Relaxation (%)	72.1	74.5 ± 4.8	79.3 ± 9.1	73.1 ± 6.5
pD_2	6.6	7.4 ± 0.3	6.7 ± 0.5	7.0 ± 0.3
+L-NAME+INDO+25mM K^+				
Max. Relaxation (%)	42.8	33.5 ± 8.0	30.1 ± 0.4	27.5 ± 9.7
pD_2	7.5	6.6 ± 0.2	6.5 ± 0.4	5.9 ± 0.3

Values are mean ± SEM. *C*, carbachol; *L-N*, L-NAME; *Indo,* indomethacin.

Vasorelaxation responses to carbachol were similar in all three groups of patients with no significant differences found in either the maximum responses or the sensitivity (pD_2). Following incubation with L-NAME, the maximum relaxation was attenuated in all groups indicating a contribution of nitric oxide to endothelium-dependent relaxation. The maximum relaxation and pD_2 data between the three groups are summarised in Table 4.3.

Table 4.3 Relaxation responses of isolated arteries to carbachol with sequential inhibition of nitric oxide, prostacyclin and endothelial hyperpolarization. One-way ANOVA and Bonferroni's post hoc test was carried out between the three patient groups.

	Healthy pregnant	*Diabetic pregnant*	*Healthy non-pregnant*	*Significance*
Internal arterial diameter (μm)	294 ± 22	296 ± 23	293 ± 26	NS
Carbachol				
Max. Relaxation (%)	92.6 ± 2.1	95.5 ± 0.9	89.9 ± 5.2	NS
pD_2	6.8 ± 0.1	7.0 ± 0.14	7.0 ± 0.2	NS
+L-NAME				
Max. Relaxation (%)	74.3 ± 3.9	76.1 ± 3.4	80.4 ± 5.4	NS
pD_2	6.6 ± 0.1	6.7 ± 0.2	6.5 ± 0.2	NS
+L-NAME+INDO				
Max. Relaxation (%)	74.4 ± 4.5	74.5 ± 3.6	74.6 ± 6.3	NS
pD_2	7.3 ± 0.2	7.2 ± 0.3	6.9 ± 0.3	NS
+L-NAME+INDO+25mM K^+				
Max. Relaxation (%)	34.2 ± 7.4	28.2 ± 5.7	28.2 ± 7.3	NS
pD_2	6.6 ± 0.2	6.2 ± 0.2	6.0 ± 0.3	NS

Values are expressed as mean ± standard error of the mean, and refer to relaxation to carbachol with the sequential additions of inhibitors. Significance assumed if $p<0.05$, one-way ANOVA and Bonferroni's post hoc test. *NS*, not significant; *INDO*, indomethacin.

The contribution of nitric oxide was approximately twenty percent in the pregnant women regardless of whether they were diabetic, and approximately eleven percent in the non-pregnant women. Endothelial hyperpolarization appears to contribute largely to vasorelaxation in all three groups.

Figure 4.1 is an experimental concentration response curve to carbachol in an isolated resistance artery obtained from an insulin-dependent diabetic woman. Figs. 4.2.1, Fig. 4.2.2 and Fig. 4.2.3 show the cumulative concentration response curves as a percentage of the maximum potassium contraction to carbachol with the sequential inhibition of nitric oxide; nitric oxide and prostacyclin; and nitric oxide, prostacyclin and endothelial hyperpolarization in each of the three study groups.

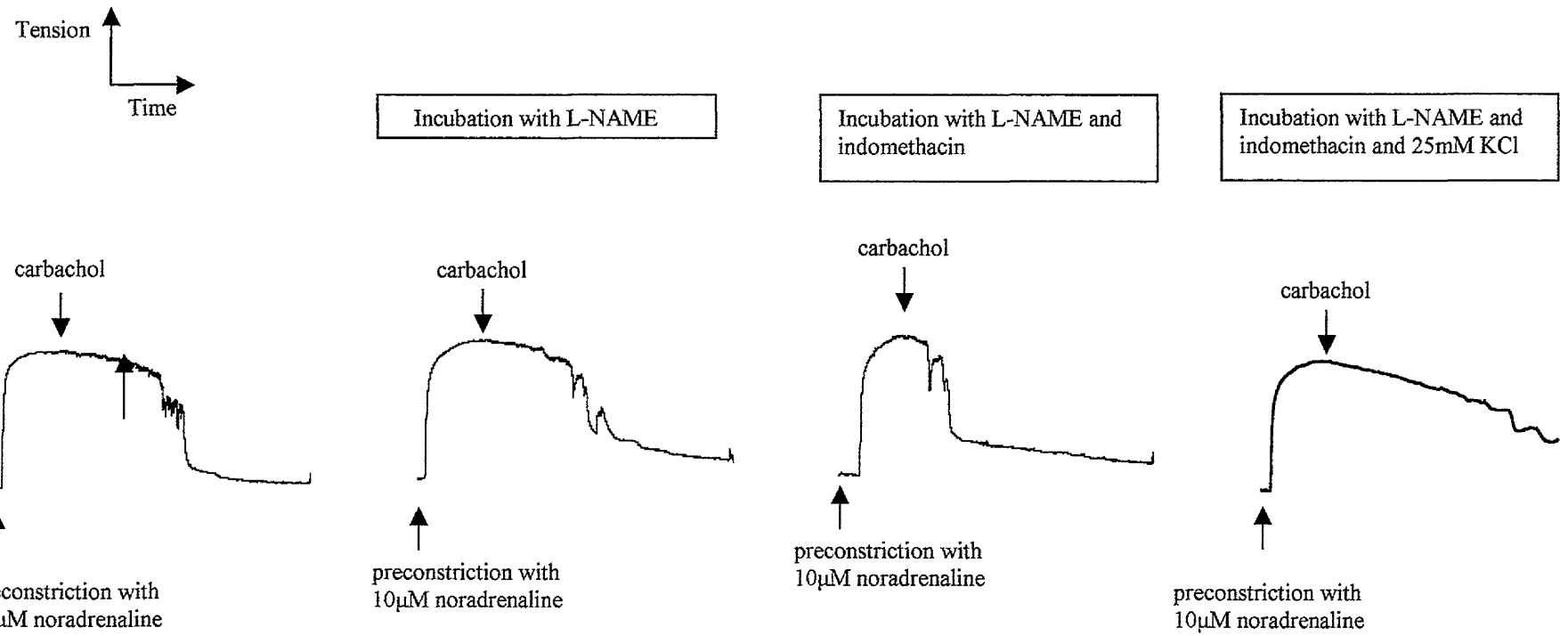


Fig. 4.1 A representative trace of a resistance artery exposed to cumulative concentrations of carbachol with the sequential inhibition of nitric oxide; nitric oxide and prostacyclin; and nitric oxide, prostacyclin and endothelial hyperpolarization.

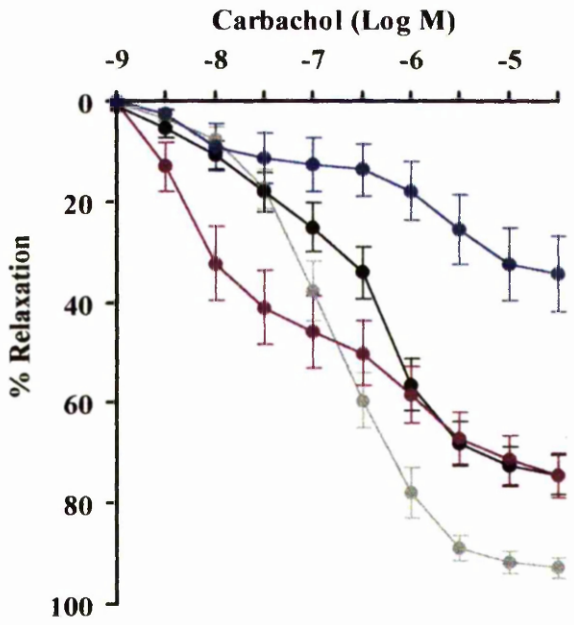


Fig. 4.2.1 Relaxation to carbachol (●) in healthy pregnant women (n=11) following incubation with N^{ω}-nitro-L-arginine methyl ester (●); N^{ω}-nitro-L-arginine methyl ester and indomethacin (●); and N^{ω}-nitro-L-arginine methyl ester, indomethacin and 25mM potassium chloride (●).

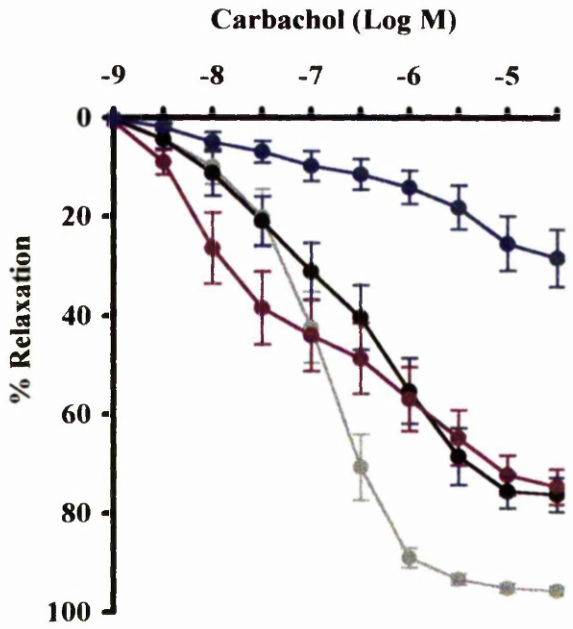


Fig. 4.2.2 Relaxation to carbachol (●) in diabetic pregnant women (n=9) following incubation with N^{ω}-nitro-L-arginine methyl ester (●); N^{ω}-nitro-L-arginine methyl ester and indomethacin (●); and N^{ω}-nitro-L-arginine methyl ester, indomethacin and 25mM potassium chloride (●).

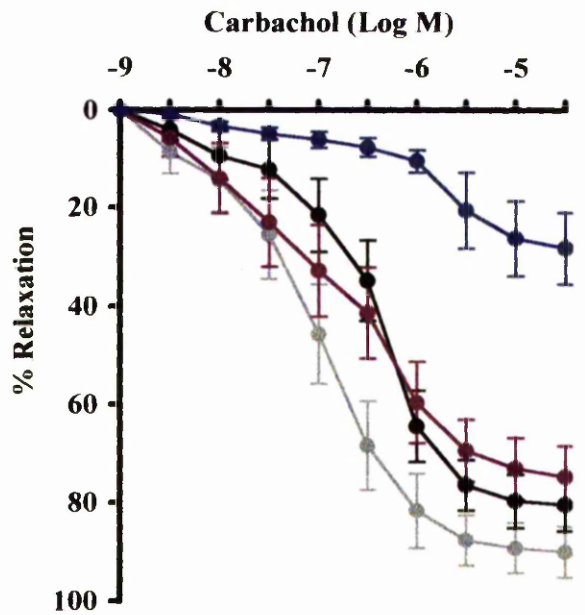


Fig. 4.2.3 Relaxation to carbachol (●) in healthy non-pregnant women (n=7) following incubation with N^{ω}-nitro-L-arginine methyl ester (●); N^{ω}-nitro-L-arginine methyl ester and indomethacin (●); and N^{ω}-nitro-L-arginine methyl ester, indomethacin and 25mM potassium chloride (●).

Fig. 4.3 is a descriptive representation of the percentage contributions of each relaxing factor in the individual patient groups.

