Insulin's role as a vascular hormone in health and disease

by

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Declaration

I declare that this thesis has been composed by myself and is a record of work performed by myself. It has not been submitted previously for a higher degree.

The work described in this thesis was carried out under the supervision of Professor JMC Connell in the Department of Medicine and Therapeutics at the Western Infirmary, Glasgow.

Stephen J Cleland

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Summary

A series of studies is described in which both clinical and basic techniques have been used to investigate insulin's role as a vascular hormone and the relationship of this to metabolic insulin sensitivity and vascular endothelial function. These techniques (described in chapter 2) have been used to investigate:

- the physiological mechanisms of insulin-mediated vasodilation (chapter 3)
- the relationship between insulin's metabolic and vascular actions (chapter 4)
- the effect on insulin sensitivity of induced endothelial dysfunction (chapter 5)
- the relationship between structural components of skeletal muscle and insulin action (chapter 6)
- the associations among insulin sensitivity, insulin vasodilation and basal endothelial function in insulin-resistant subjects (chapter 7)
- the sensitivity of platelets to agonist-induced aggregation with respect to different levels of insulin sensitivity (chapter 8)
- the role of immunogold electron microscopy in direct visualisation of GLUT4 translocation in skeletal muscle cells (chapter 9)

Chapter 3: Physiological mechanisms of insulin-mediated vasodilation in man

In this clinical physiological study, the effects on insulin/glucose-mediated vasodilation of co-infusions of L-NMMA, angiotensin II, and ouabain were studied, in a double-blind, random-order, placebo-controlled manner using bilateral venous-occlusion strain-gauge plethysmography. It was concluded that insulin-mediated vasodilation may be dependent on endothelial nitric oxide production or stimulation

of the Na^+K^+ATPase pump or both; however, this data cast doubt upon the widely-prevalent assumption that insulin-mediated vasodilation is specifically NO-dependent.

Chapter 4: Insulin-mediated vasodilation and glucose uptake are functionally linked in man

This clinical physiology study was designed to test the hypothesis that insulin-mediated vasodilation and insulin sensitivity are functionally related. Eighteen healthy volunteers attended on two separate occasions for measurement of whole-body insulin sensitivity and forearm vasodilation in response to an intra-arterial infusion of insulin and glucose. In multiple regression analysis, insulin-mediated vasodilation was, indeed, a significant independent predictor of insulin sensitivity.

Chapter 5: Systemic inhibition of endothelial nitric oxide synthesis does not impair insulin sensitivity in man.

In this clinical physiology study, an experimental state of systemic endothelial dysfunction was induced in healthy male volunteers and the effect on whole-body insulin sensitivity was assessed in a randomised double-blind, placebo-controlled manner. The results indicate that impairment of endothelial nitric oxide synthesis does not result in an impairment of whole-body insulin sensitivity, suggesting that substrate delivery to nutritive capillary beds is unlikely to be rate-limiting for insulin-mediated glucose uptake.

Chapter 6: Skeletal muscle capillary density is unrelated to insulin's vasodilator action

Skeletal muscle fibre type is known to be associated with insulin sensitivity. Since fibre type is also related to capillary density, this study set out to test the hypothesis that insulin's vasodilator action is linked with muscle capillary density, which would support the notion that skeletal muscle structure and function may be a significant 'third factor' accounting for the observed association between insulin's metabolic and vascular actions. Following immunohistochemical staining of sections of muscle biopsy samples from 13 healthy volunteers in whom insulin sensitivity and local insulin/glucose-mediated vasodilation had already been measured, it was concluded that the quantity of capillary endothelium in muscle is unlikely to be a significant determinant of insulin's vascular action.

Chapter 7: Insulin action and endothelial function in type 2 diabetes and essential hypertension

Twenty seven men participated in this clinical physiology study (9 controls, 9 hypertensives and 9 type 2 diabetics) which was designed to characterise insulin action and basal endothelial function. There was a trend for reduced insulin-mediated vasodilation in type 2 diabetic patients although statistical significance was not achieved due to high biological variability. Pooled analysis revealed a significant association between (1) insulin's metabolic and vascular actions, (2) insulin sensitivity and basal endothelial NO production, and (3) insulin-mediated vasodilation and basal endothelial NO production. Multiple regression analysis with basal endothelial NO production as the dependent variable revealed that a model

including insulin-mediated vasodilation and age accounted for over 40% of the variance. The fact that insulin sensitivity did not persist as an independent predictor is supportive of the hypothesis that insulin-stimulated endothelial NO production is a key intermediate mechanism linking insulin-mediated glucose uptake with basal endothelial function.

Chapter 8: Sensitivity of washed platelets to thrombin-induced aggregation is not associated with whole-body insulin sensitivity

Previous studies have demonstrated that platelet aggregation is increased in association with insulin resistance. However, it remains unclear whether this feature is intrinsic to platelet function or secondary to influencing factors in plasma. In the present study, a washed platelet preparation was used to determine sensitivity of platelets to thrombin-induced aggregation in the absence of plasma. There was no association between platelet aggregation and either insulin sensitivity or insulin-mediated vasodilation. This negative result might be due to differences in technique from previous studies and supports the notion that plasma factors associated with insulin resistance exert influences on platelet function rather than there being intrinsic platelet defects secondary to insulin resistance.

Chapter 9: Direct visualisation of glucose transporter protein GLUT4 in skeletal muscle using immunogold electron microscopy

In this pilot study, a collaboration was initiated with experts in membrane biochemistry and cell ultrastructure with a view to developing the technique of immunogold electron microscopy for use in direct visualisation of key cellular

proteins involved in the insulin signalling pathway. The primary aim of this ongoing work is to demonstrate and quantify GLUT4 translocation to myocyte t-tubules in response to insulin stimulation. Once this technique is established, it is planned to explore mechanisms of GLUT4 translocation to provide clues for possible defects which may underlie cellular insulin resistance.

Conclusions

- Insulin is a vasoactive hormone.
- Mechanisms of insulin-mediated vasodilation include stimulation of endothelial NO production and membrane hyperpolarisation via stimulation of Na^+K^+ATPase.
- Insulin-mediated vasodilation occurs gradually over 40-50 minutes suggesting involvement of intermediate mechanisms.
- Cellular glucose uptake augments insulin-mediated vasodilation.
- Whole-body insulin-stimulated glucose uptake and local insulin/glucose vasodilation are functionally linked.
- Systemic inhibition of NO production does not impair whole-body insulin-mediated glucose uptake.
- Skeletal muscle capillary density is not associated with insulin's vascular action.
- Insulin resistance is not associated with increased sensitivity of washed platelets to thrombin-induced aggregation.
- Insulin-mediated vasodilation is preserved in older men but is blunted in parallel with reductions in insulin sensitivity.

- Vasoconstriction in response to local inhibition of NO synthase is delayed during hyperinsulinaemia compared with fasting conditions suggesting that insulin stimulates eNOS activity.
- Insulin sensitivity correlates with basal endothelial NO production in both young and older men.
- Insulin-mediated vasodilation correlates with basal endothelial NO production in older, but not in young men.
- Insulin's stimulation of endothelial NO production may be a key intermediate mechanism linking insulin sensitivity with basal endothelial function.

Chapter 1

Introduction and background

1.0 Introduction

Insulin is best known as a metabolic hormone but also exerts effects on vascular tone. Therefore, plasma insulin concentrations and/or resistance to insulin action have been proposed as potential common factors linking metabolic and cardiovascular disorders. The purpose of this thesis is to explore the complex relationship between insulin's metabolic and vascular actions and how this is related to vascular endothelial function.

1.0.1 Physiological actions of insulin [figure 1.1]

Insulin is a peptide hormone which is secreted from pancreatic β-cells into the portal circulation in response to various stimuli including glucose and amino acids. One of its main roles is to facilitate cellular uptake of these building blocks for storage as glycogen and protein. The main sites of peripheral glucose disposal are skeletal muscle (>80%) and adipose tissue (5-10%) (DeFronzo et al, 1992). Stimulation of insulin receptors in these tissues results in a complex intracellular signalling cascade (section *1.10.4*) ultimately resulting in translocation of glucose transporter proteins (GLUT-4) to the cell surface and subsequent glucose uptake. Insulin also exerts an important influence on the liver resulting in suppression of gluconeogenesis and stimulation of lipogenesis. In addition, it is a growth factor, an effect which may be mediated via IGF-1 receptors. Lastly, it stimulates cellular uptake of potassium, a mechanism which may partly explain its vascular action (section *1.3*).

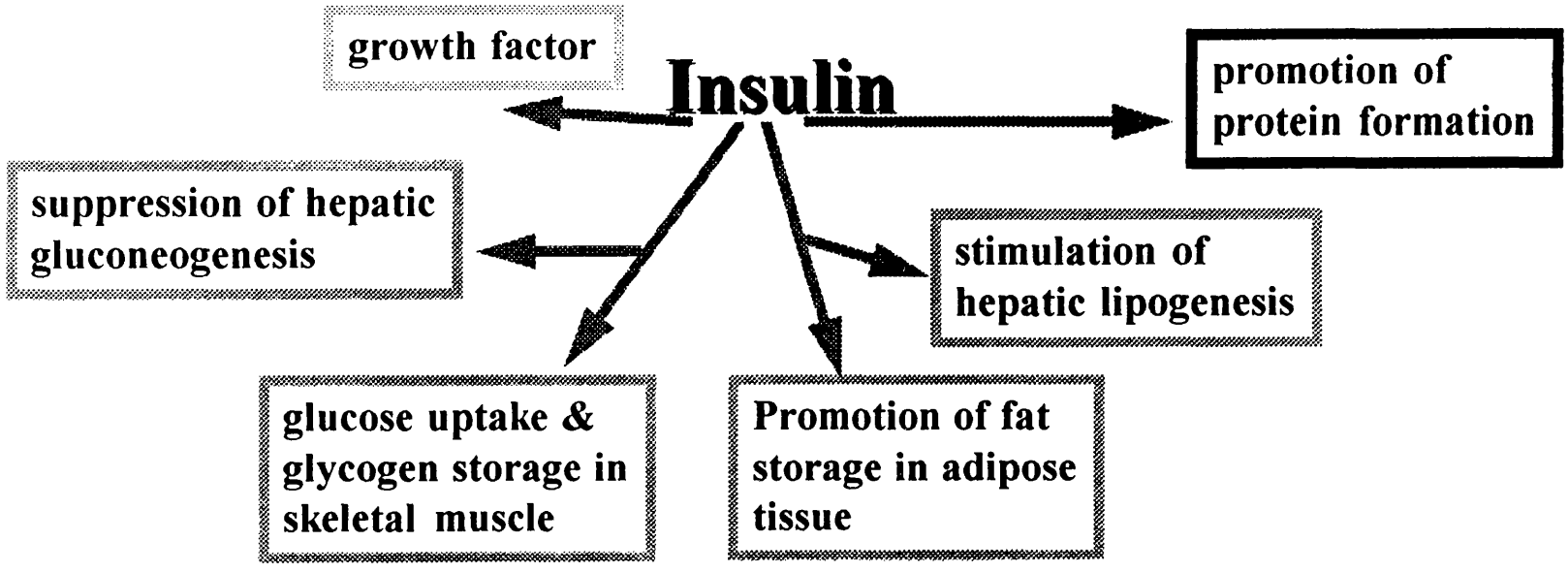


Figure 1.1
Schematic diagram summarising the main metabolic actions of insulin.

1.0.2 What is 'insulin resistance'?

The nature of 'resistance' to these actions of insulin is incompletely understood. There are several inherited syndromes of profound insulin resistance which commonly involve the insulin receptor itself. For example, in Type A Syndrome the defective receptors cannot stimulate the intracellular cascade which promotes glucose uptake and metabolism. Compensatory high insulin levels are unable to overcome the defect resulting in hyperglycaemia, but continue to exert growth effects via IGF-1 receptors causing the syndrome's characteristic features, including acanthosis nigricans (Moller & Flier, 1991).

However, in the majority of cases, the degree of resistance to insulin action is multifactorial, involving a combination of polygenic and environmental influences, resulting in considerable (2-3 fold) variation when measured in apparently healthy subjects (Hollenbeck & Reaven, 1987). Thus, insulin resistance is a continuous variable within a normal population. In the majority of subjects at the top end of this spectrum, compensatory hyperinsulinaemia maintains normal glucose tolerance, but in a proportion of insulin resistant subjects, this is an inadequate response and impaired glucose tolerance ensues. Type 2 diabetes is more likely to supervene in the context of more severe hepatic insulin resistance and β-cell failure (DeFronzo et al, 1992).

1.0.3 How is insulin resistance measured?

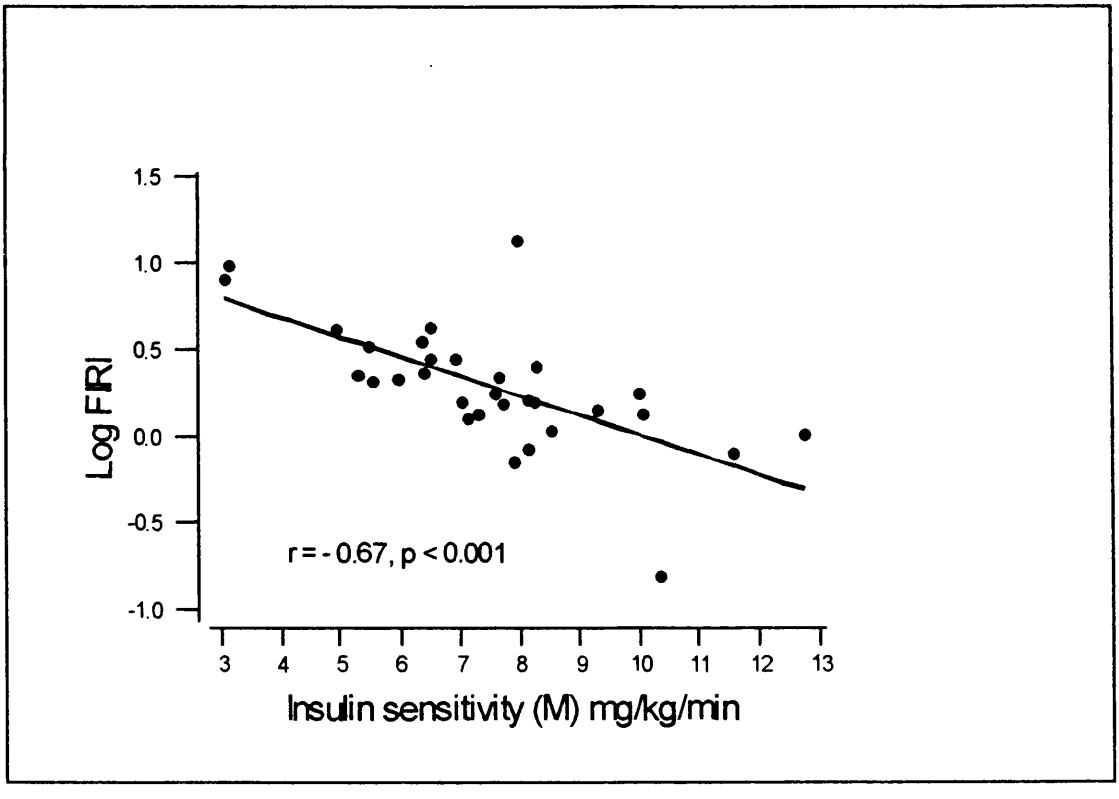
Quantification of the degree of insulin sensitivity or resistance in man involves obtaining a measurement of glucose disposal. High insulin-mediated glucose disposal

equates with high sensitivity / low resistance and vice-versa. Several techniques have been used to quantify this, including the intravenous glucose tolerance test, the minimal model technique (Bergman et al, 1987) and the insulin suppression test (Greenfield et al, 1981). However, it is generally accepted that the 'gold standard' is the hyperinsulinaemic euglycaemic clamp technique (section *2.4.1*), in which a fixed rate of insulin is infused and plasma glucose is kept constant ('clamped') by infusing 20% glucose at a variable rate. After 2 -3 hours, steady-state is achieved where the rate of glucose infusion is equal to the rate of insulin-mediated glucose disposal. Thus, insulin sensitivity is expressed as mg glucose / kg body weight / minute ('M-value'). Clearly, this technique is impractical in large-scale studies and a surrogate index tends to be used, for example 'FIRI' (fasting insulin resistance index) which equates to fasting [insulin] x fasting [glucose] divided by 25; FIRI has been shown to correlate well with clamp-derived M-values (Figure 1.2) (Cleland et al, 1996).