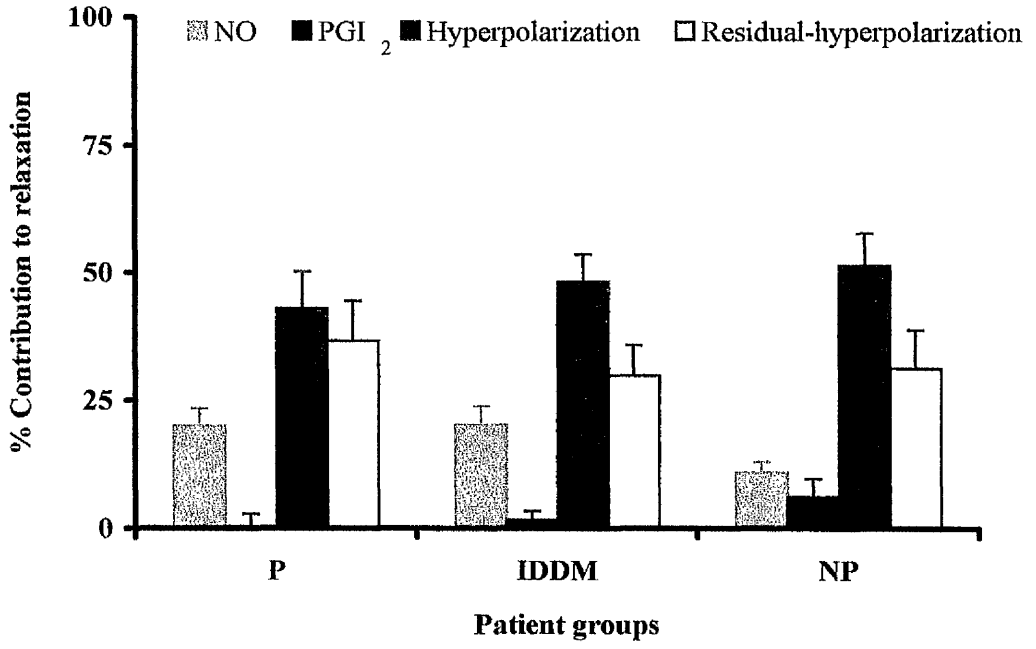


Fig. 4.3 Descriptive representation of the percentage contribution of nitric oxide (NO), prostacyclin (PGI_2) and endothelial hyperpolarization to endothelium-dependent relaxation in healthy pregnant (P, n=11), diabetic pregnant (IDDM, n=9) and healthy non-pregnant women (NP, n=7).

2. When exposed to prostacyclin, no difference in maximum relaxation or sensitivity was observed between the groups. Fig. 4.4.1 illustrates the raw experimental traces to prostacyclin in all three study groups, while Fig. 4.4.2 illustrates the maximum relaxation (%): healthy pregnant 85.8 ± 4.6%, n=14(11) *vs.* diabetic pregnant 82.5 ± 5.0%, n=12(9) *vs.* healthy non-pregnant 89.2 ± 5.4%, n=8(7). Sensitivity (pD_2): healthy pregnant 6.1 ± 0.2, n=14(11) *vs.* diabetic pregnant 6.5 ± 0.3, n=12(9) *vs.* healthy non-pregnant 5.9 ± 0.2, n=8(7).

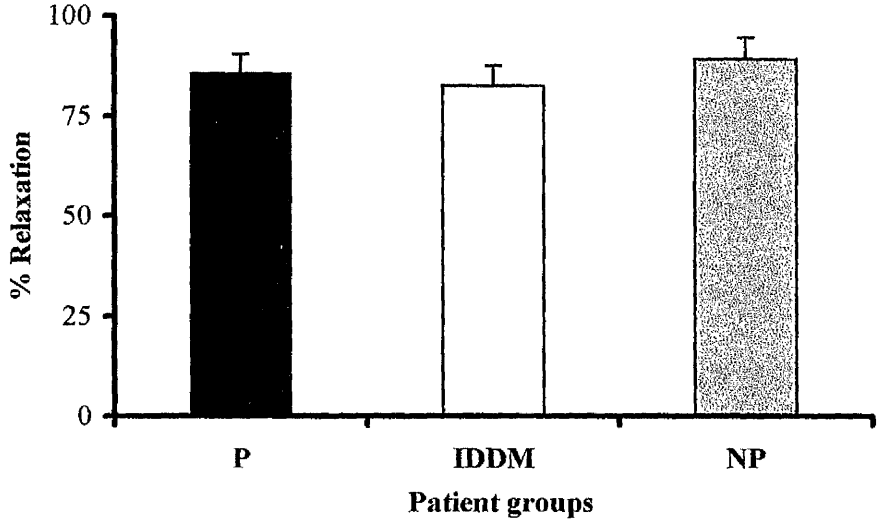


Fig. 4.4.2 Maximum relaxation to prostacyclin in isolated small arteries obtained from healthy pregnant (n=11), diabetic pregnant (n=9) and healthy non-pregnant (n=7) women.

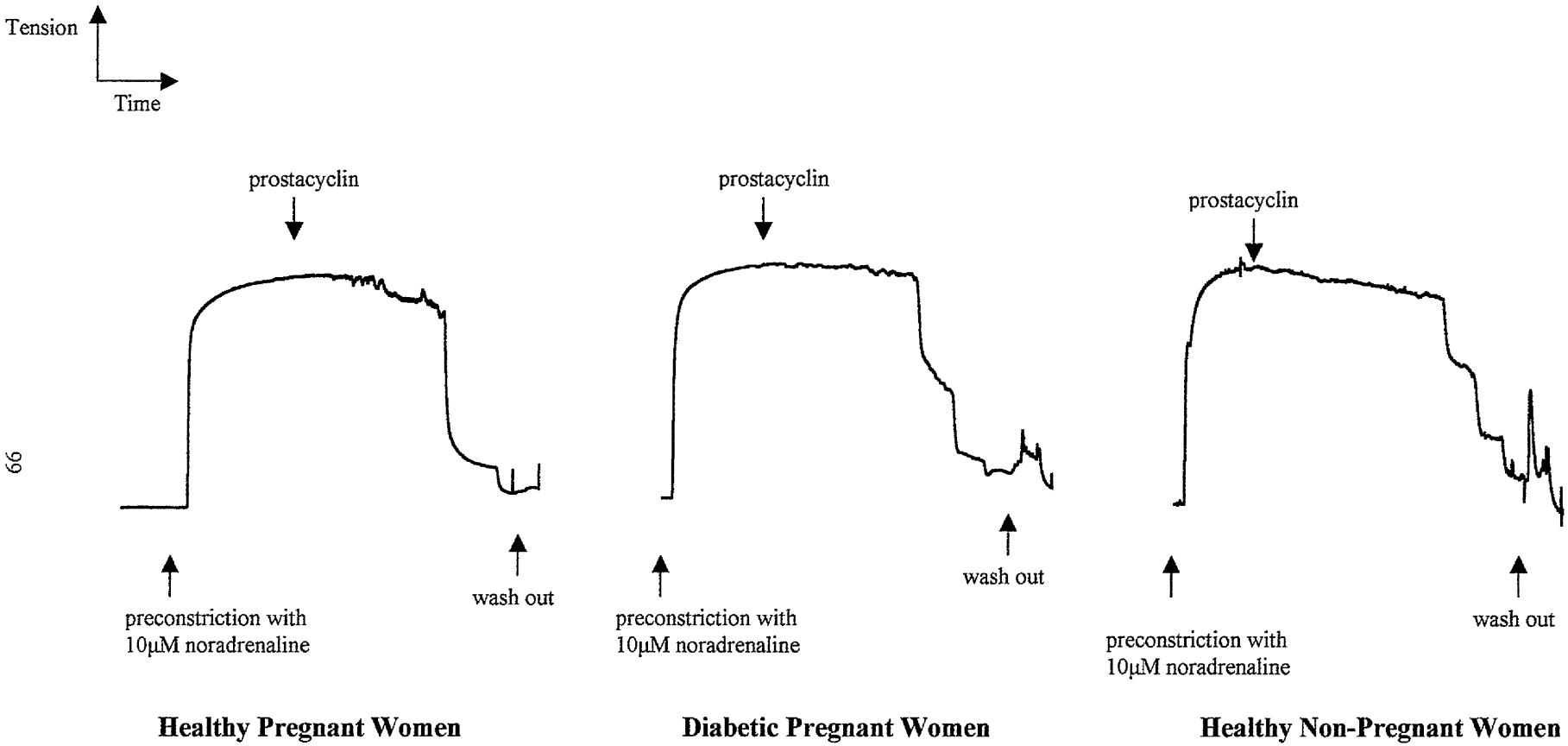


Fig. 4.4.1 Representative traces of a resistance artery exposed to cumulative concentrations of prostacyclin in each of the three study groups – healthy pregnant, diabetic pregnant and healthy non-pregnant women.

Following exposure to sodium nitroprusside, no difference in maximum relaxation or sensitivity was observed between the groups (Fig. 4.4.3 and Fig. 4.4.4). Maximum relaxation (%): healthy pregnant 92.3 ± 2.2%, n=14(11) *vs.* diabetic pregnant 95.0 ± 1.4%, n=12(9) *vs.* healthy non-pregnant 99.6 ± 0.2%, n=8(7). Sensitivity (pD_2): healthy pregnant 6.9 ± 0.2, n=14(11) *vs.* diabetic pregnant 7.3 ± 0.2, n=12(9) *vs.* healthy non-pregnant 7.3 ± 0.2, n=8(7).

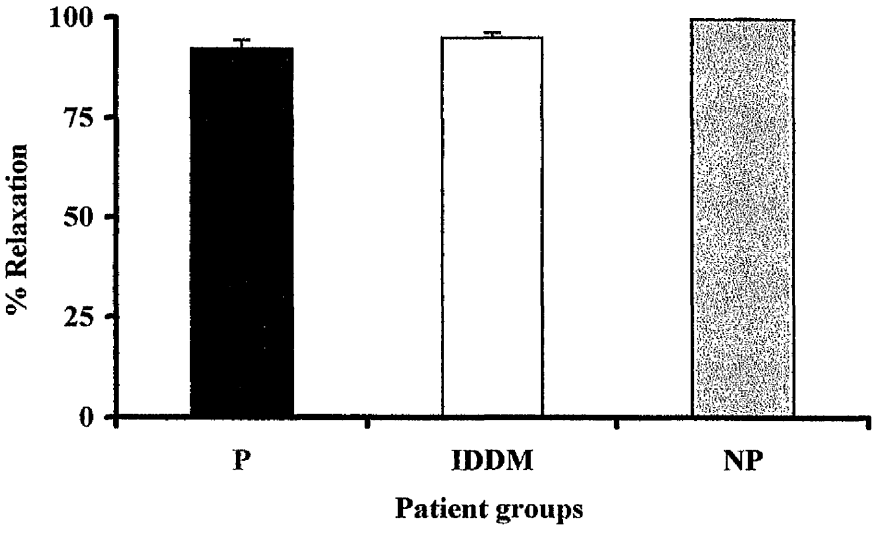


Fig. 4.4.4 Maximum relaxation to sodium nitroprusside in isolated small arteries obtained from healthy pregnant (n=11), diabetic pregnant (n=9) and healthy non-pregnant (n=7) women.