1.0.4 Insulin resistance - a beneficial physiological response?

Insulin resistance has most impact on glucose pathways (figure 1.3) resulting in hyperstimulation of fat and protein anabolic pathways by compensatory hyperinsulinaemia. It occurs as a normal physiological response in certain circumstances of physiological stress, such as major trauma, sepsis, starvation, pregnancy and puberty, with the result that essential glucose substrate is diverted from muscle metabolism and priority given to energy storage, CNS nutrition, growth and healing. Thus, in times of physical adversity, selective insulin resistance may confer an important survival advantage, which may explain why it appears so prevalent in an apparently healthy population.

Figure 1.2
Fasting insulin resistance index (FIRI) plotted against clamp-derived insulin sensitivity in 31 subjects with normal glucose tolerance



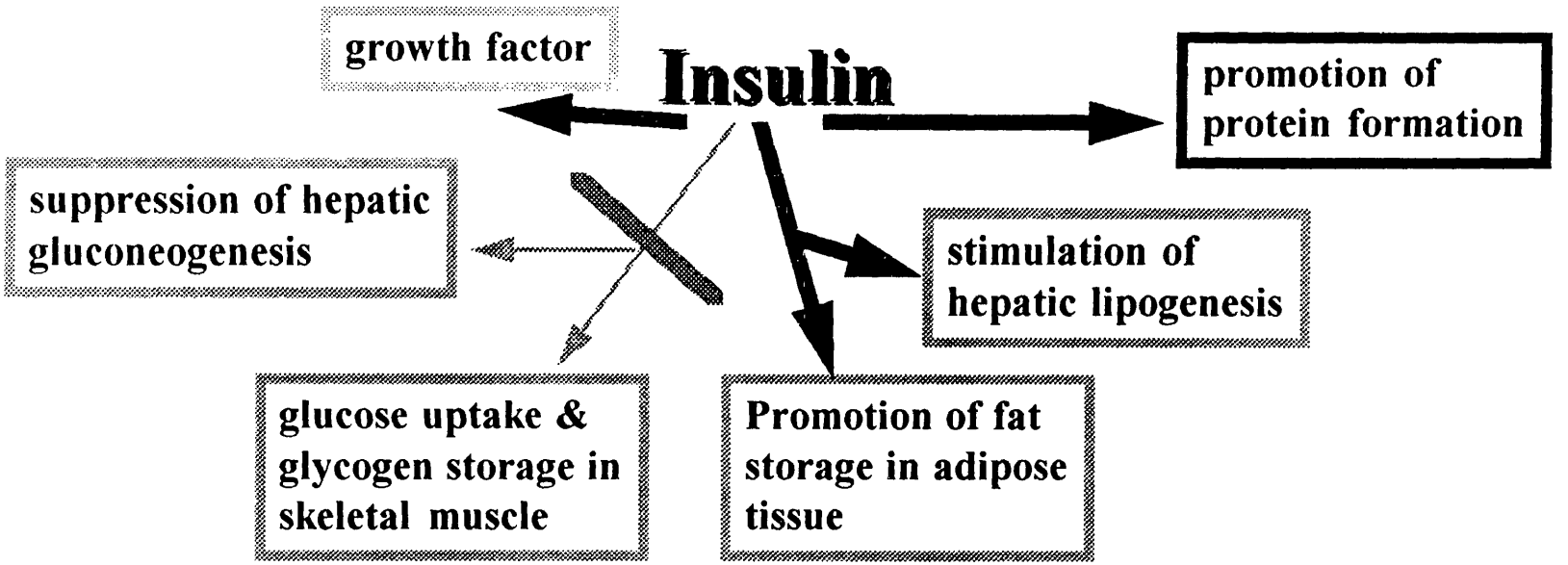


Figure 1.3
Schematic diagram illustrating the selectivity for pathways involved in glucose uptake and metabolism characterised by 'insulin resistance'. Compensatory hyperinsulinaemia results in enhancement of insulin's other actions.

1.0.5 Epidemic of insulin resistance in Western Society

Unfortunately, a survival mechanism for starvation and physical stress may be a recipe for disaster in the modern world. While life expectancy has dramatically increased in Western society, mainly due to reductions in child mortality, prevention of malnutrition, effective management of infectious disease and disease prevention policies, lifestyle issues are exerting an increasingly important influence on health. The Western diet is high in unrefined carbohydrate and saturated fat. The ready availability of food is promoting an epidemic of obesity. In an age of technology and high psychological stress, levels of physical activity have been dramatically reduced resulting in a vicious circle of inactivity, obesity and insulin resistance (figure 1.4).

There is already evidence for a global epidemic of type 2 diabetes. Currently, there are 100 million patients worldwide; it has been predicted that this figure will double by the year 2020 (O'Rahilly, 1997). The notion that Western lifestyle is the culprit responsible for this epidemic is supported by the high prevalence of type 2 diabetes in migrant groups such as South Asians, Aborigines and Polynesians (O'Dea, 1991). For example, the prevalence of diabetes in South Asians aged 40-75 in the UK is 20-30%, compared with a prevalence in the indigenous population of 2-3% (O'Rahilly, 1997). The fact that these same people groups have a high incidence of cardiovascular disease (McKeigue et al, 1988) leads us on to consider possible aetiological mechanisms which might link insulin resistance (and associated hyperinsulinaemia) with cardiovascular disease (figure 1.5).

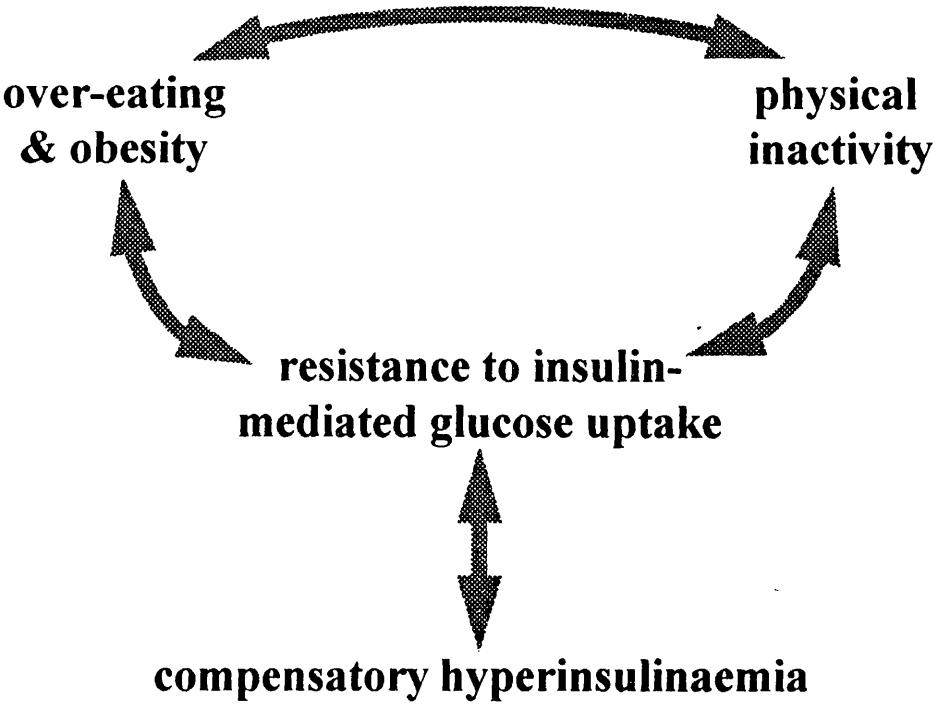


Figure 1.4
Schematic diagram illustrating the 'vicious circle' linking unhealthy lifestyle (obesity and inactivity) with insulin resistance and compensatory hyperinsulinaemia.

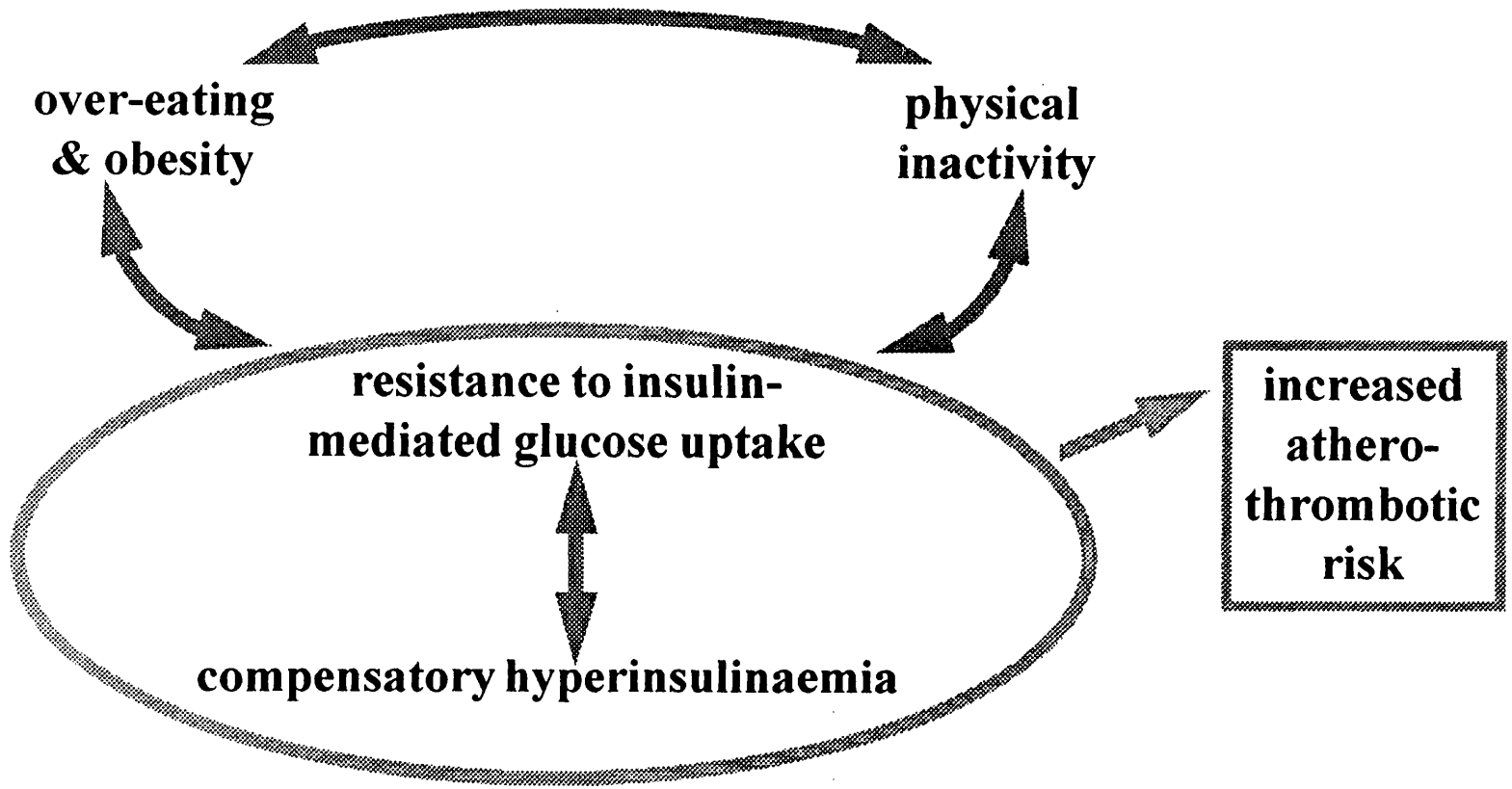


Figure 1.5
Schematic diagram showing that defective insulin action may be a key intermediate mechanism linking unhealthy lifestyle with increased atherothrombotic risk.

1.1 Endothelial function and insulin sensitivity in health and disease

1.1.1 The role of the endothelium in vascular smooth muscle control

The role of the endothelium in modifying vascular smooth muscle cell (VSMC) tone has been extensively reviewed elsewhere (Vane et al, 1990; Moncada et al, 1991; Meidell, 1994). In summary, the endothelium is responsible for local regulation of blood flow, production of growth factors and control of haemostasis. Four main enzyme systems interact to regulate blood flow; these produce the following vasoactive substances: (1) endothelin, (2) prostacyclin and thromboxane A_2 (which also exert effects on platelet adhesion), (3) angiotensin II and the kinins (which also act as local growth factors) and (4) nitric oxide (NO). These vasoactive substances produced by the endothelium operate in VSMCs by three main mechanisms: via cGMP, via cAMP, or via changes in intracellular Ca^{2+}. Endothelial cells are highly metabolically active and under stress from a variety of sources including mechanical forces, free radical oxidation, poor nutrient supply and blood-borne toxins (figure 1.6). Endothelial NO production is arguably the most important mechanism for protection against endothelial dysfunction and disruption which are central to the process of atherothrombosis. Not only is NO a potent vasodilator, it also acts as an anti-inflammatory factor, an anti-oxidant, and an anti-thrombotic agent as well as regulating local production of growth factors and involvement in autoregulatory control (Griffith et al, 1987; Griffith et al, 1990) (figure 1.7) A fine balance of sophisticated control mechanisms is required to ensure maintenance of local blood flow, blood fluidity and vessel architecture. Any reduction in endothelial NO production may predispose to atherothrombosis and subsequent vascular occlusion (figure 1.8).

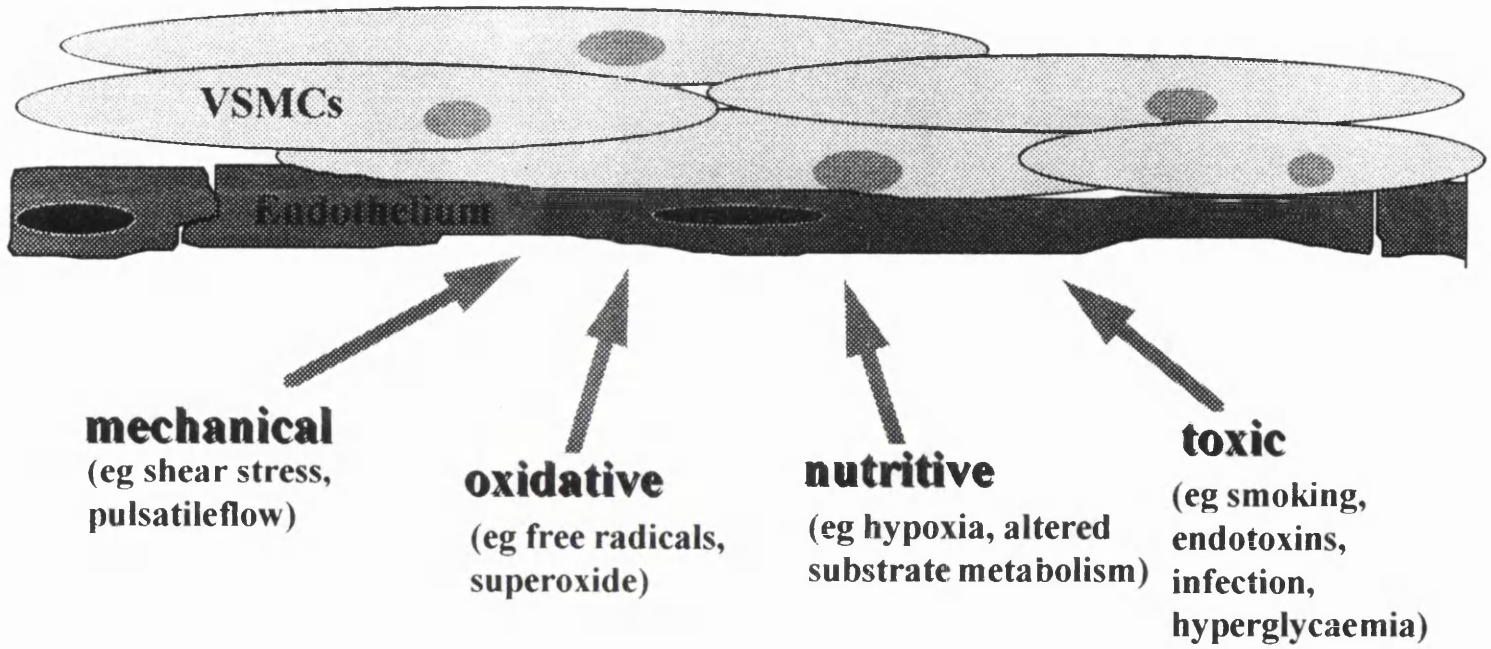


Figure 1.6
Schematic diagram summarising the different sources of stress on the endothelial cell monolayer - mechanical, oxidative, nutritive and toxic.