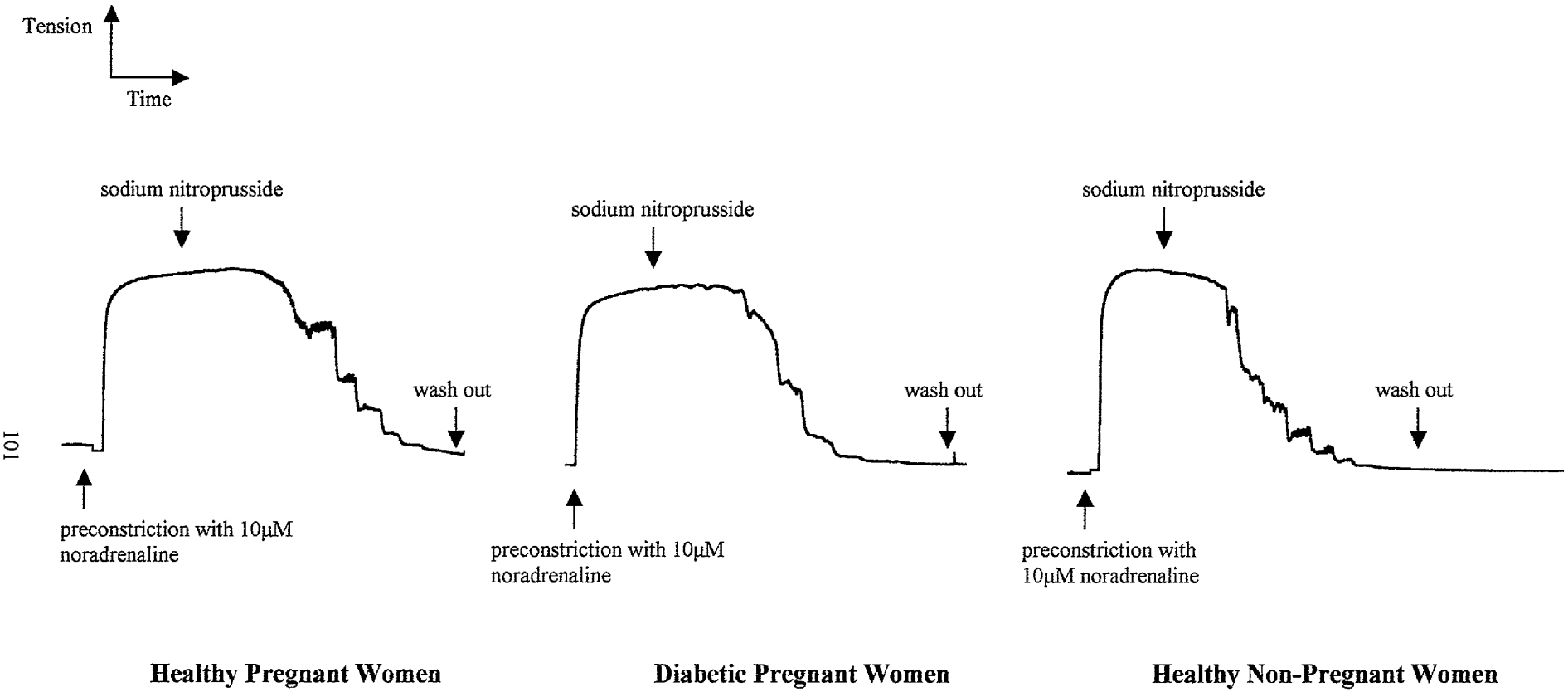


Fig. 4.4.3 Representative traces of a resistance artery exposed to cumulative concentrations of sodium nitroprusside in each of the three study groups –healthy pregnant, diabetic pregnant and healthy non-pregnant women.

3. The small degree of residual relaxation following incubation with L-NAME, indomethacin and 25mM potassium chloride was not due to incomplete blockade of nitric oxide synthase since ODQ was unable to inhibit the response further. Maximum relaxation to carbachol in the presence of L-NAME + indomethacin + 25mM potassium chloride: 16.4 ± 4.9% *vs.* L-NAME + indomethacin + 25mM potassium chloride + ODQ: 18.6 ± 4.3%, n=6(6), $p>0.05$. The observation of negligible relaxation to sodium nitroprusside tested the efficacy of ODQ. The maximum relaxation to SNP in the presence of L-NAME + indomethacin + 25mM potassium chloride + ODQ was 3.3 ± 1.4%, n=6(6). The raw experimental trace and the relaxation curves expressed as a percentage of the maximum tension following preconstriction with 10μM noradrenaline are shown in Fig. 4.5.1 and Fig. 4.5.2 respectively.

4. The combination of L-NAME, indomethacin, and the toxins –apamin and charybdotoxin, was able to fully inhibit endothelium-dependent relaxation. Maximum relaxation to carbachol in the presence of L-NAME + indomethacin + apamin + charybdotoxin: 1.9 ± 0.8%, n=6(6) (Fig 4.6.1 and Fig 4.6.2).

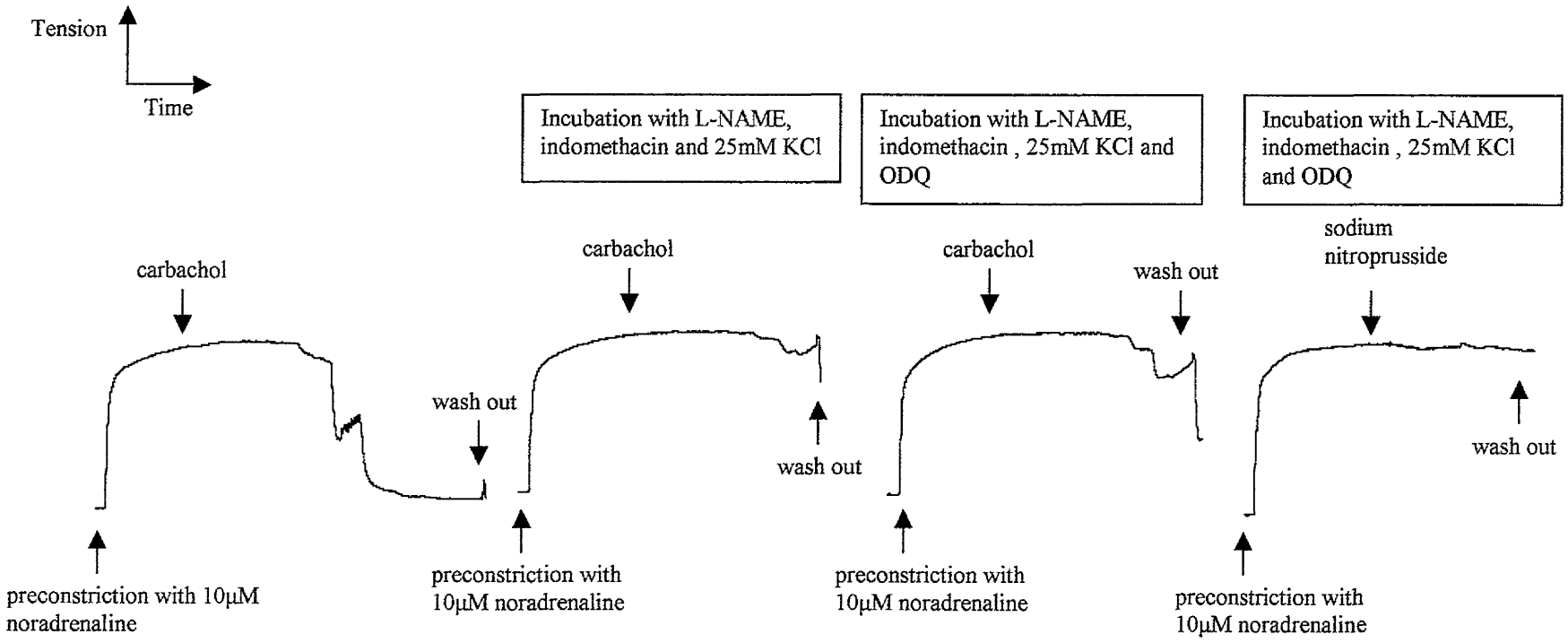


Fig. 4.5.1 A representative trace of a resistance artery obtained from a healthy pregnant woman exposed to cumulative concentrations of carbachol (a) in the absence of any inhibitors; (b) in the presence of L-NAME, indomethacin and 25mM KCl; and (c) L-NAME, indomethacin, 25mM KCl and ODQ. The last trace is the same artery exposed to cumulative concentrations of sodium nitroprusside in the presence of L-NAME, indomethacin, 25mM KCl and ODQ.

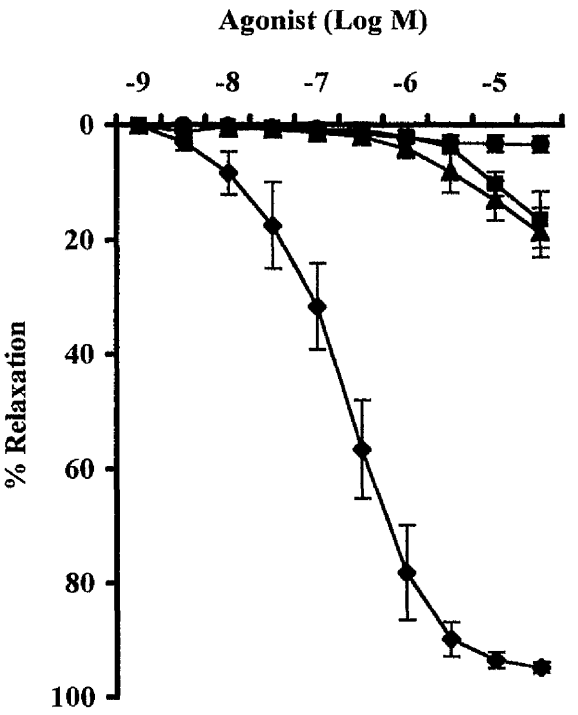


Fig. 4.5.2 Relaxation to carbachol (◆) in healthy pregnant women (n=6) following incubation with N^{ω}-nitro-L-arginine methyl ester, indomethacin and 25mM potassium chloride (■), N^{ω}-nitro-L-arginine methyl ester, indomethacin, 25mM potassium chloride and oxadiazoloquinoxalin (▲). Relaxation to sodium nitroprusside following incubation with N^{ω}-nitro-L-arginine methyl ester, indomethacin, 25mM potassium chloride and oxadiazoloquinoxalin (●).

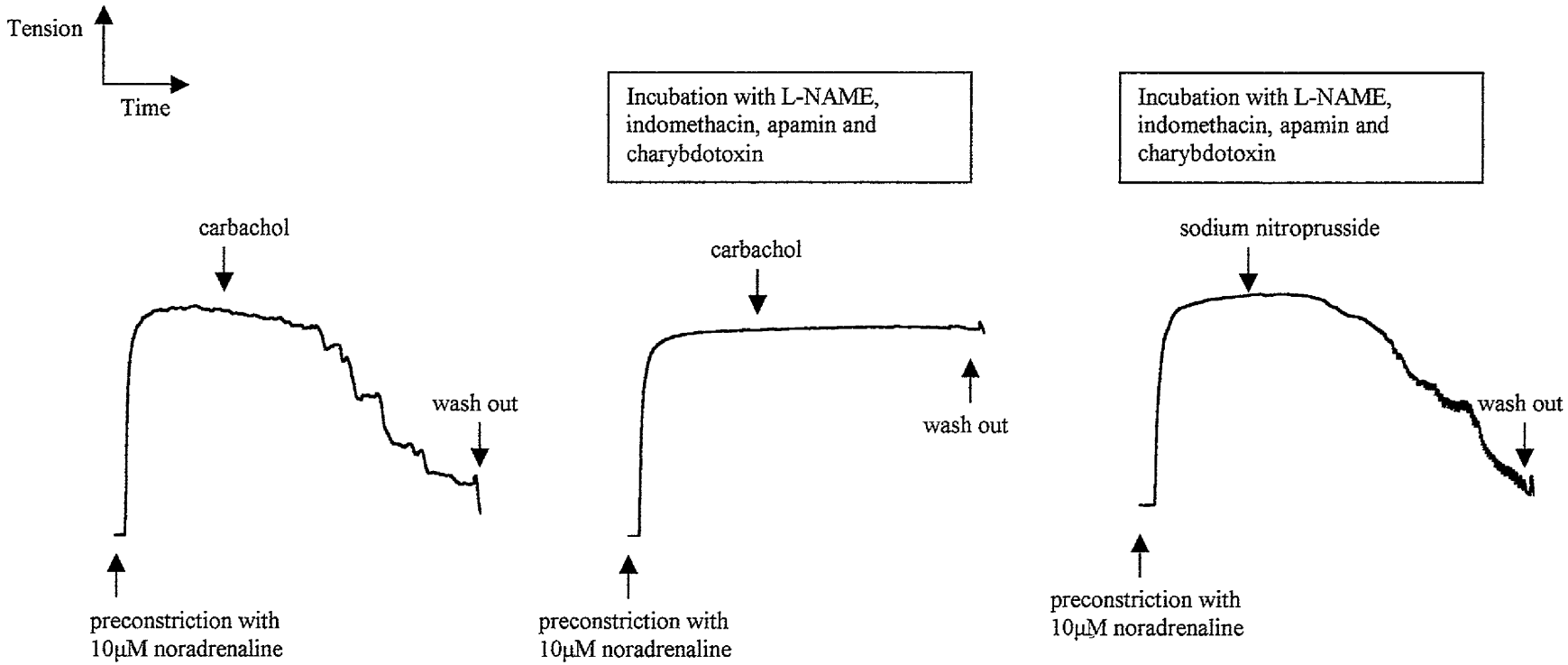


Fig. 4.6.1 A representative trace of a resistance artery obtained from a healthy pregnant woman exposed to cumulative concentrations of carbachol in the absence of any inhibitors, and repeated following incubation with L-NAME, indomethacin, apamin and charybdotoxin. The last trace is the same artery exposed to cumulative concentrations of sodium nitroprusside in the presence of L-NAME, indomethacin, apamin and charybdotoxin.

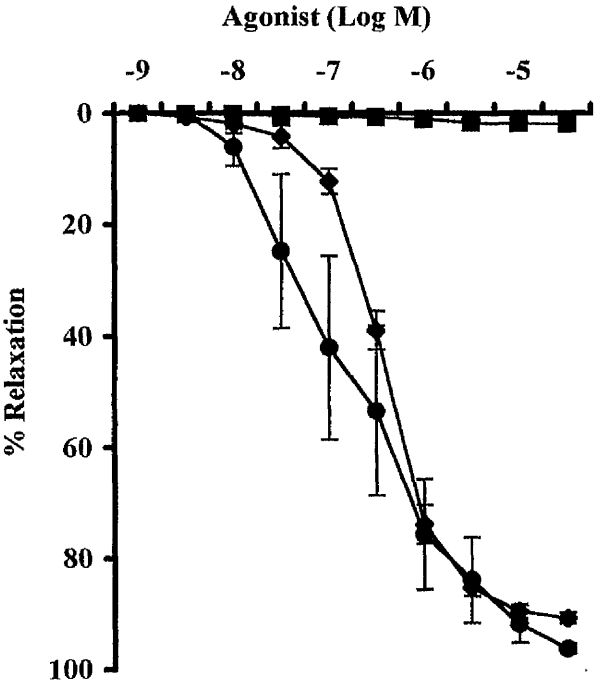


Fig. 4.6.2 Relaxation to carbachol (◆) in healthy pregnant women (n=6) following incubation with N^{ω}-nitro-L-arginine methyl ester, indomethacin, apamin and charybdotoxin (■). Relaxation to sodium nitroprusside following incubation with N^{ω}-nitro-L-arginine methyl ester, indomethacin, apamin and charybdotoxin (●).

4.3.2 Plasma lipids

1. **Triglycerides.** Plasma triglyceride levels were around three-fold higher in pregnant compared to non-pregnant women, and slightly higher in the women with diabetes. (Healthy pregnant 3.3 ± 0.5 mmol/L, Diabetic pregnant 3.7 ± 0.4 mmol/L, Healthy non-pregnant 1.1 ± 0.1 mmol/L, *p<0.05, Fig. 4.7).

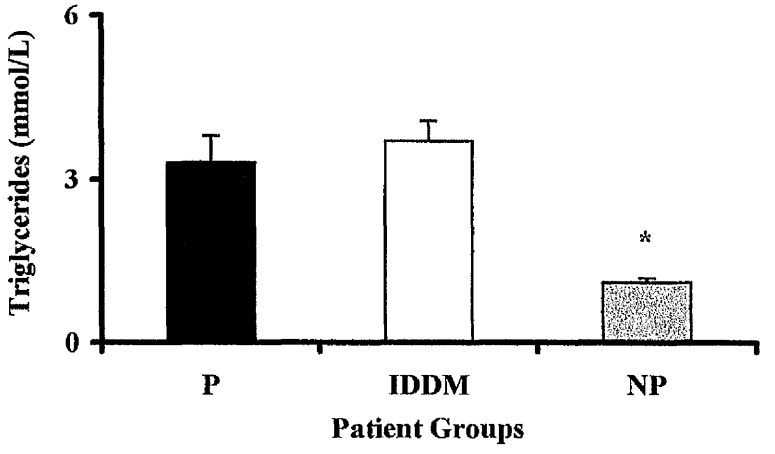


Fig. 4.7 A bar graph illustrating plasma triglyceride levels in healthy pregnant (P), diabetic pregnant (IDDM) and healthy non-pregnant (NP) women (*p<0.05).

2. **Total cholesterol.** Plasma total cholesterol levels were near double in non-diabetic and diabetic pregnant women, compared to non-pregnant women. (Healthy pregnant 7.2 ± 0.5 mmol/L, Diabetic pregnant 7.7 ± 0.3 mmol/L, Healthy non-pregnant 3.6 ± 0.2 mmol/L, *p<0.01, Fig. 4.8).

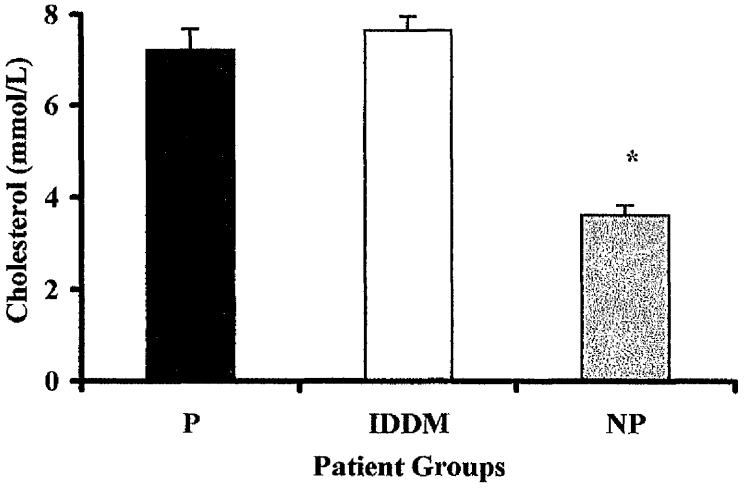


Fig. 4.8 A bar graph illustrating plasma total cholesterol levels in healthy pregnant (P), diabetic pregnant (IDDM) and healthy non-pregnant (NP) women (*p<0.01).

3. **HDL-cholesterol.** HDL-cholesterol concentrations were higher in both the women with healthy pregnancies, and to a lesser extent, in those with type 1 diabetes relative to the non-pregnant cohort, but probably due to smaller numbers such changes did not reach statistical significance. (Healthy pregnant 1.6 ± 0.1 mmol/L, Diabetic pregnant 1.4 ± 0.1 mmol/L, Healthy non-pregnant 1.2 ± 0.2 mmol/L, Fig. 4.9).

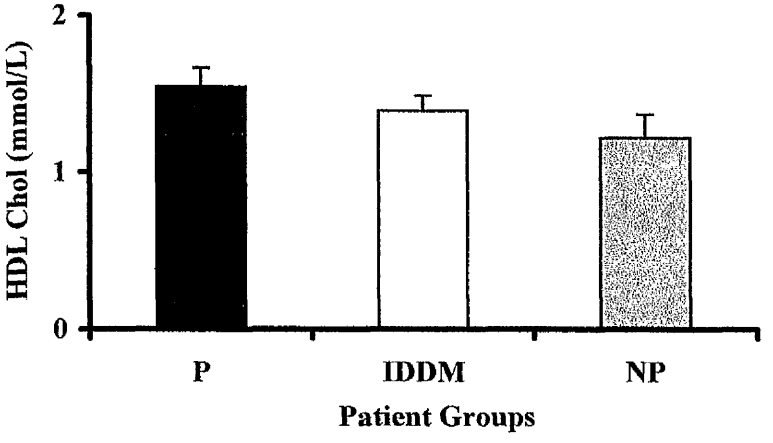


Fig. 4.9 A bar graph illustrating HDL-cholesterol levels in healthy pregnant (P), diabetic pregnant (IDDM) and healthy non-pregnant (NP) women.

4. **LDL-cholesterol.** LDL-cholesterol was approximately two to two-and-a-half times higher in the pregnant compared to the non-pregnant women. (Healthy pregnant 4.2 ± 0.4 mmol/L, Diabetic pregnant 4.6 ± 0.4 mmol/L, Healthy non-pregnant 1.9 ± 0.3 mmol/L, Fig. 4.10).

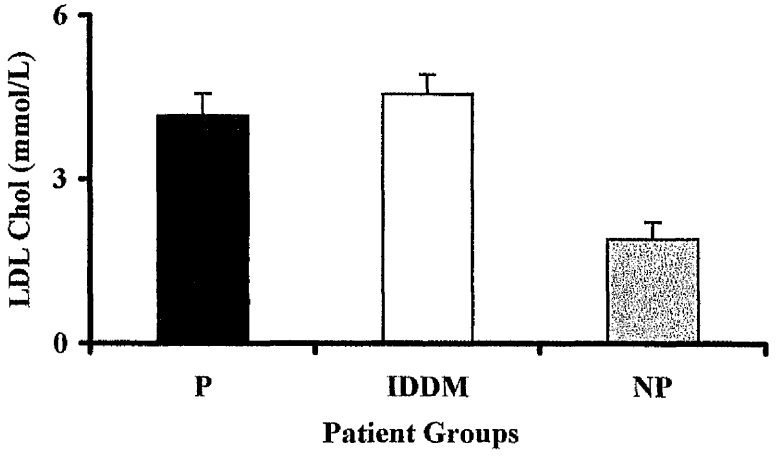


Fig. 4.10 A bar graph illustrating LDL-cholesterol levels in healthy pregnant (P), diabetic pregnant (IDDM) and healthy non-pregnant (NP) women.

4.4 Discussion

This study provides evidence that pregnant women with well-controlled pre-existing type 1 diabetes mellitus have normal endothelial and smooth muscle function in subcutaneous resistance vessels taken from the anterior abdominal wall. Furthermore, endothelial hyperpolarization appears to contribute largely to endothelium-dependent relaxation in pregnant women both with and without diabetes.

There is no apparent impairment of endothelial function in pregnant women with type 1 diabetes, which supports previous studies suggesting that endothelial function is maintained in patients with uncomplicated type 1 diabetes (McIntyre *et al.*, 2001; Smits *et al.*, 1993). These observations, in isolated subcutaneous resistance arteries obtained from the anterior abdominal wall, may be due to one of two reasons. Firstly, the assessment of endothelial function is determined by the degree of agonist-mediated vasorelaxation following pre-constriction. However, once a vessel reaches complete relaxation, any further increases in arterial luminal diameter cannot be achieved. Therefore, if present, a small degree of endothelial dysfunction may be unobservable in the diabetic women due to the increased vasodilatation associated with pregnancy. Secondly, the patients recruited were young women with a relatively shorter disease duration and tighter glycaemic control (due to closer supervision at joint medical/ante-natal clinics). This is reflected by the mean HbA_{1c} level, birthweight at delivery, and the delivery of the infants at term (>37 weeks) in this study population.