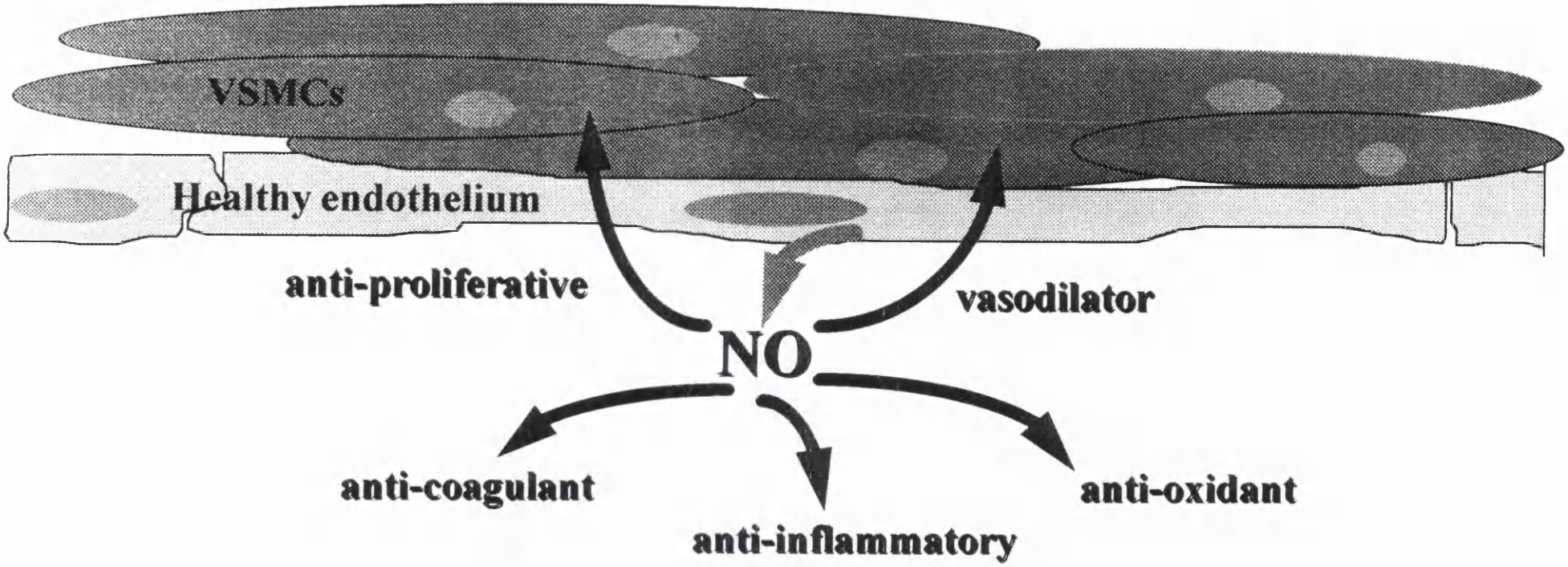


Figure 1.7
Schematic diagram summarising the varied anti-atherothrombotic properties of nitric oxide (NO) which is produced in sufficient quantity by healthy vascular endothelial cells.

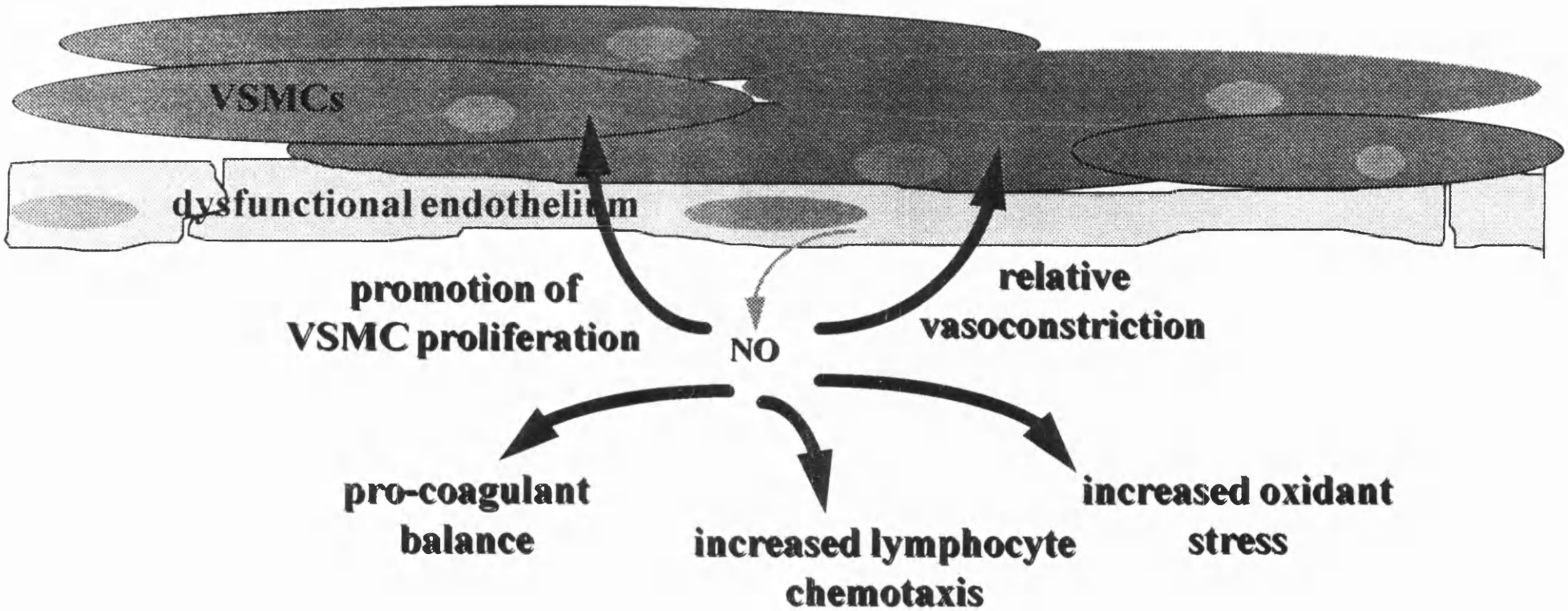


Figure 1.8
Schematic diagram summarising the pro-atherothrombotic sequelae of insufficient production of NO by dysfunctional vascular endothelial cells.

The endothelium may also be an important site of cross-talk between the metabolic and vascular requirements of the tissue. As we have seen, insulin appears to exert a significant influence on VSMC tone via endothelial mechanisms. It may be that endothelial function or dysfunction is an important factor in the link between insulin's metabolic and vascular actions. More speculatively, it is possible that insulin action on endothelial cells is important for production of adequate basal NO production - defective insulin action may result in endothelial dysfunction and predisposition to atherothrombosis. This notion is explored further in chapter 7.

1.1.2 Definition of 'endothelial dysfunction'

The term 'endothelial dysfunction' is, in practice, synonymous with the demonstration of abnormal endothelial nitric oxide production. This, in turn, can represent either abnormal NO synthesis (eg reduced activity of eNOS) or increased NO quenching (eg by superoxide or other cellular free radicals). The notion that defects in NO production go hand-in-hand with global dysfunction of other endothelial mechanisms is a common assumption for which there is very little evidence. However, it has become accepted in the scientific community that measurement of either stimulated or basal NO production is the 'gold standard' method for assessing endothelial function. Unfortunately these measurements are invasive and labour-intensive, rendering them unsuitable for large-scale studies. Limb blood flow can be assessed by a number of methods (section *1.2.2*) the most commonly used being venous-occlusion plethysmography (section *2.7.1*). A number of different compounds have been infused via a limb artery to demonstrate local vasodilation via stimulation of endothelial NO production; these include

acetylcholine (eg McVeigh et al, 1992; Panza et al, 1993b), methacholine (eg Steinberg et al, 1996) and bradykinin (eg Kelm et al, 1996; Nuutila et al, 1996). It is becoming clear that any comparisons between studies using different stimulating agents should be interpreted with caution, since each agent may have specific effects in addition to stimulation of endothelial NO; for example, there is evidence that acetylcholine may cause vasodilation partly via a NO-independent mechanism (Parsons et al, 1994). In all such protocols, it is vital to study in parallel the effects of an endothelium-independent donor of NO (eg sodium nitroprusside) which is used as an experimental control to assess sensitivity of adjacent vascular smooth muscle to NO.

An alternative approach is to assess basal endothelial NO production (eg Calver et al, 1992; Petrie et al, 1996a). The most commonly used method for this in man is measurement of the vasoconstrictor response to a local infusion of N_G-monomethyl-L-arginine (L-NMMA) which is a stereospecific substrate inhibitor of endothelial NO synthase. Vasoconstriction to L-NMMA in the human forearm is dose-dependent with maximal reduction in blood flow in the order of 30-40% (Vallance et al, 1989). Therefore, basal production of endothelial NO contributes significantly to the determination of resting blood flow. It is likely that this method of assessing endothelial function is more physiologically relevant since stimulation by pharmacological doses of agonists is artificial. However, it remains unclear whether L-NMMA has any other effects which may confuse interpretation of blood flow responses. Again, it is common practice to use an experimental control which is usually the endothelium-independent vasoconstrictor, noradrenaline.

Damaged endothelial cells release a number of products into plasma which can be measured. Thus, levels of 'endothelial markers' such as von Willebrand factor (vWF), tissue plasminogen activator (tPA), thrombomodulin, can be used to build up a profile of the severity of endothelial dysfunction (Stehouwer et al, 1997). However, in view of their lack of specificity, these markers may only be useful in large-scale studies, where the more invasive and labour-intensive procedures are not practical.

1.1.3 Association of insulin resistance and endothelial dysfunction in metabolic and cardiovascular disease

Although insulin has a variety of metabolic actions, the term 'insulin sensitivity' usually refers to the hormone's ability to dispose of glucose from the intravascular compartment into tissues, predominantly skeletal muscle and adipose tissue. Abnormalities of insulin action, including a reduction in insulin sensitivity (or 'insulin resistance'), are present not only in metabolic disorders such as type 2 diabetes (DeFronzo et al, 1992) and obesity (Ludvik et al, 1995)), but also in cardiovascular conditions such as essential hypertension (Ferrannini et al, 1987) and chronic cardiac failure (Swan et al, 1994). Notably, all these disorders are also characterised by vascular endothelial dysfunction (see *1.5.2*) (McVeigh et al, 1992; Williams et al 1996; Steinberg et al, 1996; Laine et al, 1998; Calver et al, 1992; Panza et al, 1993a; Drexler et al, 1993). The demonstration that insulin, in addition to its classical metabolic role, functions as a vasodilator, with a major component of this action being NO-dependent (Steinberg et al, 1994; Scherrer et al, 1994a) has

fuelled interest in the putative mechanisms and functional significance of insulin as a vascular hormone. Further elucidation of defects in insulin action, at both a metabolic and vascular level, may help to explain some of the pathophysiological mechanisms of the observed association between metabolic and cardiovascular diseases (De Fronzo & Ferrannini, 1991).

1.1.4 A physiological relationship between insulin sensitivity and endothelial function

In healthy non-obese subjects there is a three-fold variation in insulin sensitivity (Hollenbeck & Reaven, 1987) as measured by the hyperinsulinaemic, euglycaemic clamp technique (section *2.4.1*). Given the association of insulin resistance and endothelial dysfunction in metabolic and cardiovascular disease, in conjunction with the common observation of parallel improvement in these parameters in response to a variety of interventions, a hypothesis was generated that vascular endothelial function should explain a significant amount of the variance in insulin sensitivity in a physiological setting, *ie* there should be a correlation between these two variables across the range of insulin sensitivity found in healthy man. This hypothesis was tested by our group in a study undertaken prior to the work presented in this thesis. The results (along with others: section *1.2.5*) formed a vital platform for the studies presented in this thesis:

Fifteen healthy male volunteers, aged 21 - 35 years, completed one study day of hyperinsulinaemic euglycaemic clamp (section *2.4.1*) to measure whole-body insulin sensitivity, and two study days of forearm venous occlusion plethysmography

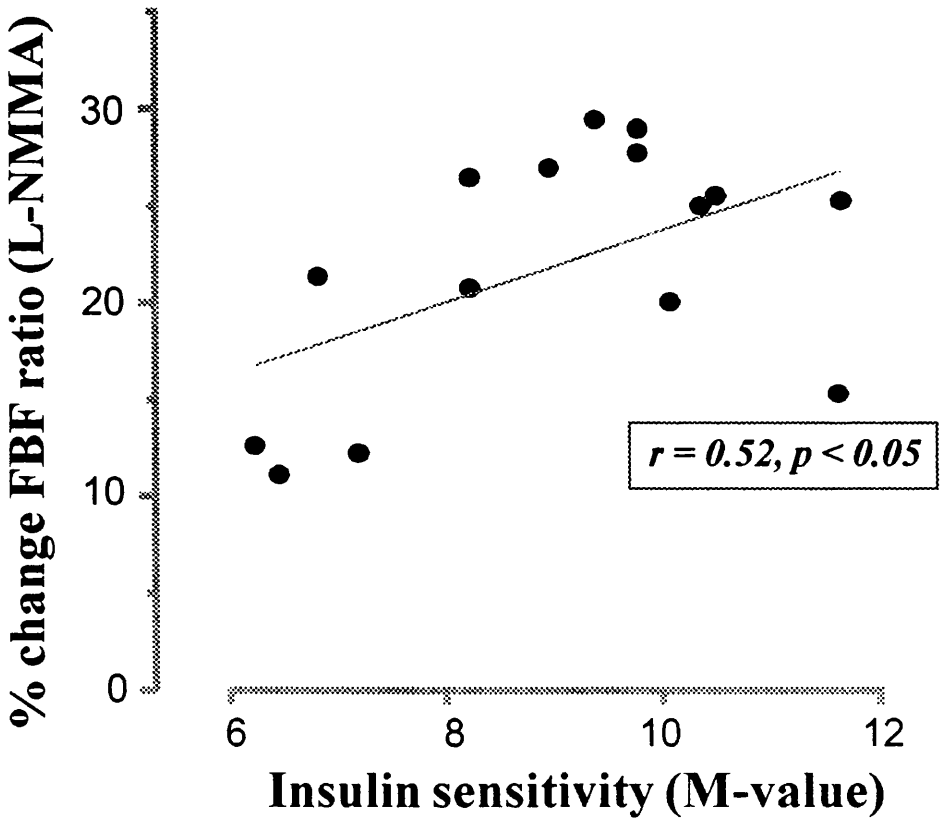
(section *2.7.1*) to measure both stimulated and basal endothelial nitric oxide production (section *1.5.2*). In univariate analysis, individual measurements of insulin sensitivity (M) were positively related to individual mean L-NMMA responses (surrogate measurements for basal endothelial nitric oxide production) ($r = 0.52$, $p < 0.05$, [figure 1.9]). However, no significant relationships were observed between M and nor-adrenaline, acetylcholine or sodium nitroprusside responses. In multiple regression analysis, in a model taking into account age, body mass index, mean arterial pressure, plasma glucose level, serum cholesterol level, alcohol intake, smoking status, family history and physical activity level, the L-NMMA response accounted for 43% of the variance in insulin sensitivity ($p < 0.01$) (Petrie et al, 1996a).

1.2 Insulin as a vasodilator hormone

1.2.1 Mechanism of insulin action in skeletal muscle

An important site of insulin's metabolic actions is skeletal muscle. This tissue has been shown to be the main site of reduced insulin-mediated glucose disposal in both essential hypertension (Natali et al, 1991) and type 2 diabetes (DeFronzo et al, 1992). Insulin's main role in skeletal muscle is to promote glucose uptake into cells for storage as glycogen. In the post-prandial period, resting muscle takes up glucose by increasing the arteriovenous fractional extraction under the influence of insulin. According to the Fick Principle, glucose uptake is a product of the arteriovenous glucose gradient and blood flow; therefore glucose would be disposed of much more efficiently if an increase in blood flow resulted in augmented substrate delivery. However, there is considerable debate in the literature concerning the rate-limiting

Figure 1.9
Basal endothelial nitric oxide production (percentage change in FBF ratio in response to intra-arterial L-NMMA) plotted against whole-body insulin sensitivity in 15 healthy male volunteers. From Petrie et al, 1996a.



step for insulin-mediated glucose uptake. If substrate delivery is the rate-limiting step (in other words, if skeletal muscle blood flow is a determinant of glucose uptake), then it can be argued that endothelial dysfunction, resulting in a relative inability of mediators, including insulin, to stimulate muscle blood flow, may be the underlying mechanism accounting for the association of atherosclerosis and other cardiovascular disorders with insulin resistance (Baron, 1994). The crux of this argument rests on insulin's role as a vasodilator hormone and also whether this action is physiologically relevant in terms of glucose disposal. Evidence for and against this will now be considered.

1.2.2 Vascular effects of systemic insulin infusion in man

Systemic hyperinsulinaemia appears to augment skeletal muscle blood flow by 20-60%, (Iannaccone et al, 1989; Laakso et al, 1990; Scherrer et al, 1994a; Anderson et al, 1991; Laakso et al, 1992; Bennett et al, 1990; Vollenweider et al, 1994) but this is by no means a universal finding (Utriainen et al, 1995; Capaldo et al, 1991; DeFronzo et al, 1985; Natali et al, 1994; Yki-Jarvinen et al, 1987; Nuutila et al, 1996). There are several possible reasons for the differing results in these studies:

- the extent of insulin's vasodilator action may be dependent on the level of systemic hyperinsulinaemia achieved. In most of the studies where insulin was shown to have a significant vasodilator effect, insulin levels were maintained in or above the high-physiological range.
- methodological differences in the measurement of limb blood flow are a source of variation. Baron's group in Iowa use a thermodilution technique to measure leg blood flow (Baron, 1994) while most European groups employ the technique of

venous-occlusion plethysmography to measure forearm blood flow (Petrie et al, 1996a; Tack et al, 1996); more recently Yki-Jarvinen and colleagues in Finland have used high-resolution positron emission tomography (PET) to investigate insulin's vascular effects and appear to conclude that insulin does, indeed, have significant vasodilator actions (Raitakari et al, 1996; Utriainen et al, 1997). In addition, the site of measurement may be important. There may be important differences between the upper and lower limb circulations in response to insulin.

- experimental design can affect the interpretation of results. For example, a double-blind, placebo-controlled, randomised design is more valid to detect small changes in blood flow than a 'before-after' experimental design. Also, the length of the protocol is of relevance, since insulin-mediated vasodilation is often not apparent until 40-50 minutes after the start of insulin infusion (Ueda et al 1998a).

1.2.3 Insulin and the sympathetic nervous system

One problem when interpreting blood flow data from studies in which insulin is infused systemically is that there is parallel stimulation of the sympathetic nervous system which has haemodynamic consequences both centrally and in the periphery. Evidence for this mechanism comes from recordings of muscle sympathetic nerve activity under conditions of insulin stimulation (Scherrer et al, 1994b). This appears to be a direct effect of insulin, and not simply baroceptor-mediated; in experimental animals with induced lesions of the anterolateral third ventricle, central sympathetic outflow in response to systemic insulin infusion is abolished (Muntzel et al, 1994). Presumably, activation of the sympathetic nervous system in combination with the peripheral vasodilatory action of insulin act to maintain blood pressure during

systemic hyperinsulinaemia. It has long been known that patients with insulin-dependent diabetes mellitus who have advanced autonomic neuropathy may become hypotensive after injecting insulin; this suggests that insulin's peripheral vasodilatory effects are preserved while the central compensatory effects are lost (Mathias et al, 1987).

In addition to its central actions, insulin appears to modulate peripheral responses at the adrenergic receptor level. Thus, it has been demonstrated that insulin augments forearm blood flow via a β-adrenergic mechanism (Lembo et al, 1996), and it has been shown that insulin attenuates both α_1 and α_2 responses in the human forearm (Sakai et al, 1993; Lembo et al, 1994). More recently Lembo et al (1997) have demonstrated that these effects of insulin on adrenergic responses are abolished by concominant inhibition of nitric oxide synthase, suggesting that nitric oxide is involved in a mediating role. However, not all of the evidence is in support of insulin's role as a modulator of peripheral sympathetic nervous system responses (Randin et al, 1994).

The time course of muscle sympathetic nerve activity in response to a systemic insulin infusion has a similar delay (slow rise over 1-2 hours) to that of insulin-mediated vasodilation (Anderson et al, 1991). In patients who have undergone sympathectomy for hyperhidrosis, the magnitude of insulin-mediated vasodilation is similar but the time to achieve steady-state is significantly reduced - the changes are all seen within the first hour (Sartori et al, 1996). This evidence suggests that insulin acts at the periphery to overcome the vasoconstictive influence of a stimulated

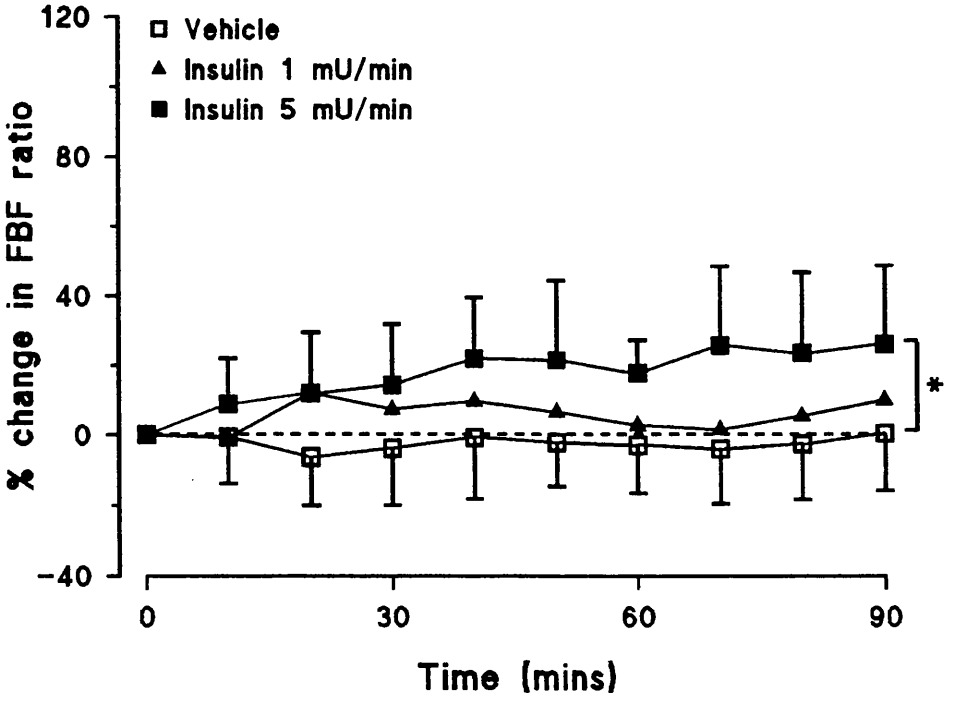
central sympathetic nervous system. Removal of this central component appears to allow insulin's vasodilator effects to take place more quickly.

1.2.4 Vascular effects of local insulin infusion in man

As outlined above, the majority of studies suggest that significant increases in limb blood flow occur in response to systemic hyperinsulinaemia, although there is marked variation in the results due to a number of confounding factors including activation of the sympathetic nervous system. In order to avoid confounding changes in systemic haemodynamics, some groups have infused insulin locally to a limb via a major artery. The majority of studies employing this technique have yielded negative results (Sakai et al, 1993; Chisholm et al, 1975; Creager et al, 1985; Natali et al, 1990); those that do demonstrate a significant augmentation of blood flow reveal, at best, a 20-25% rise (Andres et al, 1961; Gelfand & Barrett, 1987; Neahring et al, 1993). Studies conducted by our own group in the human forearm, using a double-blind placebo-controlled protocol and physiological local insulin concentrations (70-80 μU/ml), detected a 20% rise in flow after 90 minutes (figure 1.10) (Ueda et al, 1998a).

Therefore, it appears that local hyperinsulinaemia has a less marked vasodilator action than a systemic insulin infusion despite similar plasma concentrations. This is an unexpected finding if one accepts that sympathetic activation induced by systemic hyperinsulinaemia causes peripheral vasoconstriction to counter insulin's vasodilator action - in the absence of sympathetic activation one might expect an augmentation of insulin's vasodilator action. One possibility is that sympathetic stimulation is actually causing direct peripheral vasodilation, although there is very

Figure 1.10
Time course of percentage change in forearm blood flow ratio (mean ± S.D.)in response to intra-arterial infusion of (a) placebo (b) insulin 1mU/min (c) insulin 5mU/min. Double-blind, random order study design. * $p < 0.05$ From Ueda et al, 1998a.



little evidence for this.

1.2.5 Augmentation of insulin-mediated vasodilation by glucose

Another explanation may be that glucose availability may be important for insulin's vascular action. Normally, during clamp studies, 20% glucose is infused systemically along with insulin to avoid hypoglycaemia (DeFronzo et al, 1979). If a hyperglycaemic clamp is carried out, with co-infusion of somatostatin to suppress insulin secretion, no effect on limb blood flow can be demonstrated with increasing glucose levels (Baron, 1993). However, clamping glucose levels at 20mmol/l in the presence of insulin causes augmentation of limb blood flow by around 50%, compared with clamping at 5mmol/l (Baron, 1993).

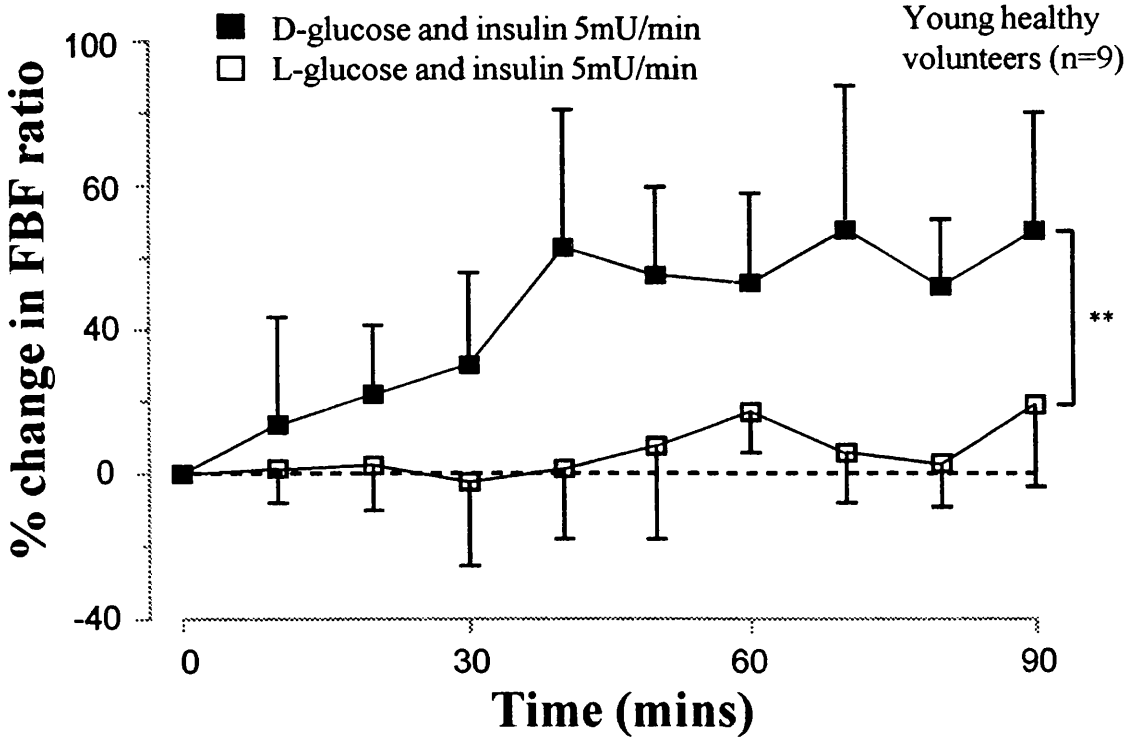
During local studies there is no risk of systemic hypoglycaemia and, therefore, it is not common practice to co-infuse glucose with insulin via a limb artery. Our own group addressed this issue by studying local insulin infusion with and without co-infusion of intra-arterial glucose, in sufficient concentration to maintain local venous euglycaemia. During this study, I learned the techniques of brachial artery cannulation and venous-occlusion plethysmography under the supervision of Drs Ueda and Petrie.

In a preliminary study, intra-arterial infusion of D-glucose at 75 μmol/min with high-physiological doses of intra-arterial insulin was shown to maintain euglycaemia in samples obtained from a deep vein of the infused arm. Furthermore, venous sampling from the other arm revealed that glucose levels remained unchanged,

confirming that there was no significant systemic effect of the local insulin infusion. We then proceeded to investigate the stereospecificity of the effect of glucose on the vasodilating effect of insulin by co-infusing D- or L-glucose (metabolically-inactive isomer) on separate study days at 75 μmol/min for 30 min with saline and subsequently for 90min with 5 mU/min of insulin (resulting in high-physiological insulin levels in deep venous effluent) in a double blind, cross over design (n=8). Blood samples were taken every 10 minutes for measurement of glucose and every 30 minutes for measurement of insulin and potassium. The infusion rate (D/L glucose plus saline) was 2 ml/min throughout. The mean±SD change in forearm blood flow (figure 1.11) during infusion of insulin with D-glucose (47.1±21.3%) was significantly greater than that during infusion of insulin and L-glucose (6.1±12.2%), p=0.01, 95% CI 23.1, 58.8). Infusion of both D- and L-glucose alone prior to insulin administration tended to reduce forearm blood flow but there was no significant difference in this response between the two treatments. There were no differences in serum potassium or insulin concentrations in the deep venous effluent between the two treatments (Ueda et al 1998a).

Therefore, augmentation of local insulin-mediated vasodilation by D-glucose is not due to an osmotic effect of glucose since we compared D-glucose with its stereo-isomer L-glucose, which is metabolically inactive. This observation suggests either that a local fall in extracellular glucose may impair insulin's vascular action or that insulin-mediated vasodilation may be more closely related to tissue glucose uptake than to insulin concentrations *per se*. Under physiological circumstances, insulin is

Figure 1.11
Percentage change in forearm blood flow (FBF) ratio over time in response to intra-arterial infusion of insulin (5mU/min) with either D-glucose (75 umol/min) or L-glucose (metabolically-inactive stereoisomer). Double-blind, random order study design. ** $p < 0.01$ From Ueda et al, 1998a.



secreted in response to a post-prandial rise in blood glucose; thus, hyperinsulinaemia in any vascular bed is likely to be accompanied by an increase in supply of substrate (glucose). If the vasodilating effect of insulin was dependent on the stimulation of tissue glucose uptake, this effect might be masked during isolated local hyperinsulinaemia. This latter suggestion may be relevant in understanding the association between endothelial function and insulin sensitivity (see below).

1.3 Mechanisms of insulin-mediated vasodilation

1.3.1 Insulin as an endothelium-dependent vasodilator

Two earlier studies conducted in man reported that insulin causes vasodilation via release of endothelial nitric oxide. In one (Scherrer et al, 1994a), prior local intra-arterial infusion of L-NMMA completely abolished insulin-mediated vasodilation in the forearm during a systemic insulin infusion. In the same study, vasoconstrictor responses to increasing doses of L-NMMA during euglycaemic hyperinsulinaemia were significantly higher than under basal conditions, while there were no differences in reponses to norepinephrine. In the other study (Steinberg et al, 1994), approximately 20% of leg blood flow was shown to be nitric oxide-dependent under basal conditions, while the value was nearer 40% during hyperinsulinaemia.