This study has also shown a preservation of smooth muscle function in pregnant women with type 1 diabetes, as responses to both prostacyclin and sodium nitroprusside were similar in all three groups of women. This implies that the normal activation of the second messenger pathways, cAMP and cGMP, in vascular smooth muscle following agonist stimulation is maintained in pregnant women with type 1 diabetes mellitus. Vascular smooth muscle tension is regulated by various vasoactive substances, which, in turn, modulate blood flow. *In vivo*, the resting tensions of smooth muscle (or the background tone) depend on a balance between endogenously produced vasodilators, such as nitric oxide, and vasoconstrictors including endothelin-1 and thromboxane A_2. The sequence of events that evokes smooth muscle responses is achieved by agonist-induced changes in the intracellular concentrations of second messenger molecules (Shaw and McGrath, 1996). The initiation of these responses is achieved by the interaction of the second messenger molecules with specific receptors located either in the plasma membrane or intracellular structures. The present experiments deal with the assessment of the cAMP and cGMP second messenger pathways via adenylate cyclase and guanylate cyclase, independent of the endothelium. Stimulation of these enzymes results in elevated intracellular concentrations of cAMP and cGMP, reduced intracellular calcium concentrations and smooth muscle relaxation. The normal vasodilatory response of the vessels studied suggests that the ability/sensitivity of the smooth muscle to respond to nitric oxide and prostacyclin is not impaired in pregnant diabetic women. As stated in the introduction, this observation is important for the correct understanding of the endothelium-dependent results, particularly those involving endothelium-derived nitric oxide and prostacyclin.

The literature has suggested an increased role of endothelium-dependent relaxing factors during pregnancy, contributing to significant vasodilatation (Greer *et al.*, 1985a; Williams *et al.*, 1997). However, previous studies have mainly concentrated on contributions by nitric oxide and prostacyclin with little emphasis on the importance of endothelium-derived hyperpolarization. These data appear to demonstrate an increased contribution of nitric oxide of approximately twenty percent in pregnant women and approximately eleven percent in non-pregnant women. However, the contribution of endothelial hyperpolarization is, qualitatively, at least twice that of nitric oxide in pregnant women, and five-fold greater in non-pregnant women, supporting other studies which suggest that endothelium-dependent hyperpolarization may be more prominent in resistance arteries (Coats *et al.*, 2001; Gerber *et al.*, 1998; Shimokawa *et al.*, 1996). It is possible, however, that the blockade of one vasodilatory pathway may result in a proportionately greater contribution from another pathway. However, limited by the constraints if time, it was not possible to assess the relative importance of endothelial hyperpolarization on its own, without inhibiting nitric oxide and prostacyclin. As yet, a precise chemical factor responsible for endothelium-derived hyperpolarization remains to be identified, but a P450 cytochrome-derivative is thought to be involved in human subcutaneous small arteries (Coats *et al.*, 2001). However, more recent evidence suggests that electrical propagation across myoendothelial gap junctions is likely to account for the endothelial hyperpolarization, and therefore the failure to identify a single chemical factor as EDHF (Coleman *et al.*, 2001a; Coleman *et al.*, 2001b; Coats *et al.*, 2001; Edwards *et al.*, 1998).

Following combined inhibition with L-NAME, indomethacin and 25mM potassium chloride, a residual relaxation of ~30% was still observed in the carbachol response, which was either due to incomplete inhibition of nitric oxide or endothelial hyperpolarization. Two assumptions were important if this dataset was to be interpreted as true residual relaxation. Firstly, it was assumed that L-NAME had successfully blocked all production of nitric oxide. Secondly, it was assumed that 25mM potassium chloride could fully eliminate endothelium-dependent hyperpolarization. Earlier studies suggested that L-NAME may not fully inhibit the nitric oxide synthase pathway (Chauhan *et al.*, 2000), and Gerber *et al.* reported that pre-incubation with 25mM potassium chloride completely abolished the effects of endothelium-derived hyperpolarization in rat mesenteric arteries (Gerber *et al.*, 1998). In this study, the efficacy of L-NAME was confirmed by incubating the vessels with L-NAME, indomethacin, 25mM potassium chloride, and oxadiazoloquinoxalin (ODQ, an inhibitor of cGMP production). In the presence of ODQ, no further inhibition of the carbachol response was observed. The efficacy of ODQ (1μM) was tested by repeating that protocol with the nitric oxide donor, sodium nitroprusside. As sodium nitroprusside only showed ~3% relaxation in the presence of ODQ, it appeared extremely unlikely that the remaining relaxation in the carbachol experiments was due to incomplete nitric oxide synthase inhibition. Similarly, to confirm the efficacy of 25mM potassium chloride as a blocker of hyperpolarization, the experiments were repeated with the potassium solution substituted with apamin (100nM) and charybdotoxin (100nM) (known to inhibit both small and large conductance calcium-dependent potassium channels and thus endothelium-dependent hyperpolarization (Edwards *et al.*, 1998). The combination of charybdotoxin and apamin abolished the residual relaxation (in the carbachol

experiments), suggesting that the previous residual relaxation had been due to the incomplete inhibition of endothelial hyperpolarization (Fig. 4.1).

The non-nitric oxide, non-prostanoid effect appears to be sensitive to the combined effects of apamin and charybdotoxin, suggesting an involvement of large and small conductance calcium-dependent potassium channels. However, potassium itself can induce a direct hyperpolarization and relax smooth muscle by either stimulating ouabain-sensitive sodium-potassium-ATPase, inward-rectifying potassium channels, or stimulate the release of a 'vasodilator mediator(s)' from perivascular nerves that induce endothelium-dependent vasodilatation (McCarron and Halpern, 1990; Knot *et al.*, 1996; Rubanyi and Vanhoutte, 1988). Pascoal and Umans explored endothelium-dependent vasodilatation in human omental resistance arteries, focusing on the action of nitric oxide-independent relaxing factors in both pregnant and non-pregnant women (Pascoal and Umans, 1996). They showed that vessels obtained from pregnant women relaxed to bradykinin in the presence of nitric oxide synthase, cyclo-oxygenase and non-selective potassium channel blockade, while vessels obtained from non-pregnant women showed minimal (12%) relaxation in the presence of the same three inhibitors. In contrast to my findings, they also suggested that endothelium-dependent vasodilatation pregnancy may be associated a hyperpolarizing vasodilator, which may not be a potassium channel factor.

The failure of 25mM potassium chloride to abolish endothelial hyperpolarization in human subcutaneous arteries when it has been shown to be effective in rat mesenteric arteries, may be due to the fact that the hyperpolarizing effect now

seems more likely to be due to electrical coupling, rather than a single identifiable chemical factor (Coleman *et al.*, 2001a; Coleman *et al.*, 2001b; Sandow and Hill, 2000). In the present study, the contribution of hyperpolarization to endothelium-dependent relaxation may therefore be more important than previously thought. In this study, it is responsible for approximately eighty percent of the total vasorelaxation response to carbachol, with no observable difference between the patient groups. However, this assumes that the mechanisms underlying vascular function act independently of each other. It is possible that inhibiting nitric oxide production may in fact enhance the potency of endothelial hyperpolarization. The role of endothelial hyperpolarization may therefore only appear to be increased in comparison to that if nitric oxide. Moreover, the earlier suggestion that prostacyclin may be an important mediator of vascular tone in pregnancy, is not supported by this data. Although prostacyclin does contribute slightly more to relaxation in non-pregnant compared to pregnant women, when compared to endothelial hyperpolarization the effect is considerably less important.

Diabetic patients who demonstrate impaired vasodilatation may reflect either underlying structural or functional abnormalities within the endothelium and/or smooth muscle. It is possible that the elevated levels of free radicals produced in poorly controlled diabetes may contribute to this dysfunction. The different manifestations of diabetes on vascular smooth muscle function appear to differ between different populations. The large majority of studies demonstrate impaired vasodilatation to endothelium-dependent agonists in the presence of preserved responses to endothelium-independent vasodilators in type 1 diabetes (Johnstone *et al.*, 1993; McNally *et al.*, 1994). However, others have also demonstrated impaired

endothelium-independent vasodilatation in diabetes (Calver *et al.*, 1992). This suggests that diabetes does not appear to cause a generalised reduction in the sensitivity of smooth muscle to endogenous vasodilators.

As expected from previous literature, levels of triglycerides (and VLDL-cholesterol), total- and LDL-cholesterol were increased in pregnant compared to non-pregnant women, as were HDL-cholesterol concentrations to a lesser extent. The co-existence of diabetes during pregnancy may affect lipid metabolism, however due to the small numbers of available plasma for lipid analysis in this study, we were unable to address this potential. It is of note, however, that others have revealed deleterious alterations in lipids with poorly controlled diabetes in pregnancy, with higher triglyceride and lower HDL-cholesterol levels relative to healthy pregnant women (Merzouk *et al.*, 2000).

Studies have demonstrated that abnormal lipid patterns (especially oxidised LDL which is raised in diabetes) can inactivate nitric oxide and result in vascular damage (Schmidt *et al.*, 1991; Bucala *et al.*, 1991; Sattar *et al.*, 1998). Furthermore, the lipid changes observed during poorly controlled diabetic pregnancies have been shown to be greater than in non-diabetic pregnancies, but in women with good control, lipid changes are comparable to normal pregnancy (Merzouk *et al.*, 2000). Plasma cholesterol and triglyceride concentrations are known to rise in pregnancy, with the increase in triglyceride concentrations being primarily due to an increase in VLDL-cholesterol concentrations (Potter and Nestel, 1979; Sattar *et al.*, 1997). Although analysis of plasma lipids was carried out on a small number of women, the apparent trend demonstrates a significant elevation of triglyceride, total- and

LDL-cholesterol in pregnant women as would be expected. These increases may be important in meeting the metabolic demands of the fetus, and to prepare the expectant mother for lactation. There did not appear to be any difference in either lipid levels or vascular function between diabetic and non-diabetic pregnant women, which may reflect the relatively tighter degree of glycaemic control and the apparent presence of uncomplicated disease in these women.

No significant difference was observed in the HDL-cholesterol levels between the diabetic/non-diabetic pregnant and the non-pregnant women, which may be due to the small number of women studied. The literature suggests that mean HDL-cholesterol levels peak at 28 weeks gestation, and are significantly higher in pregnant compared to the non-pregnant women (Sattar *et al.*, 1996). Other studies have also suggested that the increase in HDL-cholesterol may be due to the elevated oestrogen levels in pregnancy, promoting the hepatic synthesis of HDL and reducing hepatic lipase activity (Sattar and Greer, 1999; Desoye *et al.*, 1987).

4.5 Conclusions

This study provides evidence that pregnant women with well-controlled pre-existing type 1 diabetes have both normal endothelial and smooth muscle function in isolated subcutaneous abdominal resistance arteries. Endothelial hyperpolarization appears to contribute largely to endothelium-dependent relaxation in human subcutaneous arteries, and is not altered by pregnancy or the presence of diabetes in pregnancy. Furthermore, there did not appear to be any suggestion of abnormal lipid patterns in the diabetic women, which may reflect the importance of

good metabolic control. The physiological increase in peripheral vasodilatation in pregnancy may contribute to the maintenance of vascular function and tissue perfusion in pregnant diabetic women. Although underlying diabetes does have some effect on the pregnant mother, fears of endothelial dysfunction leading to damaging vascular disorders are probably unfounded in well-controlled diabetics.

Chapter Five

The Effect of Endothelin-1 on Vasoconstrictor Responses in Pregnant Women with Type 1 Diabetes Mellitus

5.1 Introduction

The preceding chapters of this thesis have examined the vasodilatory effect of insulin on subcutaneous resistance arteries in healthy pregnant women, and endothelium-dependent relaxation in pregnant women with and without type 1 diabetes mellitus. As well as vasodilator mechanisms, the assessment of vascular function should also involve the study of vessel responses to endogenous vasoconstrictors, such as nordarenaline and endothelin-1.