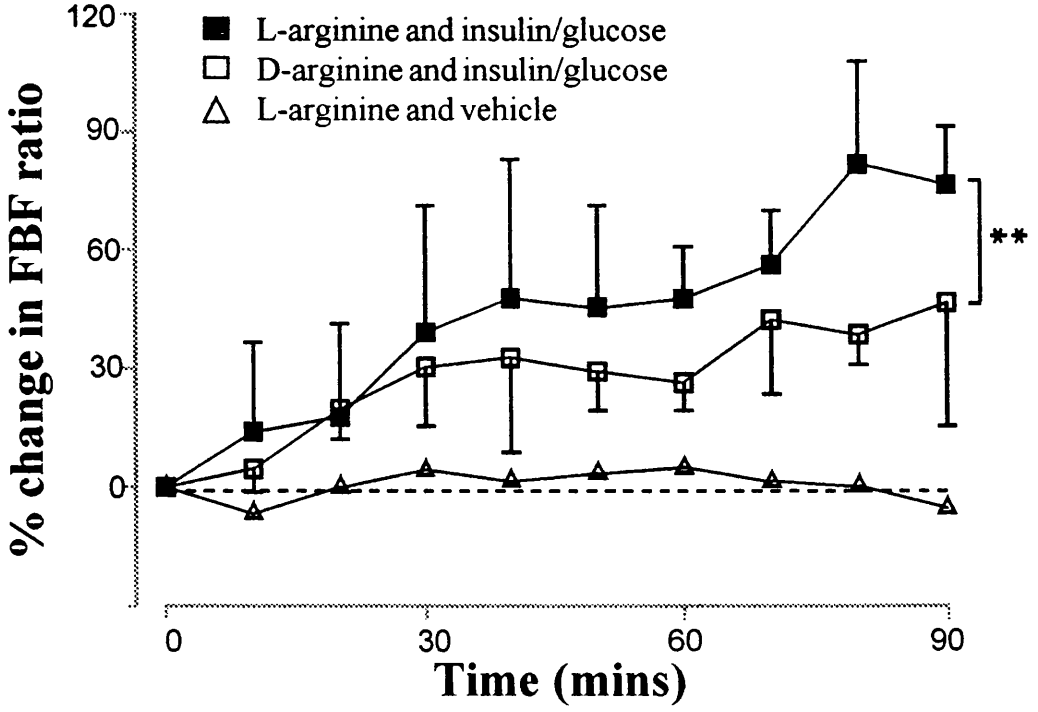
More recently, a number of studies have supported the notion that insulin is an endothelium-dependent vasodilator. It has been noted in children that a bolus of intravenous insulin causes an increase in nitric oxide production, as measured by urinary nitrite/nitrate excretion (Tsukahara et al, 1997). Furthermore, using multiple regression analysis, it has been shown in man that limb blood flow response to local nitric oxide synthase inhibition (a measure of basal nitric oxide production), together

with the degree of forearm muscularity, explained over 50% of the variation in insulin-mediated blood flow, as measured by PET scanning (Utriainen et al, 1996a). It has also been demonstrated that forearm vasodilation secondary to local intra-arterial infusions of insulin can be completely abolished by co-infusion of L-NMMA in man (Van Veen et al, 1997). A number of *in vitro* studies provide supportive evidence: in rat vessels, insulin's ability to attenuate adrenergic preconstriction was abolished by prior incubation with L-NMMA (Wu H-Y et al, 1994) while, more recently, insulin was shown to vasodilate rat cremasteric arterioles *in vitro*, an effect which was inhibited by either incubation with L-NMMA or endothelial removal (Chen & Messina, 1996). However, in the hamster cremasteric microcirculation insulin retained its ability to cause vasodilation despite blockade of nitric oxide synthase (McKay & Hester, 1996), suggesting that endothelium-dependent mechanisms are unlikely to be the only explanation for insulin-mediated vasodilation in certain vascular beds.

The mechanisms of insulin-stimulated nitric oxide production remain to be fully elucidated. In this regard, our own group has recently demonstrated that concomitant intra-arterial infusion of L-arginine significantly augments the vasodilator response to local insulin/glucose infusion compared with control (intra-arterial D-arginine) (Ueda et al, 1998b). This suggests that insulin-stimulated uptake of L-arginine (the substrate of nitric oxide synthase) by endothelial cells may lead to an increase in nitric oxide production. In this study eight healthy male volunteers (aged 20-40 years) underwent intrabrachial infusions of L-arginine or D-arginine (40 μmol/min, hydrochloride salts) along with insulin (5mU/min) and glucose (75

μmol/min) for 90 minutes on two separate days in a double-blind random-order crossover design. On a third day, the same subjects received L-arginine alone (40 μmol/min) for the same time period. Forearm blood flow was measured using bilateral strain-gauge venous-occlusion plethysmography (section *2.7.1*). The vasodilator response to L-arginine with insulin/glucose was significantly greater than that observed with D-arginine and insulin/glucose (figure 1.12). Infusion of L-arginine alone had no effect on forearm blood flow. Local steady-state insulin concentrations were approximately 500 pmol/L (high physiological) in both insulin/glucose conditions. Local L-arginine concentrations were approximately 900 μmol/L (nine-fold excess) at steady-state in both L-arginine conditions. From these results it appears that during local hyperinsulinaemia (similar to post-prandial conditions), L-arginine (but not D-arginine) administration produces stereospecific vasodilation. The mechanism for this effect is unclear since it is known that L-arginine, which is the substrate for the classical pathway of NO synthesis (via stereospecific NO synthase), is present in high concentrations in endothelial cells; thus, administration of additional L-arginine should not affect blood flow in health (Chowienczyk & Ritter, 1997). It has been suggested that a non-enzymatic pathway for the generation of NO may explain the unexpected vasodilator effects of arginine (Chowienczyk & Ritter, 1997). Thus, formation of NO from either enantiomer of arginine and hydrogen peroxide in conditions of local oxidative stress may account for reports of normalisation by L-arginine of vascular responses in states of endothelial dysfunction (Drexler et al, 1991), and of non-stereospecific vasodilator effects of arginine at high concentrations (MacAllister et al, 1995). However, the results from our study demonstrating a clear stereospecific effect of L-arginine

Figure 1.12
Time course of percentage change in forearm blood flow ratio in 9 healthy male volunteers in response to intra-arterial infusion of (a) insulin, D-glucose & L-arginine (b) insulin, D-glucose & D-arginine (c) L-arginine & vehicle. Double-blind, random order study design. ** $p < 0.01$ From Ueda et al, 1998b.



would be more consistent with the notion that L-arginine transport into endothelial cells may be rate-limiting during conditions of active production of NO. This notion is supported by *in vitro* studies in cultured endothelial cells, which show that insulin stimulates system y^+, a transporter system which facilitates cellular uptake of L-arginine (Sobrevia et al, 1996). The prolonged time-course of insulin-mediated vasodilation observed in most *in vivo* studies would be compatible with the hypothesis that insulin promotes *de novo* synthesis of the system y^+ transporter.

Alternatively, there is evidence that insulin causes a rise in intracellular Ca^{2+} levels in rat aortic endothelial cells with subsequent stimulation of constitutive nitric oxide synthase (Han et al, 1995). One possible mechanism for this depends on insulin's stimulation of endothelial Na^+-K^+ ATPase: as there are no voltage-gated Ca^{++} channels in endothelial cells, hyperpolarisation leads to an increase in intracellular [Ca^{++}] (Luckhoff & Busse, 1990) which may activate the Ca^{++}-dependent enzyme eNOS.

An alternative opinion is that insulin does not stimulate endothelial nitric oxide production *per se* but rather, in some way, modulates the responsiveness to agents such as acetylcholine. Taddei et al (1995) proposed that insulin was not a significant vasodilator, but was able to shift the blood flow dose-response curve to acetylcholine; more recently, the same group reported a correlation (albeit weak) in a group of hypertensive patients between insulin-mediated vasodilation and the slope of the acetylcholine dose-response curve (Natali et al, 1997). However, studies on human gluteal vessels in vitro revealed that after 20 minutes of incubation with

insulin, the acetylcholine dose-response curves remained unchanged (McNally et al, 1995), and this casts doubt on the idea that insulin effects are mediated through this indirect mechanism

Finally, there is evidence that insulin stimulates endothelial vasoactive agents other than nitric oxide. During systemic hyperinsulinaemia, insulin caused increased circulating levels of 6-keto PGF-1α and decreased levels of endothelin - interestingly, this effect was observed only in women (Polderman et al, 1996), and the significance is uncertain.

In chapter 3, a study is presented which sets out to explore the physiological mechanisms of insulin-mediated vasodilation and to establish the role of endothelial nitric oxide production in comparison with other putative mechanisms for insulin's vascular action.

1.3.2 Direct effects of insulin on vascular smooth muscle cells

Insulin has long been known to stimulate a variety of ion pumps (such as Ca^{2+}-ATPase, Na^{+}-K^{+} ATPase and the Na^{+}-H^{+} exchanger) on VSMCs thus causing hyperpolarisation, closure of voltage-gated calcium channels and vasodilation (Sowers, 1996). Recent work supports the notion of insulin acting as a vasodilator via effects on membrane potential: insulin and insulin-like growth factor-1 (IGF-1) have both been shown to stimulate Na^{+}-K^{+} ATPase activity independently of nitric oxide synthase activation (Gupta et al, 1996; Standley et al, 1997). In addition, inhibition of Na^{+}-K^{+} ATPase activity by ouabain in the human forearm has been

shown to abolish insulin-mediated vasodilation (Tack et al, 1996). In addition, an increase in intracellular Ca^{2+} levels in VSMCs and platelets has been demonstrated in conjunction with insulin resistance (Baldi et al, 1996), the proposed mechanism being abnormal modulation of the Ca^{2+}-ATPase pump (Zemel et al, 1992). Furthermore, addition of insulin to cultured VSMCs causes a transitory rise in intracellular Ca^{2+} levels in VSMCs with subsequent attenuation of Ca^{2+} entry. Based on these results it has been proposed that insulin may stimulate constitutive nitric oxide synthase in VSMCs (Touyz et al, 1994), thereby reducing contractility.

1.4 Are insulin's metabolic and vascular actions functionally related ?

1.4.1 Concept of metabolic and vascular 'cross-talk'

In recent years the concept of metabolic and vascular 'cross-talk' has emerged (Clark et al, 1995). In relation to insulin and cardiovascular physiology this concept is teleologically attractive and has been supported by data from several recent studies. Theoretically, 'cross-talk' couples tissue blood flow (nutrient supply) with its specific metabolic needs. In his review, Clark et al (1995) advance the notion of 'nutritive' and 'non-nutritive' capillary networks in skeletal muscle, blood flow to which is controlled by specific functional vascular shunts sensitive to the tissue's metabolic requirements. It is, of course, already known that changes in the immediate local environment during exercise can influence blood supply, for example pH, and levels of K^+ or CO_2, and it is conceivable that in the anabolic state, for example during insulin-stimulated glucose uptake, blood flow is altered to facilitate glucose uptake to the most appropriate areas. This could occur via a

specific vascular effect of insulin at an arteriolar level, or secondary to a product of intracellular glucose metabolism.

1.4.2 Evidence supporting a functional association between insulin sensitivity and insulin-mediated vasodilation

It has been demonstrated, using doses of insulin ranging from physiological to pharmacological, that there is a dose-dependent effect of insulin on skeletal muscle glucose uptake. This may be due to a combination of increased fractional glucose extraction and increased limb blood flow (Laakso et al, 1990). In a series of studies from Baron's group, clamp-derived insulin sensitivity (whole-body glucose uptake) was significantly correlated with insulin-mediated vasodilation (Baron et al, 1995) but not with arteriovenous fractional extraction of glucose (Baron, 1994), implying that insulin-mediated glucose uptake is more closely associated with insulin's ability to stimulate blood flow. Furthermore, it has been demonstrated that vascular sensitivity to insulin, as assessed by relaxation in preconstricted dorsal hand veins, is closely associated with whole-body insulin sensitivity, suggesting a functional link between these two processes (Feldman et al, 1995).

Results from a number of *in vitro* studies provide evidence that glucose uptake may be important in promoting VSMC relaxation (Graier et al, 1993; Kahn et al, 1995; Sobrevia et al, 1996). Two important *in vivo* studies have already been cited above: insulin-mediated vasodilation appears to be progressively augmented by increasing levels of glycaemia in man (Baron, 1993) and we have recently demonstrated that insulin's local vasodilator action is significantly augmented by co-infusion with

physiological D-glucose; this does not occur with the stereo-isomer L-glucose, which is metabolically inactive (Ueda et al, 1998a).

In further support of metabolic and vascular coupling of insulin action are recent data in hypertensive patients demonstrating a correlation between insulin-mediated blood flow and whole-body insulin sensitivity (Natali et al, 1997; Baron & Steinberg, 1997). However, Yki-Jarvinen and colleagues were unable to demonstrate any relationship between insulin sensitivity and forearm vasodilation in response to systemic hyperinsulinaemia in healthy subjects (Utriainen et al, 1996a; Utriainen et al, 1996b). In support of this conclusion, the same group used positron emission tomography and [^{18}F]fluoro-2-deoxy-D-glucose uptake to demonstrate a dissociation of insulin-stimulated blood flow and glucose uptake in thigh skeletal muscle; however, despite this finding, they demonstrated a significant relationship between the insulin-induced increase in leg blood flow and whole-body insulin sensitivity (Raitakari et al, 1996). In a recent study, in which similar methods were used, the same group showed that insulin-stimulated muscle blood flow co-localized with regional glucose uptake, suggesting that insulin redirects flow to areas where it stimulates glucose uptake and, therefore, that the vascular and metabolic actions of insulin may, indeed, be functionally coupled (Utriainen et al, 1997).

1.4.3 Evidence questioning the physiological relevance of any functional link between insulin's metabolic and vascular actions

There is also a body of data which does not support the notion that tissue glucose uptake and skeletal muscle blood flow are functionally related. In one study, when

human volunteers were fed a mixed physiological meal, arteriovenous glucose extraction was found to be the main determinant of limb glucose uptake; this rose by a factor of five, while blood flow increased by only 16% (Mijares & Jensen, 1995). In another study, no significant increases in forearm blood flow were observed during an oral glucose tolerance test (Kelly et al, 1988). The results of these two studies question the physiological significance of any functional link between glucose uptake and blood flow.

Furthermore, dissociation of insulin-mediated glucose uptake from blood flow was observed in a study in which both limb blood flow and muscle sympathetic nerve activity were stimulated to similar degrees at different systemic insulin concentrations, ranging from 100 to 400pmol/l (Anderson et al, 1991). Moreover, fructose, which causes an increase in cellular metabolism without stimulation of insulin secretion, did not result in any increase in blood flow or muscle sympathetic nerve activity in human volunteers (Vollenweider et al, 1993). This result, of course, does not rule out the possibility that the coupling signal for promotion of blood flow is unique to intracellular glucose metabolism.

In summary there is evidence both for and against the existence of a functional relationship between insulin-mediated glucose uptake (insulin sensitivity) and insulin-mediated vasodilation, although the balance of recent evidence supports the idea that insulin-stimulated blood flow is positively related to peripheral glucose uptake by skeletal muscle. How physiologically relevant this relationship is remains a matter for debate. What also remains unclear is how any functional coupling between

insulin's metabolic and vascular actions might be of pathophysiological relevance in the link between metabolic and cardiovascular disease. These questions are further explored in chapters 4 and 7 where detailed clinical physiology studies are carried out on healthy volunteers as well as patients with either essential hypertension or type 2 diabetes.

Given that insulin-mediated vasodilation is, at least in part, endothelium-dependent, it is possible that endothelial function plays a pivotal role in metabolic and vascular 'cross-talk'. The following sections will go on to discuss the importance of endothelial function (or dysfunction) with respect to metabolic and vascular control mechanisms.

1.5 Does endothelial dysfunction cause relative insulin resistance ?

1.5.1 Reduced substrate delivery to skeletal muscle secondary to vascular endothelial dysfunction - evidence for and against

Vascular endothelial dysfunction could, in theory, be related to insulin resistance in a number of ways (Anderson & Mark, 1993; Baron, 1994; Petrie et al, 1996a; Pinkney et al, 1997). Blunting of the endothelium-dependent component of insulin-mediated vasodilation could reduce substrate delivery to target tissue resulting in a reduction in insulin-stimulated glucose uptake. In support of this hypothesis it has been demonstrated in obese subjects (Laakso et al, 1990) and in patients with type 2 diabetes (Laakso et al, 1992) and IDDM (Baron et al, 1991) that there is blunting of insulin-mediated vasodilation in parallel with reductions in whole-body insulin sensitivity. However, the physiological relevance of these studies has been

questioned. In many of these clamp studies, pharmacological doses of insulin were used, and in those involving subjects with type 2 diabetes, glucose values were clamped at the relatively hypoglycaemic value of 5mmol/l. In another study, in which glucose levels were clamped at fasting levels in subjects with type 2 diabetes, no blunting of the blood flow response to insulin was observed (Dela et al, 1995). It has been suggested that substrate delivery is only likely to be rate-limiting for glucose disposal at supra-physiological insulin levels maintained for many hours and therefore does not explain the physiological association between endothelial function and insulin sensitivity (Yki-Jarvinen, 1995).