Endothelin-1 (ET-1) is an endothelium-derived vasoconstrictor peptide that contributes to basal vascular tone, and is also thought to play a role in pregnancy, both from a maternal (utero-placental function) and fetal (embryogenesis) perspective (Bobik *et al.*, 1990; Douglas and Ohlstein, 1997). Its potent vasoconstrictor properties have implicated it in the pathogenesis of diseases associated with abnormal vascular function, such as pre-eclampsia and hypertension (Wolff *et al.*, 1996a; Lind *et al.*, 1999)

It has been suggested that the pathogenesis of diabetic micro-angiopathy involves an initial increase in micro-vascular blood flow leading to micro-vascular sclerosis and disturbed autoregulation (McAuley *et al.*, 2000). Alterations in the vasoconstrictor response to ET-1 in diabetes may therefore result in hyperperfusion and subsequent micro-vascular damage. Elevated insulin levels have been linked to increased endothelin receptor expression, possibly by up-regulating endothelin receptors (Wu *et al.*, 2000), while vasoconstrictor responses to ET-1 have been shown to be enhanced in insulin resistant arteries due to an enhanced expression of

ET receptors and underlying endothelial dysfunction (Katakam *et al.*, 2001). Hyperinsulinaemia and insulin resistance may result in an imbalance between the action of nitric oxide and endothelin-1, thereby increasing vascular tone and vascular dysfunction. It is possible that the elevated plasma ET-1 levels seen in diabetic patients may be one of the factors contributing to the development of the vascular complications of the disease. Plasma levels have been shown to remain constant throughout pregnancy, with similar levels seen in non-pregnant women, but doubled in pregnant women with diabetes (Wolff *et al.*, 1997). This may be an important finding due to the fact that pregnant women with diabetes are at an increased risk of developing pre-eclampsia compared to their non-diabetic counterparts.

As physiologically elevated levels of insulin are seen in healthy pregnant women, the interaction between insulin and ET-1 may be amplified in pregnancy, especially in the presence of (pre-pregnancy) diabetes. Although I have demonstrated a preservation of endothelial and smooth muscle function in this particular group of type 1 diabetic pregnant women, changes in the response of subcutaneous resistance arteries to ET-1 may still be demonstrated in these women. This study aims to investigate the effect of type 1 diabetes on the vasoconstrictor response to ET-1 in pregnant women. It is hypothesised that isolated arteries obtained from pregnant women with diabetes will demonstrate differences in both maximum response and sensitivity when exposed to ET-1 compared to non-diabetic pregnant women.

5.2 Methods

5.2.1 Study groups

Local Ethical Committee approval was granted, and the study carried out in accordance with the Declaration of Helsinki (1996). Written informed consent was obtained prior to caesarean section in nine non-diabetic pregnant women and seven diabetic pregnant women; and prior to laparotomy in five non-diabetic non-pregnant women. All women were normotensive and matched for maternal and gestational age. Diabetic patients were well controlled on insulin, and did not require significant manipulation of their regimes with advancing gestation. Further patient characteristics including blood pressure, birthweight, HbA_{1c} levels and disease duration are summarised in Table 5.1.

Standardising recruitment to include only women having abdominal surgery enabled the same vascular bed to be studied. The indications for caesarean section in the healthy pregnant women were fetal cardiac anomaly (one), breech presentation (one), multiple pregnancy (one) and previous caesarean section (six). The indications for caesarean section in the diabetic pregnant women were previous caesarean section (two), fetal distress (four) and failed induction (one). All non-pregnant women underwent elective gynaecological surgery.

Table 5.1 Characteristics of patients recruited into the study investigating effect of endothelin-1 on isolated small arteries.

	Healthy pregnant	*Diabetic pregnant*	*Healthy non-pregnant*	*Significance*
Age (years)	31 ± 1 (n=9)	29 ± 2 (n=7)	37 ± 3 (n=5)	S
Gestational age (weeks)	39.4 ± 0.3	37.3 ± 0.4	–	S
Birthweight (g)	3677 ± 163	4040 ± 271	–	NS
Blood pressure (systolic) (mmHg)	120 ± 4	127 ± 5	126 ± 5	NS
Blood pressure (diastolic) (mmHg)	74 ± 3	78 ± 2	79 ± 4	NS
Smokers	2/9	0/7	1/5	–
HbA_{1c} (%)	–	6.2 ± 0.4	–	–
Duration of diabetes (years)	–	16.6 ± 1.9	–	–

Values are expressed as mean ± standard error of the mean. n represents the number of women recruited. Significance assumed if $p<0.05$, one-way ANOVA and Bonferroni's post hoc test. *S*, significant; *NS*, not significant; *HbA_{1c}*, glycosylated haemoglobin.

5.2.2 Experimental protocols

The standard methodology of small vessel wire myography has been explained in detail in Chapter 2. Only protocols and solutions specific to this chapter have been described.

Small arteries ~280μm were dissected free from the biopsies of subcutaneous fat obtained at laparotomy. The vessels isolated achieved a mean relaxation of 70.1 ±

2.8% in healthy pregnant women, 71.9 ± 7.2% in diabetic pregnant women, and 71.7 ± 3.2% in non-pregnant women.

5.2.3 Pharmacological protocols

Two pharmacological protocols were used:

Study 1 – Cumulative contraction curves (CRCs) were performed to noradrenaline (1nM to 30 μM).

Study 2 – CRCs to ET-1 (1pM to 0.3μM) were carried out.

5.2.4 Drugs and solutions

All drugs were obtained from Sigma, Poole, UK, and made up in distilled H_20. PSS and KPSS were made up as described in Chapter 2.

5.2.5 Statistical analysis

Contractile responses to potassium were expressed as isometric tension in mN/mm. Responses to noradrenaline and ET-1 were expressed as a percentage of each individual vessel's maximum potassium contraction, allowing for the standardization of responses between vessels. Data are expressed as the mean ± standard error of the mean (SEM). The number of experiments is represented as n(N), where n is the number of vessels and N the number of patients.

In this study, maximum contraction and sensitivity (pD_2 – negative log of the concentration required to produce 50% of the maximum response) were compared using Student's *t* test, with a p value <0.05 being accepted as statistically significant.

5.3 Results

As there were no differences in vessel responses between the smokers and non-smokers, all results were combined (Table 5.2).

Table 5.2 Summary of maximum responses ($\%K^+$) and sensitivity (pD_2) to endothelin-1 in smoking and non-smoking women.

	Healthy pregnant		*Healthy non-pregnant*	
	Smokers	***Non-smokers***	***Smokers***	***Non-smokers***
n	2	7	1	4
Max. Response (%)	134.6 ± 11.9	147.6 ± 15.1	111.0	99.9 ± 10.3
pD_2	5.4 ± 0.3	5.5 ± 0.1	4.9	5.4 ± 0.3

Values are mean ± SEM.

There was also no significant difference in the maximum response to K^+ or noradrenaline between the three groups of women. These results are summarised in Fig. 5.1 and Fig. 5.2.

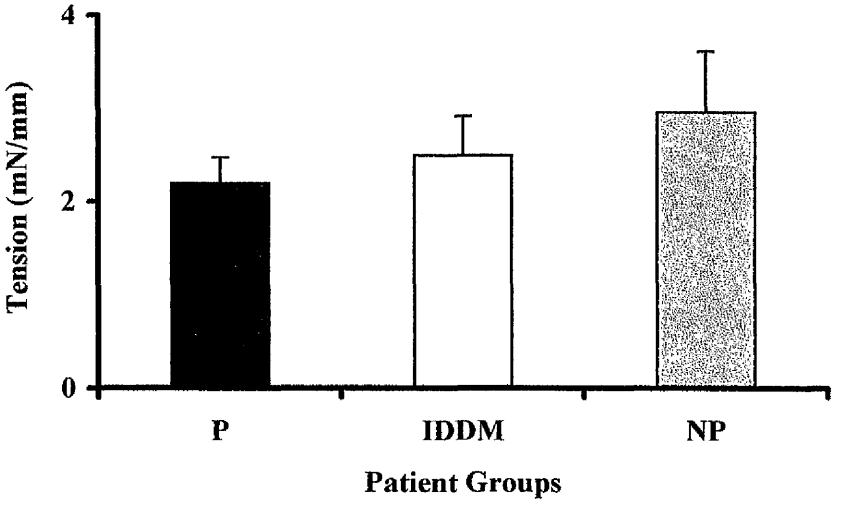


Fig. 5.1 A bar graph of the maximum vessel responses to potassium (K^+) in healthy pregnant (P), diabetic pregnant (IDDM) and healthy non-pregnant (NP) women.

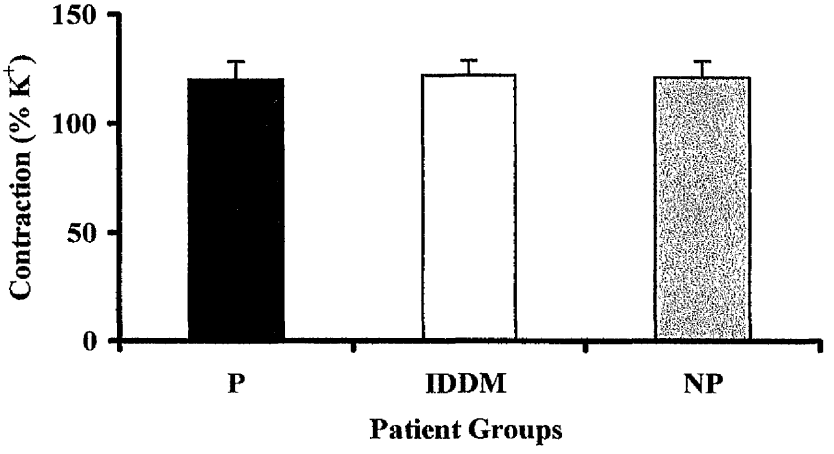


Fig. 5.2 A bar graph of the maximum vessel responses to noradrenaline in healthy pregnant (P), diabetic pregnant (IDDM) and healthy non-pregnant (NP) women.

The raw experimental traces of resistance arteries exposed to cumulative concentrations of endothelin-1 in each of the three study groups –healthy pregnant, diabetic pregnant and healthy non-pregnant women are shown in Fig 5.3.

The maximum response to ET-1 was significantly increased in pregnant compared to non-pregnant women [Maximum response (%K^+) non-diabetic pregnant: 142.4 ± 12.4%, n=9(9) *vs.* non-pregnant: 101.8 ± 8.8%, n=6(5) *p<0.05, Fig. 5.4]. No difference in the maximum response was seen between the diabetic and non-diabetic pregnant women [Maximum response (%K^+) non-diabetic pregnant: 142.4 ± 12.4%, n=9(9) *vs.* diabetic pregnant: 138.3 ± 6.8%, n=8(7)].