One approach to resolve this issue is to manipulate limb blood flow during hyperinsulinaemia and assess whether insulin-mediated glucose uptake is affected. There is some evidence from these studies that skeletal muscle blood flow may be a determinant of insulin-mediated glucose uptake; infusion of L-NMMA into the femoral artery during a clamp reduces insulin-mediated stimulation of blood flow (via inhibition of endothelial nitric oxide production) resulting in a 25% decrease in limb glucose uptake (despite a 50% increase in arteriovenous fractional glucose extraction) (Baron et al, 1995). This observation is consistent with the notion that 20-30% of insulin-mediated glucose uptake is blood flow dependent. In the same study, whole-body insulin sensitivity correlated significantly with the decrement in limb blood flow as a result of L-NMMA infusion; the authors' interpretation of this was that blood flow is more important in determining glucose uptake in insulin sensitive individuals (Baron et al, 1995).

However, similarly designed experiments have produced contrary results. In human studies, forearm blood flow has been augmented by 100% using intra-arterial adenosine (Natali et al, 1994) and bradykinin (Nuutila et al, 1996) without a detectable effect on forearm glucose uptake. These results provide evidence against a significant role for muscle blood flow in determining glucose disposal, but it should be borne in mind that infusates used to alter blood flow may also have metabolic properties which affect glucose uptake. This is likely to be the case with bradykinin (Dietze et al, 1996) and may partly explain why bradykinin antagonists administered to rats cause a significant reduction in insulin-mediated glucose disposal (Kohlman et al, 1995).

A recent controlled study, in which PET scanning was used to measure forearm blood flow, showed no defect in insulin-stimulated muscle blood flow in a group of patients with type 2 diabetes, implying that insulin's metabolic function is impaired but its vascular action is preserved in this condition (Utriainen et al, 1997). In a group of patients with essential hypertension, calf blood flow measured during systemic hyperinsulinaemia was not directly related to insulin sensitivity and there was no difference compared with a group of healthy matched controls (Hunter et al, 1997). On the other hand, Wascher and colleagues (1997) found that insulin-mediated forearm vasodilation was blunted in subjects with either obesity or type 2 diabetes. Co-infusion of L-arginine not only normalised the vasodilator responses to insulin, but also improved insulin sensitivity. However, these data should be interpreted with caution since intravenous L-arginine may elicit systemic insulin release (Guigliano et al, 1997).

The study described in chapter 5 was designed to test whether whole-body insulin sensitivity is affected by artificially inducing a state of temporary endothelial dysfunction - any reduction in insulin-mediated glucose disposal would support the notion that tissue blood flow and substrate delivery were rate-limiting to glucose uptake and, therefore, that endothelial dysfunction might be a significant determinant of insulin resistance.

1.5.2 Other potential mechanisms for damaged endothelium to affect glucose uptake

There are other theoretical mechanisms by which endothelial dysfunction could cause insulin resistance. For example, reduced activity of the endothelial enzyme lipoprotein lipase could result in dyslipidaemia with subsequent effects on insulin-mediated glucose metabolism (Ahn et al, 1993). Insulin must traverse the endothelial barrier to exert its metabolic effects and therefore dysfunctional transport mechanisms could limit insulin's metabolic effectiveness (Yang et al, 1989).

1.6 Does a reduction in insulin-mediated glucose uptake affect endothelial function ?

Rather than being a consequence of endothelial dysfunction, it is more tempting to conclude on current evidence that peripheral insulin resistance is a cause of this defect (Stehouwer et al, 1997). A number of putative mechanisms for this have already been alluded to above and these will now be expanded.

1.6.1 Hepatic insulin resistance, increased oxidised lipid sub-fractions and endothelial dysfunction

Hepatic insulin resistance is associated with a shift in substrate metabolism resulting in increased serum concentrations of free fatty acids, triglycerides and oxidised LDL-cholesterol (Reaven et al, 1993; see section 1.9.1 for further explanation of possible mechanisms). If oxidation of circulating LDL (particularly small dense LDL) occurs at an endothelial level, the oxidised particles generated have the potential to cause endothelial dysfunction (and hence atherogenesis) either by direct cellular damage or by quenching nitric oxide production (DiCorleto & Soyombo, 1993; Davda et al, 1995). Furthermore, there is recent evidence that increased post-prandial circulating concentrations of triglyceride-rich particles and remnant particles may be deleterious to the endothelium (Vogel et al, 1997; Plotnick et al, 1997). One interesting speculation is that insulin, secreted post-prandially, plays a role in boosting endothelial nitric oxide production to buffer this temporary increase in lipid-induced oxidant stress.

1.6.2 Glucose uptake, ATP generation and endothelial function

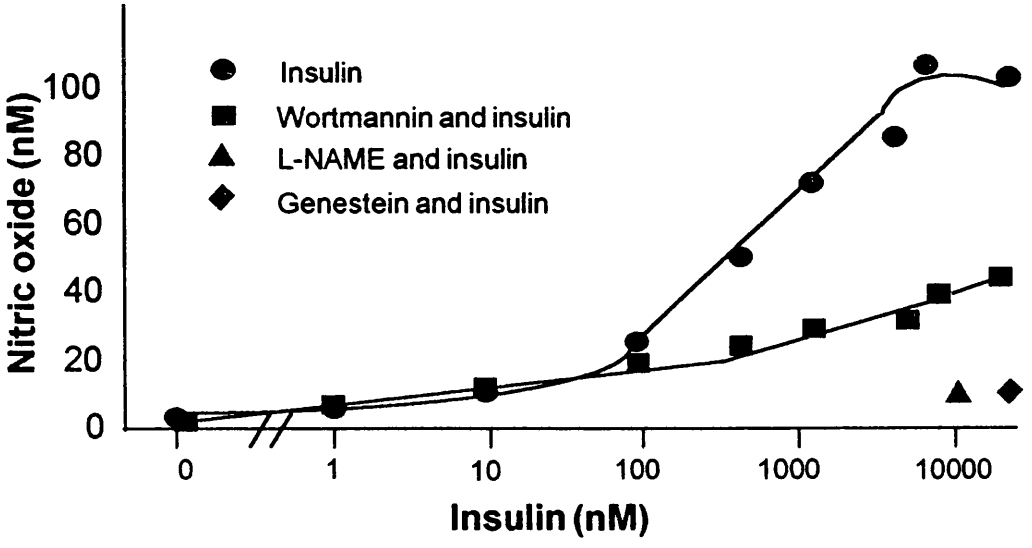
There are a number of putative mechanisms by which cellular glucose uptake could be linked with vascular tone. One hypothesis is that insulin-stimulated glucose uptake may determine blood flow in response to the hormone by causing an increase in ATP production, as a consequence of aerobic glycolysis. This will provide energy for membrane ion pumps (for example Na^+-K^+ ATPase) resulting in hyperpolarisation and changes in Ca^{2+} fluxes in both endothelium and vascular smooth muscle cells leading to vascular relaxation by mechanisms discussed above.

Furthermore, if nitric oxide synthase activity is enhanced by stimulation of ATP-dependent ion pumps such as Na^+-K^+ ATPase, insulin-stimulated glucose uptake may result in increased nitric oxide production as a consequence of endothelial cell glucose metabolism; conversely, reduced glucose uptake would be predicted to lead to diminished nitric oxide production. Additionally, since another consequence of increased intracellular glucose levels is shunting through the pentose monophosphate pathway, which produces NADPH, an essential co-factor for nitric oxide synthase (Wu G et al, 1994), any reduction in glucose uptake might indirectly affect NOS activity. Finally, of special note is a study demonstrating nitric oxide production secondary to insulin stimulation in endothelial cell culture and subsequent blocking of this in the presence of wortmannin, which inhibits PI 3-kinase, an essential enzyme involved in intracellular insulin-stimulated glucose transport and metabolism (Zeng & Quon, 1996) (figure 1.13). This suggests that there is a common post-receptor pathway for insulin's metabolic and vascular actions which may partly explain the functional link between them.

1.6.3 Chronic hyperglycaemia, insulin resistance and endothelial dysfunction - a special case in type 2 diabetes

Chronic hyperglycaemia has deleterious effects on both insulin sensitivity and endothelial function. In type 2 diabetes, glucotoxicity exacerbates peripheral insulin resistance by down-regulating post-insulin-receptor signalling and by impairing β-cell function (Richter et al, 1988; Rosetti et al, 1990; Yki-Jarvinen, 1995). Glucose activates endothelial nitric oxide synthase, by triggering a rise in intracellular Ca^{2+} levels (Graier et al, 1993) and by stimulating the system y^+ transporter for L-arginine

Figure 1.13
Direct measurement of nitric oxide production in human umbilical vein cell culture in response to insulin. Wortmannin inhibits PI3-kinase; L-NAME inhibits NOS; genestein inhibits tyrosine kinase. From Zeng & Quon, 1996.



(Sobrevia et al, 1996); this may explain the observation of increased microvascular flow in the early stages of diabetes. However, chronic hyperglycaemia appears to cause down-regulation of insulin's stimulatory effect on system y^+ (Sobrevia et al, 1996). Lastly, advanced glycosylation end-products (AGEs), which form in an environment of chronic hyperglycaemia, cause endothelial damage, probably by stimulating the production of oxygen free radicals (Brownlee et al, 1988; Poston & Taylor, 1995).

1.6.4 Insulin resistance and endothelial dysfunction - a potential positive feedback cycle

If both mechanisms to relate insulin sensitivity and endothelial function discussed above are valid, there is a theoretical potential for a damaging positive feedback cycle. Defective metabolic pathways associated with insulin resistance may result in a reduction in basal endothelial NO production and relative endothelial dysfunction. This, in turn, may reduce the efficacy of vasodilation to nutrient vascular beds resulting in further impairment of insulin-mediated glucose uptake and compensatory hyperinsulinaemia. Thus, a vicious cycle is created (figure 1.14).

1.7 Are insulin sensitivity and endothelial function both influenced by a 'third factor' ?

The danger of interpreting data demonstrating consistent association of two variables is that there might be an underlying confounding factor (or factors) influencing each variable. One way to take account of this is to employ multiple

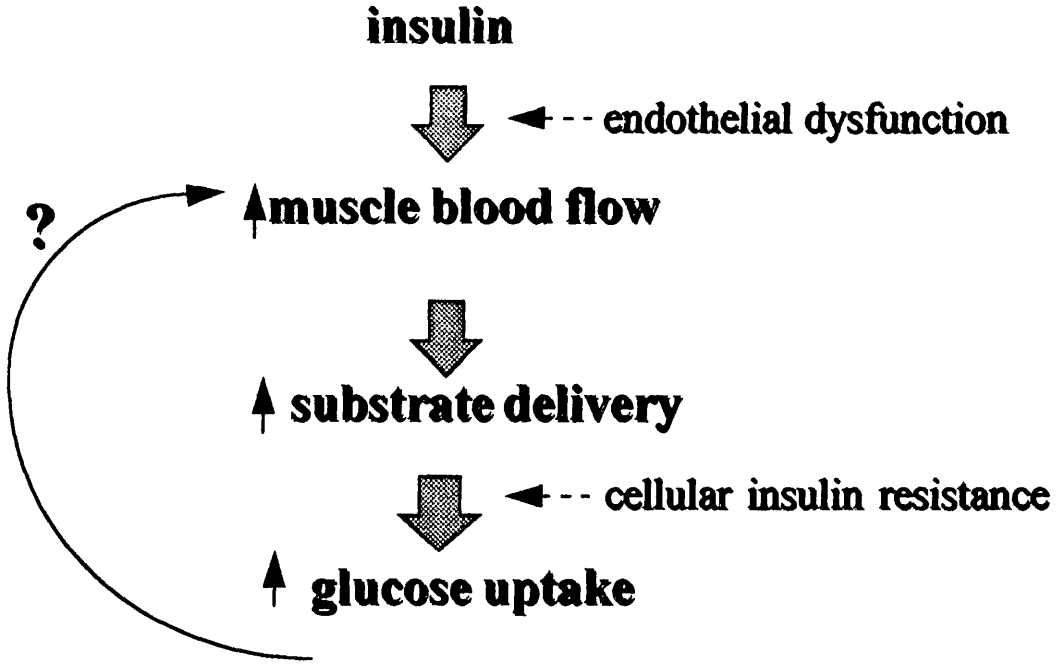


Figure 1.14
Schematic diagram outlining a hypothetical mechanism for a potential positive feedback cycle linking cellular insulin resistance and endothelial dysfunction. This is based on two premises: (1) skeletal muscle blood flow is rate-limiting for cellular glucose uptake and (2) cellular glucose uptake and metabolism determines muscle blood flow via an endothelium-dependent mechanism.

regression analysis, a technique which has been used in several of the studies presented in this thesis. This type of analysis is limited by the number of factors measured - it is always possible that a potential 'third factor' explains an observed relationship but simply has not been measured. Three of the most attractive candidates for 'third factors' in the insulin action / endothelial function association are discussed in greater detail:

1.7.1 The role of skeletal muscle structure and function in metabolic and cardiovascular disease

Associations have been noted between skeletal muscle fibre types and both metabolic and cardiovascular disorders. Subjects with obesity (Lilloja et al, 1987; Wade et al, 1990; Simoneau & Bouchard, 1993), central obesity (Lilloja et al, 1987; Krotkiewski & Bjorntorp, 1986), hypertension (Juhlin-Dannfelt et al, 1979; Frisk-Holmberg et al, 1983) and type 2 diabetes (Lilloja et al, 1987) have a reduced capillary density, a predominance of type IIB glycolytic, fast-twitch fibres and a relative lack of type I oxidative, slow-twitch fibres. The oxidative capacity of skeletal muscle fibres is correlated with capillary density and fibre type is a determinant of blood flow and peripheral resistance; in muscles composed of predominantly type I fibres, there is evidence of increased basal blood flow (Frisk-Holmberg et al, 1981).

The relationships among fibre type, capillary density, haemodynamics and metabolism are illustrated by the effects of exercise. Chronic aerobic exercise training results in a shift from type IIB to type IIA fibres (Anderson & Henriksson,

1977a) and also an increase in muscle capillary density (Anderson & Henriksson, 1977b; Krotkiewski et al, 1983). It is possible that the increase in oxidative capacity and promotion of skeletal muscle blood flow associated with exercise could mediate the observed improvement in insulin sensitivity and reduction in total peripheral resistance. Thus, physical inactivity is associated with hypertension (Paffenbarger et al, 1983), while chronic aerobic exercise training results in significant improvements in blood pressure control (Tipton, 1991). Aerobic training reduces insulin resistance (Holloszy et al, 1987) but anaerobic training does not have the same beneficial effect (Yki-Jarvinen & Koivisto, 1983).

In addition, there are interesting comparisons between effects of the ageing process on skeletal muscle structure and function and disease states. Advancing age results in inactivity and concomitant reduced muscle mass, a relative shift to type IIB fibre types and a decrease in maximal aerobic capacity. It is generally accepted that blood pressure and total peripheral resistance rise with age; this is paralleled by a fall in insulin-mediated glucose uptake (Shimoketa et al, 1991). These metabolic and cardiovascular conditions tend to have a familial pattern and, likewise, the structure of skeletal muscle is largely inherited (Komi & Karlsson, 1979); furthermore, relatives of patients with both type 2 diabetes (Nyholm et al, 1996) and essential hypertension (Beatty et al, 1993) had 15-20% lower values than controls for predicted maximum oxygen consumption.

The possibility that skeletal muscle structure and function accounts for the observed relationship between insulin's metabolic and vascular actions is explored further in chapter 6.