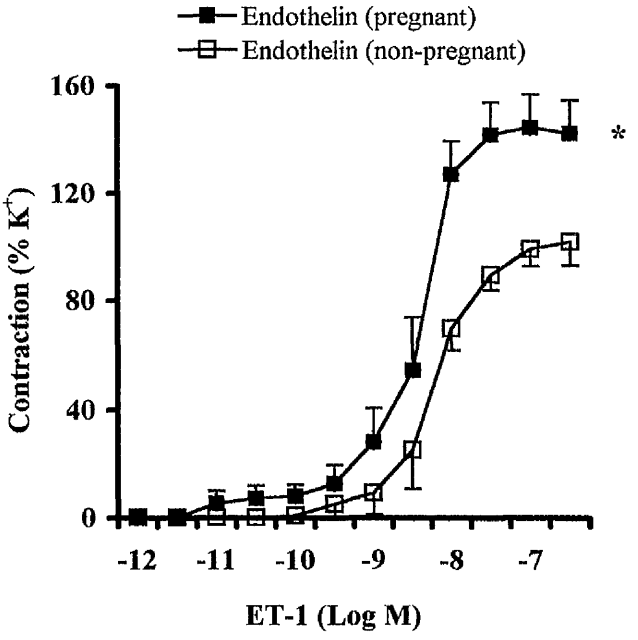
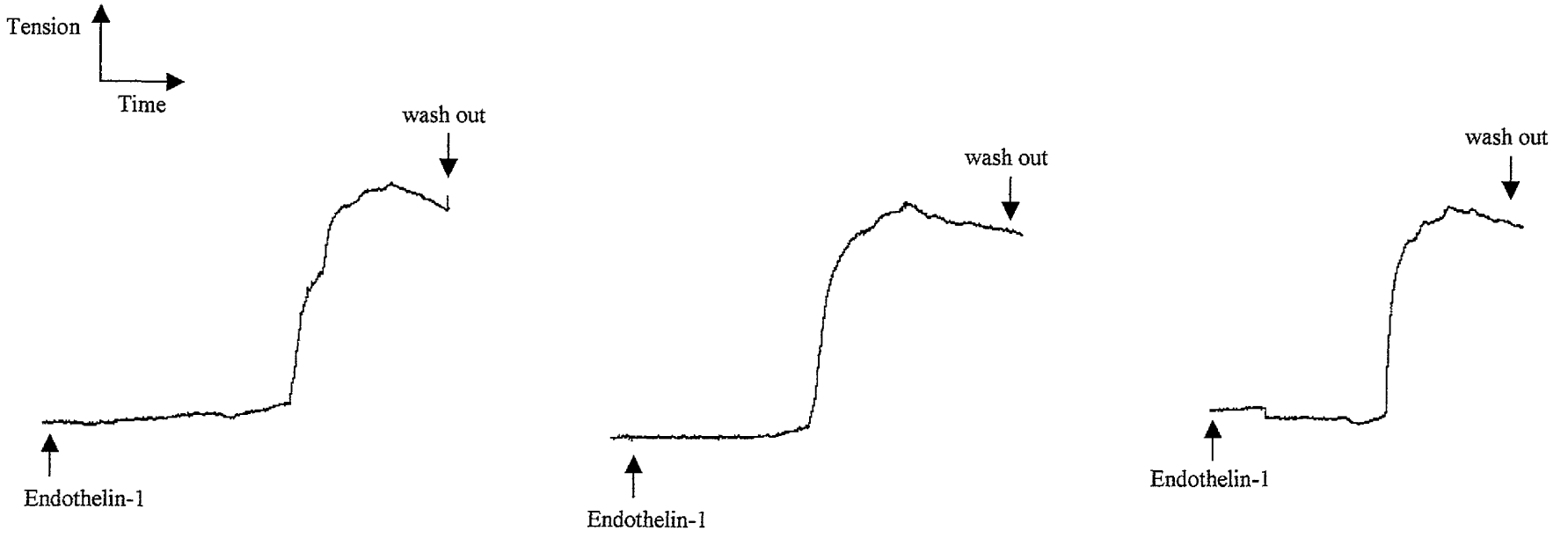


Fig. 5.4 The effect of endothelin-1 (ET-1) on isolated small arteries obtained from healthy pregnant and non-pregnant women (*p<0.05).



Healthy Pregnant Women **Diabetic Pregnant Women** **Healthy Non-Pregnant Women**

Fig. 5.3 Representative traces of resistance arteries exposed to cumulative concentrations of endothelin-1 in each of the three study groups – healthy pregnant, diabetic pregnant and healthy non-pregnant women.

Sensitivity to ET-1 was significantly reduced in the pregnant diabetic women compared to the non-diabetic pregnant women [pD_2 non-diabetic pregnant: 5.5 ± 0.1, n=9(9) *vs.* diabetic pregnant: 4.8 ± 0.2, n=8(7) *p<0.05, Fig. 5.5]. No difference in sensitivity was seen between the healthy pregnant and non-pregnant women [pD_2 non-diabetic pregnant: 5.5 ± 0.1, n=9(9) *vs.* non-pregnant: 5.3 ± 0.2, n=6(5)].

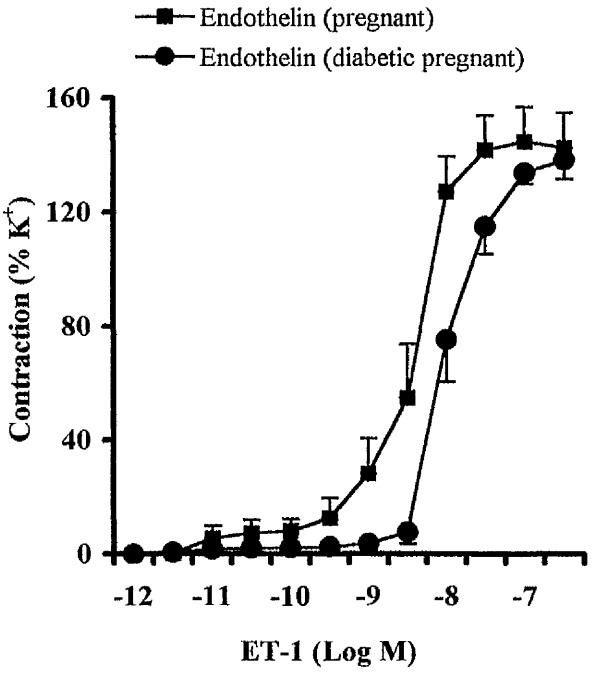


Fig. 5.5 The effect of endothelin-1 (ET-1) on isolated small arteries obtained from healthy pregnant and diabetic pregnant women (pD_2, *p<0.05).

5.4 Discussion

The main findings in this study were a significant increase in the maximum vasoconstriction response to ET-1 in pregnant compared to non-pregnant women, and a significant reduction in sensitivity to ET-1 in pregnant women with type 1 diabetes compared to healthy pregnant women.

Endogenous ET-1 appears to contribute to the regulation of basal vascular tone and systemic blood pressure by a balanced activation of ET_A and ET_B receptors, stimulating both vasoconstriction directly and vasodilatation via nitric oxide release (Haynes and Webb, 1998). Studies have demonstrated ET-1 as a significant regulator of placental blood flow, and an important factor in fetal development (Sabry *et al.*, 1995; Kohnen *et al.*, 1997; Kurihara *et al.*, 1994; Clouthier *et al.*, 1998). Furthermore, it is also thought to have a role in placental growth due to its mitogenic properties, and in the closure of umbilical vessels at birth (Fant *et al.*, 1992; Nisell *et al.*, 1990). Plasma ET-1 levels have been shown to be significantly elevated in conditions associated with vascular dysfunction such as diabetes mellitus, pregnancy-induced hypertension and chronic cardiac failure (Wolff *et al.*, 1997; Kamoi *et al.*, 1990; Kiowski *et al.*, 1995). In one of the largest reported longitudinal studies, plasma levels of ET-1 in pregnancy were observed to be in the picomolar range, although no measurements have been possible of local or tissue levels, which may have more significance (Wolff *et al.*, 1997). As the vascular effects of ET-1 are either autocrine or paracrine, tissue levels are of greater importance compared to plasma levels in terms of their influence on the resistance vasculature. In this study, supraphysiological concentrations of ET-1 were used to

demonstrate the pharmacological effects of the peptide in the different patient groups, which give an indication of the maximum response that can be achieved by the tissue and may not reflect the effect found at physiological concentrations.

As there were no differences in responses either to potassium or noradrenaline, the results of this study are specific to ET-1. This suggests that any alteration in ET-1 response is more likely to be secondary to changes in receptor expression and not the second messenger pathway within the smooth muscle (McIntyre *et al.*, 2001). This study shows a significant increase in the maximum vasoconstrictor response to ET-1 in pregnancy, reflecting the ability of the vessels to respond to the peptide. This finding suggests that the normal vascular changes in pregnancy may be partly modified by the presence of ET-1. The non-pregnant women recruited into the study were slightly older than the pregnant women. However, this is unlikely to significantly alter vascular function, as the age difference was less than ten years, and all the non-pregnant women were pre-menopausal, with no increased incidence in smoking.

In contrast, the findings in pregnant diabetic women show a different type of effect, with an attenuation of vascular tissue sensitivity to ET-1, but no change in maximum vasoconstrictor response. This implies that vessels obtained from pregnant diabetic women require a higher concentration of ET-1 to produce a similar response, which may reflect vascular dysfunction. However, the diabetic women in our study achieved good glycaemic control, as reflected by the mean HbA_{1c} level, birthweight at delivery, and the delivery of the infants at term (>37 completed weeks of gestation). It is possible that the reduction in sensitivity

observed in pregnant diabetic women may be secondary to a down-regulation of ET_A receptor signalling in response to a rise in plasma ET-1 levels. Wolff *et al.* demonstrated that resistance vessels pre-treated with the ET_A receptor antagonist, BQ-123, showed a similar shift in the response curve to the right and a reduction in sensitivity to ET-1 (Wolff *et al.*, 1996b). Previous studies have also demonstrated changes in vascular tissue sensitivity to other endogenous vasoconstrictor peptides during pregnancy. Gant *et al.* showed that healthy pregnant women were remarkably resistant to the pressor effects of infused angiotensin II (Gant *et al.*, 1973), and that the likely explanation for this reduction in sensitivity was the increased plasma angiotensin II concentrations seen during pregnancy (Robertson *et al.*, 1971).

The absence of a group of young non-pregnant type 1 diabetic women does not allow the effect of pregnancy on ET-1 responses in type 1 diabetic women to be studied. However, there would be significant difficulty in recruiting a sufficient number of young diabetic women undergoing laparotomy, and it would have been necessary to obtain the vessels from gluteal biopsies.

In a recent study by McIntyre *et al.*, vascular responses to ET-1 were studied in a mixed sample of both male and female type 1 diabetic and non-diabetic subjects (McIntyre *et al.*, 2001). They reported an increase in ET-1 sensitivity with no change in maximum response in the diabetic group, and suggested that these findings may be due to either a down-regulation of ET_{B1} receptors, or an up-regulation of ET_A/ET_{B2} receptors. Another possibility would be the increased production of ET-1 resulting from a reduction in tissue sensitivity due to vascular

damage. However, this type of response is much less well understood. The greater importance of the vasoconstrictor pathway suggests that the elevated plasma ET-1 concentrations seen in diabetics is more likely to result in a *down-regulation* of ET_A/ET_{B2} receptors, and be observed as a *reduction* in tissue sensitivity to ET-1. Unfortunately, ET-1 levels were not measured in this study.

Insulin has been shown to increase ET-1 production, receptor synthesis and gene expression (Frank *et al.*, 1993; Hu *et al.*, 1993). Wolff *et al.* reported constant plasma ET-1 levels throughout pregnancy similar to non-pregnant women, but significantly elevated in diabetic pregnant women (Wolff *et al.*, 1997). If insulin increases ET-1 production, one would expect elevated plasma ET-1 levels in healthy pregnant women who are physiologically insulin resistant and 'hyperinsulinaemic'. Therefore, the raised ET-1 levels seen in diabetics are unlikely to be due to exogenous insulin administration, but rather to diabetes *per se*. The role of glucose in the vascular effects of ET-1 is unclear. Studies have reported both increased and decreased ET-1 release in endothelial cell cultures following glucose administration (Yamauchi *et al.*, 1990; Hattori *et al.*, 1991; Ferri and De-Mattia, 1995).

5.5 Conclusions

This study has shown that pregnancy is associated with a greater maximum response to ET-1, while diabetes reduces ET-1 sensitivity in the peripheral vasculature. These important observations suggest that the presence of disease may

significantly alter vessel sensitivity to circulating peptides, and result in an increased disposition to developing micro-vascular disease.