1.7.2 Distribution of adiposity, hepatic insulin resistance and endothelial dysfunction

One issue which is emerging from the recent literature is the possible confounding role of obesity/dyslipidaemia when variables such as endothelial function and insulin sensitivity are measured in patients with conditions such as essential hypertension and type 2 diabetes. For example, it was demonstrated in one study that body mass index accounted for 50-60% of the variance of leg blood flow response to methacholine during a hyperinsulinaemic clamp. The more obese the subject, the more this response was blunted (Steinberg et al, 1996); of particular interest was the observation that a group of type 2 diabetic patients with similar mean BMI exhibited no further blunting of endothelium-dependent vasodilation. Furthermore, the same group have recently shown that blunting of methacholine-stimulated leg blood flow was induced by systemic free fatty acid infusion with co-infusion of somatostatin to suppress insulin secretion. When the protocol was repeated in the presence of insulin, this blunting of endothelial function was reversed (Steinberg et al, 1997). This result is supported by results of a study in which the free fatty acid, oleic acid, was shown to inhibit nitric oxide synthase in endothelial cell culture (Davda et al, 1995). Thus it seems that dyslipidaemia *per se* may be a causal factor for both endothelial dysfunction and insulin resistance, and this phenomenon may explain some of the conflicting results when patients with metabolic and cardiovascular

disorders are studied without careful control for variations in body mass index. When patient groups are studied in chapter 7, great care has been taken to match for age and BMI.

1.7.3 The role of endogenous corticosteroids in metabolic and cardiovascular disease

Endogenous and exogenous glucocorticoid excess is associated with hypertension, insulin resistance and central adiposity. Glucocorticoids are known to decrease insulin-mediated glucose uptake (Holmang & Bjorntorp, 1992), and mediate the increased insulin requirement associated with stress and infection in diabetic patients; they also inhibit endothelium-dependent vasodilation in animal models (Walker et al, 1995). Proliferation of capillary endothelium induced by insulin in rat skeletal muscle is abolished by administering concomitant corticosterone (Holmang et al, 1996), and insulin-mediated vasodilation in man can be abolished by administration of oral dexamethasone 48 hours prior to a clamp study (Scherrer et al, 1993). Possible mechanisms for this include central modulation of sympathetic outflow (via effects on neuropeptide Y or corticotrophin releasing hormone) and peripheral effects on insulin-mediated glucose uptake, with consequent effects on endothelial NOS activity. Skeletal muscle fibre types also appear to be modified by corticosteroids; in Cushing's syndrome there is a predominance of type II fibres (Rebuffe-Scrive et al, 1988). Furthermore, sensitivity to glucocorticoids in man, as assessed by dermal vasoconstrictor responses, correlate significantly with reduced glucose tolerance, insulin resistance and increased levels of plasma triglycerides (Walker et al, 1996). Therefore, enhanced availability of cortisol at its receptor (either through increased

production, local synthesis or impaired metabolism) may account for the association of insulin action, endothelial function and skeletal muscle morphology; in fact it has been suggested that 'metabolic syndrome X' is, in effect, sub-clinical Cushing's syndrome (Bujalska et al, 1997).

1.8 Insulin resistance and endothelial function in essential hypertension

1.8.1 Associations of insulin resistance / hyperinsulinaemia with hypertension

The association of essential hypertension with hyperinsulinaemia was first noted over 30 years ago (Welborn et al, 1966). Since then, a number of cross-sectional epidemiological studies have confirmed the association between blood pressure and insulin levels (Haffner et al, 1992a; Modan et al, 1985; Zavaroni et al, 1989; Jiang et al, 1993; Feskens et al, 1995), although the results of a number of studies dispute the significance of this association (eg Asch et al, 1991; Dowse et al, 1993; Muller et al, 1993). Results from this type of epidemiological study appear to depend on how rigorously the investigators control for confounding factors, especially body mass index, distribution of adiposity and ethnicity. While it is clearly important to take account of confounding factors, it is equally important not to control for a closely-related co-variable since a false-negative result may occur - this may be the case for body mass index (section *1.8.2*). Prospective studies have shown that individuals with hyperinsulinaemia have a higher risk of going on to develop hypertension (Haffner et al 1992b; Bao et al, 1996). In addition, subjects with impaired glucose tolerance in 1968 had a significantly increased risk of being hypertensive 20 years later (Salomaa et al, 1991). However, not all groups are in agreement (Vaccaro et al, 1996).

What has become clear is that hyperinsulinaemia *per se* does not directly elevate blood pressure; for example, artificial chronic elevation of insulin levels in dogs does not affect blood pressure (Brands et al, 1991) and patients with insulinoma do not tend to have hypertension (Fujita et al, 1992). The probable explanation for these findings is that hyperinsulinaemia is only associated with hypertension when it occurs as a compensatory response to defects in insulin-mediated glucose uptake (or insulin resistance) mainly in skeletal muscle. Whole-body insulin-mediated glucose uptake, measured using the euglycaemic hyperinsulinaemic clamp technique is reduced in patients with essential hypertension (Ferrannini et al, 1987; Lind et al, 1995). Whilst it remains unclear if there is a causal association between these parameters, it is of interest to note that insulin resistance is present in normotensive offspring of hypertensive patients (Beatty et al, 1993; Endre et al, 1994), suggesting that the metabolic defect precedes blood pressure elevation.

1.8.2 Obesity as a possible confounding factor

It has been suggested that obesity is a confounding factor which can account for an apparent relationship between insulin action and blood pressure, since it is well recognised that insulin resistance is closely associated with elevations in body mass index (BMI) (Jarrett, 1992). To address this issue, clamp data on 333 subjects were pooled from a number of European centres so that meaningful multivariate analyses could be carried out. In a model simultaneously accounting for sex, age, BMI and fasting insulin level, both systolic and diastolic blood pressures remained significantly inversely related to insulin sensitivity (Ferrannini et al, 1997). In addition, age was

positively related to blood pressure levels independently of insulin sensitivity, whereas BMI was not; in other words, the association of BMI with blood pressure is likely to be mediated via changes in insulin sensitivity (Ferrannini et al, 1997).

1.8.3 Mechanisms linking insulin action with blood pressure

What mechanisms underlie the relationship between insulin action and blood pressure? As already discussed above, insulin's physiological effect on the cardiovascular system is two-fold. Firstly, it acts as a peripheral vasodilator (Laakso et al, 1992; Anderson et al, 1991; Baron, 1993; Natali et al, 1997; Utriainen et al, 1997; Ueda et al, 1998a) mainly in skeletal muscle vascular beds. Secondly, it has pressor effects mainly via stimulation of the sympathetic nervous system (Anderson et al, 1991) and enhancement of renal sodium absorption (Ferrannini et al, 1993). The net effect is a balance of pressor and depressor effects and blood pressure is maintained. Therefore, if either the pressor effect is enhanced or the depressor effect is blunted, blood pressure increases. In obese subjects there is evidence for both an enhanced sympathetic drive in response to hyperinsulinaemia (Scherrer et al, 1994b) as well as blunting of insulin-mediated vasodilation (Laakso et al, 1990) thus providing a possible mechanism for hypertension. In patients with essential hypertension, there is recent evidence to suggest that insulin-stimulated muscle blood flow is impaired (Laine et al, 1998), a result which concurs with previous findings that insulin-induced vasodilation is correlated with blood pressure (Baron, 1993, Feldman et al, 1993). However, supraphysiological doses of insulin were used in these studies; when physiological doses were used for shorter periods no correlation was found between insulin-mediated vasodilation and blood pressure

(Anderson et al, 1991; Hunter et al, 1997). Therefore, it remains unclear whether blunting of insulin-mediated vasodilation contributes to hypertension via increased peripheral vascular resistance.

1.9 Insulin resistance and atherosclerosis (figure 1.15)

1.9.1 Insulin action, blood pressure and cardiovascular risk

Reaven (1988) speculated that defects in insulin action may be involved in mediating the atherosclerotic complications of hypertension. A recent study evaluated the importance of the association of insulin resistance with blood pressure in terms of cardiovascular risk. In multivariate analysis (Ferrannini et al, 1997), when diastolic BP values were simultaneously related to age, insulin sensitivity and fasting insulin levels only 18% of the variability of diastolic BP was explained; furthermore, in a group where the mean±SD value for insulin sensitivity was 34±11.8 μmol/min/kg, the predicted impact on blood pressure of a decrease in insulin sensitivity of 10 μmol/min/kg was +1.4mmHg (similar to that associated with a 10 year difference in age). At first glance one might conclude that insulin resistance is a fairly insignificant contributor to the problem of hypertension and cardiovascular risk. However, at a population level, it has been calculated that a small (2mmHg) increase in arterial BP will result in a 17% increase in incidence of cerebrovascular disease and a 10% increase in incidence of ischaemic heart disease (Collins et al, 1990).

The question of whether hyperinsulinaemia / insulin resistance affects cardiovascular outcome continues to be a source of controversy. Several longitudinal studies have implicated hyperinsulinaemia as an independent risk factor for coronary heart disease

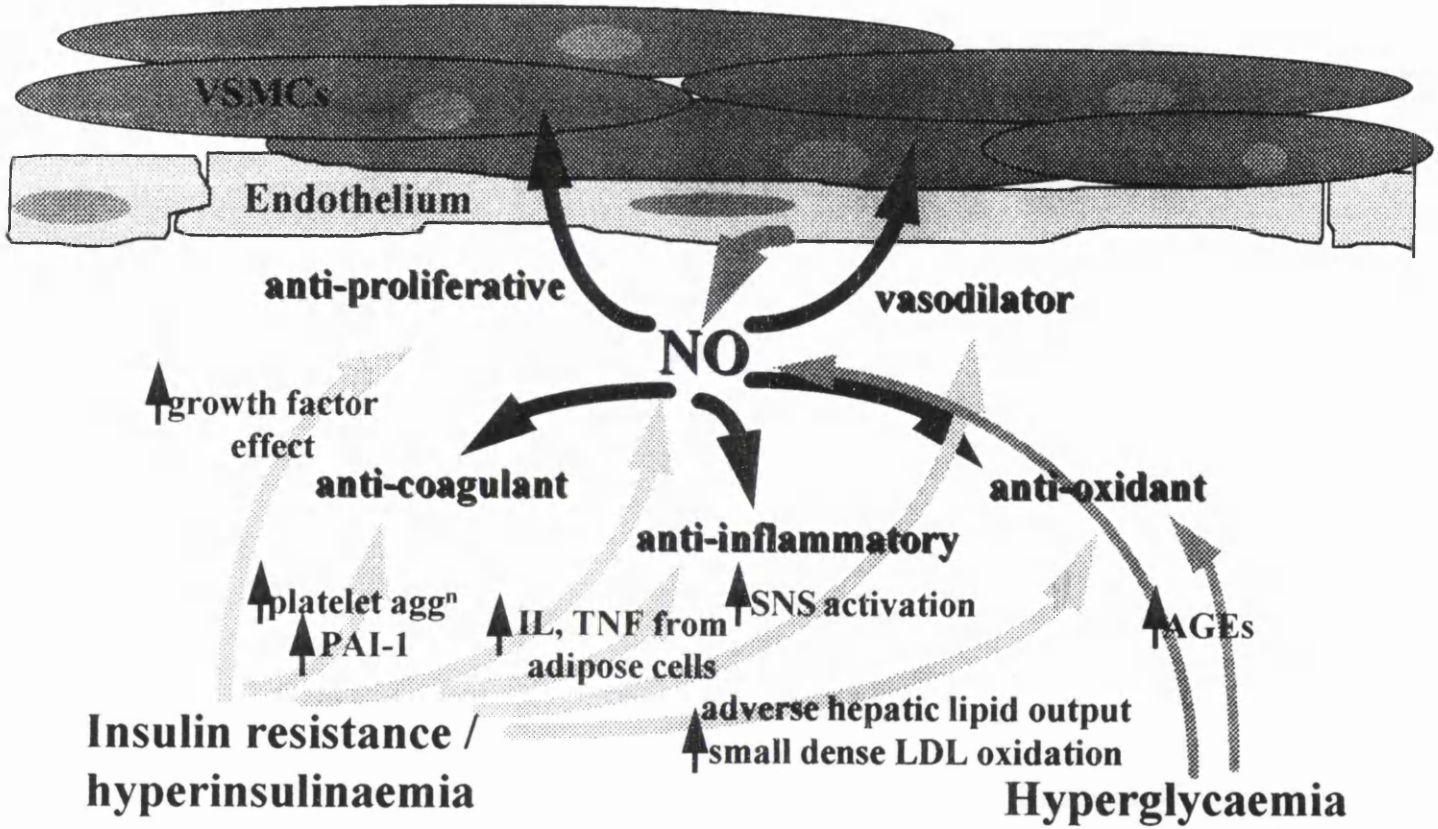


Figure 1.15
Putative mechanisms by which insulin resistance, hyperinsulinaemia and hyperglycaemic may contribute to the atherothrombotic process either directly or via a reduction in nitric oxide's protective effects on the vascular endothelium.

(reviewed in Morris et al, 1994; Despres et al, 1996). However, there are a number of negative studies (eg Ferrara et al, 1994; Orchard et al, 1994; Yarnell et al, 1994). Again, interpretation of results depends on the process of accounting for co-variates in multivariate analyses. For example, Yarnell et al (1994) corrected for serum triglyceride concentrations and body mass, and concluded that there was no significant independent association between fasting insulin levels and ischaemic heart disease. However, if defects in insulin action are causally linked with obesity and hepatic lipid handling, correcting for these co-variates may mask a valid association between insulin action and atherosclerotic risk. Defects in lipoprotein metabolism are known to be involved in the pathogenesis of atherosclerosis (Ross 1993). Insulin plays a major role in lowering plasma triglyceride levels mainly by suppression of VLDL1 and apolipoprotein B production (Malmstrom et al, 1997a). Furthermore, it has been demonstrated that this suppression is defective in association with insulin resistance, thus providing a mechanism for hypertriglyceridaemia secondary to defective hepatic insulin action (Malmstrom et al, 1997b). Plasma triglyceride levels are related, in turn, to levels of small dense LDL which increase oxidative stress at an endothelial level, thus predisposing to atherosclerosis (Paolisso et al, 1996).

Insulin, by virtue of its action as a growth factor, may play a direct role in atherogenesis at the level of vascular smooth muscle (Stout, 1989). Furthermore, there is evidence that insulin levels may contribute to increased re-stenosis rates after coronary angioplasty (Sobel, 1996), suggesting a direct role for insulin in the process of atherogenesis.

1.9.2 Insulin and platelet aggregability

When atherosclerotic plaques rupture, intimal damage ensues. Platelets are stimulated to form aggregates which adhere to the damaged area. These platelet aggregates may increase in size until they block the flow of blood, resulting in thrombosis. Anit-platelet drugs inhibit platelet aggregation and have proven efficacy in reducing thrombotic events (Antiplatelet Trialists Collaboration, 1994).

In newly-diagnosed type 2 diabetes there appears to be significant platelet hyperaggregation in whole blood (Mandal et al, 1993; Menys et al, 1995). After metabolic control of blood glucose there is significant reduction in platelet aggregability, suggesting that both hyperinsulinaemia and hyperglycaemia may be influencing aggregation mechanisms (Mandal et al, 1993). The role of hyperinsulinaemia and insulin resistance on platelet glucose transport and on the metabolic function of platelets is poorly understood. In one study, platelet aggregation was measured in healthy volunteers before and after intravenous insulin: aggregation was significantly reduced after insulin suggesting that insulin may have important anti-aggregatory properties (Kahn et al, 1993). One group has used platelet-rich plasma (PRP) to study the relationship between insulin and platelet aggregability: firstly, they demonstrated an anti-aggregatory effect of insulin to platelet stimulation by collagen or ADP in PRP from healthy volunteers (Trovati et al, 1994). They also measured cGMP production and concluded that insulin's anti-aggregatory effect was via a cGMP-dependent mechanism. They went on to show that this effect was significantly impaired in the insulin resistant states of obesity and type 2 diabetes (Trovati et al, 1995) and, recently, they demonstrated that lean

patients with type 2 diabetes exhibited normal insulin anti-aggregation (Anfossi et al, 1998), suggesting that insulin resistance is the most important underlying mechanism for this effect rather than hyperglycaemia. The L-arginine/nitric oxide pathway has been known for some time to be an important mechanism regulating aggregation in platelets (Radomski et al, 1990), and it appears likely that insulin's anti-aggregatory effect is mediated via production of nitric oxide and consequent elevated levels of cGMP (Trovati et al, 1996; Trovati et al, 1997). Whether stimulation of nitric oxide production is a direct effect of insulin or as a result of insulin-stimulated transport of L-arginine or D-glucose remains to be elucidated. Unfortunately, there are a number of methods of studying platelet aggregation (PRP, 'washed' platelets, and whole blood) and a number of different aggregatory stimuli (ADP, collagen, thrombin, sodium arachidonate and platelet-activating factor). Results from one method are often not reproduced using alternative methods and there are questions concerning the physiological relevance of some of the methods used. However, evidence from these limited and preliminary studies would indicate that platelet aggregability is increased in conditions of insulin resistance and this may be a key factor in terms of predisposition to thrombotic vascular disease.