Chapter Six

Final Conclusions

6.1 Summary

Extensive research has been carried out investigating vascular function in both type 1 and type 2 diabetics. The complexity of the aetiology and progression of diabetes has led to a wide range of observations that appear to depend on patient age, disease duration, level of glycaemic control, the co-existence of micro-vascular complications, the species, and even the vascular bed studied. Studies in type 1 diabetics have demonstrated both impaired and normal endothelial function, whereas in type 2 diabetics and in type 2 post-menopausal diabetics only abnormal function has been reported. The complications associated with diabetes, particularly those of micro-vascular origin, are amplified in pregnancy, and can lead to devastating pregnancy outcomes for both the mother and the fetus. This contributes significantly to patient morbidity and the demands on health care. Many of the problems associated with diabetic pregnancies are thought to stem from damage to the small vessels that control peripheral resistance, and ultimately tissue perfusion. There has been very little published literature on vascular function in younger women with type 1 diabetes, especially during pregnancy. This thesis has examined some of the factors involved in the control of vascular function in subcutaneous resistance arteries from pre-pregnancy type 1 diabetic pregnant women.

Pregnancy is an insulin resistant state, where resulting hyperinsulinaemia becomes necessary to maintain maternal normoglycaemia and normal fetal development. A significant physiological maternal vasodilatation develops, evident as early as five weeks gestation, that becomes more pronounced in the second trimester. This

occurs as a consequence of vast increases in blood volume and cardiac output needed to maintain the fetal-placental circulation, and may be due, in part, to an increase in nitric oxide and prostacyclin production.

The first part of this thesis examines the vasodilatory effect of insulin on resistance arteries obtained from physiologically insulin resistant pregnant women. The study demonstrates that insulin is able to significantly attenuate noradrenaline-induced vasoconstriction in healthy pregnant women independent of the endothelium. This suggests that insulin may partially contribute to the physiological vasodilatation observed in pregnancy through changes in vascular smooth muscle tone.

Women with type 1 diabetes require higher levels of exogenous insulin during pregnancy to maintain maternal and fetal health, and to compensate for the physiological insulin resistance that develops. These women are at a higher risk of developing pregnancy-induced hypertension, and may experience rapid deterioration of pre-existing micro-vascular complications, compared to non-diabetic women. Chapter 4 investigates both endothelial and smooth muscle function in subcutaneous resistance arteries in pregnant women with and without diabetes, and demonstrates not only normal vascular function in the diabetic women, but that endothelial hyperpolarization appears to contribute greatly to the control of vascular tone in these arteries. The mechanisms underlying the regulation of vascular function in diabetics are multi-factorial, and may depend on disease progression, evolution and control. An abnormal lipid profile, seen more commonly in diabetics, can also contribute to endothelial damage. Various lipid markers, such as triglycerides, total-, HDL- and LDL-cholesterol, have been

investigated in the different groups of women, and suggest that this particular group of diabetic women do not have abnormal lipid profiles when compared to non-diabetic pregnant women. This supports the *in vitro* myography assessments of vascular function.

Diseases affecting tissue responses to vasoactive substances can be observed, *in vitro*, as changes in vascular sensitivity to chosen agonists. The effect of endothelin-1 (ET-1) on vasoconstrictor responses in diabetic pregnant women was examined. ET-1, one of the most potent endogenous vasoconstrictors known, is synthesised primarily by the vascular endothelium and has been implicated in hypertension and cardiac disease, and in the increased incidence of pre-eclampsia seen in diabetic women. Besides its vasodilatory effects, insulin is also known to stimulate ET-1 release, which may be critical in diabetic pregnancies. This study shows that pregnancy is associated with a marked increase in the maximum ET-1 response, while the presence of diabetes in pregnancy results in a significant reduction in tissue sensitivity to ET-1. These observations suggest that ET-1 may be important in regulating peripheral resistance in pregnancy, and may contribute to the increased disposition of diabetics to developing arterial disease.

There were several technical drawbacks with the study. Firstly, using a 95%O_2/5%CO_2 mixture results in a partial pressure of O_2 well outside the physiological range (>600mmHg). This hyperoxic environment can be problematic due to potential superoxide ion generation, which can be a source of variability, particularly in the study of nitric-oxide-mediated responses. However, the reason behind the use of this 'gassing' mixture is to maintain the pH of the particular

physiological salt solution used in the organ bath during the *in vitro* study of isolated resistance arteries at 37°C. Historically, this experimental condition has been commonly used for studies on vascular (and endothelial) function, therefore, despite its limitations comparison with other studies is easier to address. Secondly, another limitation of using wire myography as a means of investigating the functional characteristics of resistance arteries is the inability to satisfactorily mimic normal physiological changes, such as alterations in intraluminal pressure and shear stress. An alterative method of assessing small vessel function that would take this factor into account is pressure myography (Coats *et al.*, 2001;Doughty *et al.*, 2000). Thirdly, the total number of patients recruited was much higher than the number from which biopsies were actually obtained. A coded sticker on their case files identified all recruited patients, alerting admitting medical/midwifery staff of their participation in the study. However, several of the recruited women who had emergency caesarean sections were missed because of the urgency of delivery. Furthermore, a few women delivered out of hours at a time when laboratory access could not be arranged. Other problems included difficulty in isolating subcutaneous arteries from women who had previous abdominal surgery. Scar tissue is extremely difficult to dissect, and the resistance arteries tended to either be too small, or were embedded in thick fibrous tissue that was very difficult to remove without vessel damage. Furthermore, abdominal subcutaneous fat appears to be less vascular compared to, gluteal subcutaneous fat, for example, posing problems in arterial identification and isolation.

Further investigation of the action of insulin following endothelial denudation should ideally also have been carried out in the non-pregnant group, as this may

have helped explain some of the mechanisms involved. Similarly, studying a group of type 1 diabetic non-pregnant women would have given further indication of the effects of pregnancy and diabetes on the control of vascular function, but was unfortunately not included in the study. Although this was considered, there was difficulty recruiting sufficient age-matched non-pregnant women and young type 1 diabetic women undergoing laparotomy within the period of research. The alternative solution of performing gluteal biopsies on these young diabetic women had not been originally planned and therefore organisational elements such as research unit booking and ethical permission were not in place.

Other omissions were serial measurements of plasma endothelin-1 levels and glycosylated haemoglobin, which, along with other blood analyses could have been related to the *in vitro* assessments of vascular function, and provided valuable information.

6.2 Future Research

Although this study has identified some of the factors important in the control of vascular tone in human subcutaneous resistance arteries, greater mechanistic insight is still required. The following areas based on the work in this thesis are worthy of further research.

6.2.1 The mechanism(s) underlying the vasodilatory effect of insulin in pregnancy

The vasodilatory effect of insulin is maintained in pregnancy, and attenuates vasoconstriction via an endothelium-independent mechanism. Its mode of action may possibly be secondary to activation of the Na^+/K^+-ATPase or Ca^{2+}-ATPase pumps, or via the β-adrenoceptors as previously suggested. The identification of the mechanism(s) underlying this effect of insulin would involve a step-wise inhibition of each of the known possible pathways, following endothelial denudation.

The ability of insulin to attenuate vasoconstriction responses has been shown to vary depending on the patient group and vascular bed studied. The extension of this study to include type 1 diabetic non-pregnant women is important. This would outline the effect of both pregnancy and diabetes on insulin action, and may provide some insight into the pathophysiology surrounding the increased incidence of pregnancy-induced hypertension in diabetic women.

6.2.2 Alterations in endothelin-1 receptor expression/density in pregnant women with diabetes

Pregnant women with and without type 1 diabetes have demonstrated significant differences in vascular responses to endothelin-1 (ET-1), with a potentiation of the maximum ET-1 response in healthy pregnant compared to non-pregnant women,

and a reduction in sensitivity to ET-1 in diabetic compared to non-diabetic pregnant women.

This merits further research, with changes in receptor density/expression, alterations in receptor signalling or modifications in peptide uptake mechanisms being possible explanations for these observations. ET-1 has been implicated in pre-eclampsia, hypertension and cardiac failure. A greater understanding of ET-1 pharmacology in pregnancy and diabetes may provide potential therapeutic targets aimed at reducing the incidence of hypertension in pregnant women with and without diabetes.

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Appendix I

Publications

The following have resulted from work based on the text of this thesis.

Full papers

1. **Ang C**, Hillier C, MacDonald A, Cameron AD, MacDonald A, Greer IA, Lumsden MA. Insulin-mediated vasorelaxation in pregnancy. *BJOG* 2001; **108(10)**: 1088-1093.

2. **Ang C**, Hillier C, Cameron AD, Greer IA, Lumsden MA. The effect of type 1 diabetes mellitus on vascular responses to endothelin-1 in pregnant women. *J Clin Endocrinol Metab* 2001; **86(10)**: 4939-4942.

3. **Ang C**, Hillier C, Johnston F, Cameron AD, Greer IA, Lumsden MA. Endothelial function is preserved in pregnant women with well-controlled type 1 diabetes. *BJOG*, 2002 (in press)

Review articles

1. **Ang C**, Lumsden MA. Diabetes and the maternal resistance vasculature (review). *Clin Sci* 2001; **101(6)**: 719-729.

Abstracts

1. **Ang C**, Hillier C, Cameron AD, Greer IA, Lumsden MA. EDHF in pregnant women with and without type 1 diabetes (abstract). *Scott Med J* 2001; **46(4)**: 125.

2. **Ang C,** Hillier C, Cameron AD, Greer IA, Lumsden MA. Sensitivity to endothelin is reduced in pregnant diabetic women (abstract). *J Soc Gynecol Invest* 2001; **8(suppl. 1)**: 116A.

3. **Ang C**, Hillier C, Cameron AD, Greer IA, Lumsden MA. Endothelin-induced vasoconstriction is increased in pregnancy (abstract). *J Soc Gynecol Invest* 2001; **8(suppl. 1)**: 116A.

4. **Ang C**, MacDonald A, Johnston F, Cameron AD, Greer IA, Hillier C, Lumsden MA. The effect of insulin on small vessel contractility in normal pregnancy (abstract). *J Int Angio* 2000; **19(2, suppl. 1)**: 33.

5. **Ang C**, MacDonald A, Johnston F, Cameron AD, Greer IA, Hillier C, Lumsden MA. Insulin resistance in pregnancy does not abolish the effect of insulin on small vessel contractility (abstract). *J Endocrin* 2000; **164**: P94.

Appendix II

Presentations

Oral presentations

1. Endothelin-mediated responses in pregnant diabetics.

   Presented to the Glasgow Obstetrical and Gynaecological Society.

   Glasgow, UK. 17 January 2001.

2. Alterations in endothelin-mediated responses in pregnancy and diabetes.

   Presented to the Scottish Society of Experimental Medicine.

   Edinburgh, UK. 27 October 2000.

3. Vascular effects of insulin in pregnancy.

   Presented to the Scottish Society of Experimental Medicine.

   Glasgow, UK. 26 May 2000.

4. The effect of insulin on small vessel contractility in normal pregnancy.

   Presented at the 19th World Congress of the International Union of Angiology.

   Ghent, Belgium. 4 May 2000.

Poster presentations

1. EDHF in pregnant women with and without type 1 diabetes. (Second prize winner)

   Presented to the Scottish Society of Experimental Medicine.

   Aberdeen, UK. 4 May 2001.

2. Sensitivity to endothelin is reduced in pregnant diabetic women.

   Presented at the 48th Annual Meeting of the Society for Gynecologic Investigation. Toronto, Canada. 14-17 March 2001.

3. Endothelin-induced vasoconstriction is increased in pregnancy.

   Presented at the 48th Annual Meeting of the Society for Gynecologic Investigation. Toronto, Canada. 14-17 March 2001.

4. Insulin resistance in pregnancy does not abolish the effect of insulin on small vessel contractility.

   Presented at the 19th Joint Meeting of the British Endocrine Societies with the European Federation of Endocrine Societies.

   Birmingham, UK. 13-16 March 2000.