In chapter 8, pilot data are presented on insulin action and platelet aggregability. The technical experience gained from collecting these pilot data has allowed a number of well-designed platelet aggregation studies to be initiated.

1.9.3 Insulin resistance and cardiovascular disease - the clinical relevance?

Having identified insulin resistance as a potential independent cardiovascular risk factor and having proposed lifestyle intervention and insulin-sensitising drugs as potential risk-reduction strategies, one might argue that the issue of insulin resistance still remains more of academic interest than realistic clinical relevance. For although weight loss and aerobic exercise have been shown to improve insulin sensitivity, attempts in clinical practice to instigate major lifestyle changes are usually disappointing (NIH Technology Assessment Conference Panel, 1993, Eriksson et al, 1997), and the initial experience of insulin-sensitising drugs has been marred by the possibilty of serious side-effects, exemplified by the recent withdrawal of troglitazone in the UK due to case reports of hepatic disease.

However, it is becoming clear that insulin-resistant subjects benefit more from traditional risk-reduction management for cardiovascular disease and, therefore, it may be that the main clinical relevance of insulin resistance is to help target those people in whom we should attempt to reduce cardiovascular risk more aggressively. For example, analysis of the type 2 diabetic sub-groups in a number of recent blood pressure lowering trials has revealed a 2 - 3 fold increase in relative risk reduction for cardiovascular events (UKPDS Study Group, 1998; Hansson et al, 1998, Curb et al, 1996) irrespective of the class of anti-hypertensive agent used. Furthermore, aiming for a target diastolic BP of < 80mmHg compared with < 90mmHg resulted in a 50% further reduction in cardiovascular event rate in type 2 diabetic patients (Hansson et al, 1998) emphasising the importance of aggressive risk reduction. In

addition, it has long been known that diabetic patients benefit more than non-diabetic counterparts from thrombolysis following myocardial infarction as well as from cessation of smoking in terms of reduction of cardiovascular morbidity and mortality.

Physicians must not ignore the main root cause of insulin resistance and its sequelae. While adherence to lifestyle advice is disappointing in adults, efforts must be directed, firstly, at prevention of obesity and inactivity (especially in children and young adults) and, secondly, at improving counselling methods to help tackle the difficult stages of major behaviour change. American public health physicians having already been forced to address these issues faced with the continuing 'triumph of obesity' (Foreyt & Goodrick, 1995).

1.9.4 Treatment of insulin resistance and endothelial dysfunction in metabolic and cardiovascular disease

(a) Non-pharmacological approaches

Evidence from studies where either non-pharmacological or therapeutic interventions were studied may provide useful pointers to the mechanisms underlying the links between metabolic and vascular disease. Aerobic exercise is known to increase insulin sensitivity (Rodnick et al, 1987; Kahn et al, 1990; Ikeda et al, 1996; Erikkson et al, 1997) probably via a variety of cellular mechanisms (Mikines et al, 1989; Houmard et al, 1991). Weight loss is associated with favourable changes in blood pressure (Ikeda et al, 1996) and endothelial function (Steinberg et al, 1996) possibly via a combination of increased insulin sensitivity,

reduced sympathetic drive (Scherrer et al, 1994b) and beneficial changes in hepatic lipogenesis (section *1.6.1*), and aerobic exercise results in blood pressure lowering (Halbert et al, 1997), an effect which is probably mediated by changes in metabolism, although the exact mechanisms involved remain unclear (Ikeda et al, 1996). Exercise-induced improvements in endothelial function have also recently been demonstrated, both in healthy subjects (Kingwell et al, 1997) and in patients with chronic heart failure (Hornig et al, 1996).

(b) Pharmacological approaches

Various pharmacological approaches to both metabolic and cardiovascular disease have been shown to affect insulin sensitivity or endothelial function or both. Data on the effects of anti-hypertensive drugs on insulin sensitivity are confusing. On the one hand, it has been proposed that ACE inhibitors and α-blockers improve insulin sensitivity (Pollare et al, 1989a; Vuorinen-Markkola & Yki-Jarvinen, 1995; Giorda et al, 1995) and that thiazide diuretics and β-blockers reduce insulin sensitivity (Murphy et al, 1982; Pollare et al, 1989b). On the other hand, there are a number of well-designed controlled studies which report no significant metabolic effects of ACE inhibitors, thiazides or cardioselective β-blockers (Heinemann et al, 1995; Harper et al, 1994; Harper et al, 1995; Hunter et al, 1998).

Lipid lowering therapy has been shown to improve endothelial function (Stroes et al, 1995; Anderson et al, 1995), and there is some evidence to suggest that pravastatin lowers insulin levels in patients with hypercholesterolaemia and hypertension (Chan et al, 1996) although it is of interest that hypercholesterolaemia *per se* does not

appear to be associated with insulin resistance (Karhapaa et al, 1993). Hypercholesterolaemia-induced endothelial dysfunction can be reversed by administering L-arginine, the precursor of nitric oxide (Tsao et al, 1994; Clarkson et al, 1996), presumably because the rate-limiting step is the formation of nitric oxide which is rapidly quenched by superoxide anions. However, in hypertension L-arginine does not appear to have a beneficial effect on endothelial function (Panza et al, 1993b) while in IDDM it may be of some benefit (Poston & Taylor, 1995).

Insulin-sensitising agents, such as the thiazolidinedione derivatives, appear to exert their effects on post-receptor binding steps in the transduction of the insulin response (Kotchen, 1996). Limited data in man appear to suggest that these agents also cause significant reduction of blood pressure (Nolan et al, 1994, Ogihara et al, 1995). *In vitro* data using pioglitazone suggest that insulin's vascular effects are modified: agonist-induced vasoconstriction is blunted while stimulated endothelium-dependent vasodilation is augmented (Kotchen et al, 1996). The most likely mechanism for these effects is a reduction in VSMC intracellular Ca^{2+} (Pershadsingh et al, 1993; Zhang et al, 1994). However, it is also possible that troglitazone exerts beneficial effects by an anti-oxidant action. Structurally, it is very similar to vitamin E, a natural anti-oxidant which has been shown to improve both endothelial function and insulin action. For example, vitamin E attenuated the blunting of acetylcholine-induced vascular relaxation in diabetic rats (Keegan et al, 1995), and in man it appears to improve insulin action in both normal subjects and those with type 2 diabetes (Paolisso et al, 1993). Furthermore, patients with myocardial infarction had fewer additional coronary events on pharmacological doses of vitamin E compared

with placebo (Stephens et al, 1996). In addition, the related anti-oxidant vitamin C improves insulin action in both normal subjects and diabetics (Paolisso et al, 1994), as well as improving endothelial function (Ting et al, 1995).

Thus, in general terms, endothelial function and insulin sensitivity are often affected in parallel by a variety of non-pharmacological and therapeutic interventions employed in both metabolic and cardiovascular disorders. However, this is not always the case; for example, fish oil appears to improve dyslipidaemia (Goh et al, 1997) and endothelial dysfunction (Chin & Dart, 1994) while exerting an adverse effect on insulin sensitivity (Rivellese et al, 1996).

1.10 Mechanisms of cellular insulin resistance

Insulin stimulates glucose disposal in muscle and adipose tissue. In muscle, this is achieved via a co-ordinated program of insulin-stimulated cellular events leading to increased glucose transport and increased activities of the enzymes involved in glycogen synthesis. The mechanism by which insulin controls both glucose transport and glycogen synthesis is becoming increasingly well understood. It has long been established that skeletal muscle is the primary site of post-prandial glucose uptake stimulated by insulin; furthermore, it is clear that muscle from individuals with insulin resistance exhibits blunted rates of insulin-stimulated glucose transport and glucose metabolism to glycogen. (reviewed in Reaven 1988 & De Fronzo et al, 1992).

1.10.1 Insulin-stimulated glucose transport and the role of GLUT-4

Insulin stimulates glucose uptake into adipocytes and muscle by virtue of the specific expression of an insulin-regulatable glucose transporter, GLUT4. The translocation of this transporter from an intracellular site to the plasma membrane in insulin-exposed cells is responsible for the large increase in glucose transport observed. Under basal conditions in both fat and muscle, >90% of GLUT4 is sequestered intracellularly (Slot et al, 1991; Slot et al, 1997). It has been suggested that the majority of the intracellular GLUT4 is housed in vesicular structures similar to small synaptic vesicles observed in neuroendocrine cells (Slot et al, 1991; Slot et al, 1997; Martin et al, 1998). Indeed, the fusion of GLUT4-containing vesicles with the plasma membrane appears to be mechanistically similar to the fusion of synaptic vesicles with presynaptic membranes, as related proteins have been shown to be involved in both fusion reactions (see for example Cain et al, 1992; Martin et al, 1996; Volchuk et al, 1996; Martin et al, 1998).

Skeletal muscle is the main tissue involved in the insulin-induced stimulation of glucose uptake. Studies using euglycaemic-hyperinsulinemic clamps have established that when insulin is maintained in the high physiological range, most of the infused glucose is taken up by skeletal muscle and converted to glycogen (James et al, 1986; Kraegen et al, 1993). It has long been established that insulin treatment, exercise or electrical stimulation all rapidly stimulate glucose transport in skeletal muscle, and that these effects are mediated by increased V_{max} for glucose transport (reviewed in Zorzano et al, 1996). Recent studies have established that GLUT4 translocation in

muscle is sufficient to account for the effects of insulin on glucose transport (Lund et al, 1995; Zorzano et al, 1996; Lund et al, 1997).

Glucose transport into skeletal muscle is rate limiting for the pathway for glucose utilisation. Individuals with type 2 diabetes exhibit insulin resistance in skeletal muscle (Pedersen et al, 1990). As muscle accounts for up to 40% of the body mass, this insulin resistance is of singular importance in the development of hyperglycaemia. A primary defect in insulin-stimulated glucose transport has been identified in such individuals, which, together with defective glucose phosphorylation, underlies the insulin resistance observed in this tissue (Eriksson et al, 1992; Häring and Mehnert, 1993; Bonadonna et al, 1996).

1.10.2 Subcellular localisation and translocation of GLUT4 in skeletal muscle.

Muscle fibres contain highly specialised organelles associated with the contractile properties of this tissue. For example, there are deep invaginations of the plasma membranes of muscle cells which interact with the terminal cisternae of the sarcoplasmic reticulum, the t-tubules. T-tubules possess insulin receptors, and thus may be a potential site for translocation of glucose transporters in response to insulin in addition to the sarcolemma (Zorzano et al, 1996). There is some debate regarding the relative contributions of translocation of GLUT4 to the sarcolemma and t-tubular regions. Biochemical and immuno-EM evidence has clearly demonstrated insulin-stimulated translocation of GLUT4 to both these sites in rat skeletal muscle (reviewed in Zorzano et al, 1996). The present consensus suggests that t-tubules contain more GLUT4 per unit membrane than the sarcolemma after insulin

treatment, suggesting some heterogeneity in the translocation process to these different plasma membrane domains (reviewed in Zorzano et al, 1996). (In chapter 9, a pilot collaborative venture is described in which the technically-demanding technique of immunogold electron microscopy was set up with a view to studying GLUT4 translocation in human skeletal muscle.)

Immunolocalisation of GLUT4 in both human (Friedman et al, 1991) and rat (Rodnick et al, 1992) skeletal muscle has indicated that GLUT4 is associated with intracellular membranes, corresponding to a perinuclear compartment located close to the Golgi, between myofibrils, and in vesicular structures close to both the sarcolemma and the T-tubules. Like the situation in adipocytes, skeletal muscle GLUT4 does not co-localise with the *trans* Golgi reticulum marker, TGN38.(Martin et al, 1996). Similarly, proteins known to be resident in GLUT4-containing vesicles in adipocytes, such as VAMP2, cellubrevin, vp165 and SCAMPs have also been identified in GLUT4-containing vesicles in skeletal muscle (see for example Martin et al, 1996; Martin et al, 1998). Despite the profound differences in subcellular architecture and methods of subcellular fractionation, insulin-responsive GLUT4-containing vesicles from these tissues exhibit very similar sedimentation coefficients, buoyant densities and protein composition, suggesting that the compartment(s) to which GLUT4 is(are) targeted in muscle and fat are highly related structures (see Hanpeter et al, 1995; Kandror et al, 1995).

1.10.3 The SNARE hypothesis and GLUT4 translocation

The SNARE hypothesis describes a series of protein-protein interactions which regulate the docking and fusion of specific membrane compartments in regulated exocytosis (Südhof, 1995). It is now well accepted that GLUT4 translocation shares mechanistic similarity with regulated exocytosis, hence the SNARE hypothesis represents a useful framework within which to consider aspects of GLUT4 cell biology. SNARE protein homologues have been shown to function in regulated and constitutive exocytosis in a variety of cell types, suggesting that they may be general fusion proteins. To that end, the proteins VAMP2 and Syntaxin-4 have been identified in GLUT4-expressing cells, and shown to be required for GLUT4 translocation using a variety of techniques (see for example Cain et al, 1992; Martin et al, 1996; Martin et al, 1998). It has been demonstrated that the main GLUT4-storage organelle in adipocytes is enriched for VAMP2 and that VAMP2 and syntaxin 4 play central roles in GLUT4 translocation in response to insulin in adipocytes (Martin et al. 1996, 1998). Thus, using adipocytes as a paradigm for insulin-stimulated GLUT4 translocation, an important role for VAMP2 and syntaxin 4 would be expected in skeletal muscle. To date, there is no information on either the expression levels or the co-localisation of VAMP2 and GLUT4 in human skeletal muscle.

1.10.4 Insulin signalling

Insulin activates a range of cellular signalling pathways via the intermediary of multi-phosphorylated protein substrates for the insulin receptor, IRS-1 and IRS-2 (White

& Kahn, 1994; Withers et al, 1998). These proteins serve as focal points for the assembly of a complex array of signalling enzymes in response to insulin binding its receptor. Insulin stimulated glucose transport in both muscle and fat requires the activation of phosphatidylinositol 3'-kinase (Backer et al, 1992). The activation of this lipid kinase leads to the activation of protein kinase B and the subsequent activation of glycogen synthase kinase -3. These enzymes are thought to be key players in insulin-stimulated glucose disposal (Okada et al, 1994). Although some studies of PI3 kinase levels in rodent models of diabetes have been published (see Shepherd et al, 1996) relatively few data are available from human muscle samples for these enzymes.

1.11 Aims of thesis and hypotheses to be tested

Following on from work previously carried out in our department, namely the association of insulin sensitivity with endothelial function in healthy subjects (Petrie et al, 1996a) and the demonstration that co-infusion of D-glucose augments local insulin-mediated vasodilation (Ueda et al, 1998a), the aims of this thesis were as follows:

- to explore the mechanisms of local insulin/glucose-mediated vasodilation
- to examine the relationship between local insulin/glucose-mediated vasodilation and whole-body insulin sensitivity
- to assess the effect on whole-body insulin sensitivity of systemic inhibition of endothelial function

- to demonstrate whether skeletal muscle fibre type and capillary density might be candidates for a 'third factor' to explain the association between insulin's metabolic and vascular effects
- to extend these studies to groups of patients with either essential hypertension or type 2 diabetes (as well as age- and BMI-matched healthy volunteers)
- to establish a robust 'washed platelets' model with which to study mechanisms of insulin-mediated inhibition of platelet aggregation
- to initiate a pilot study using the technique of GLUT4 immunogold electron microscopy with the ultimate aim of demonstrating insulin-stimulated GLUT4 translocation in intact human skeletal muscle cells

Thus, the following hypotheses were generated and subsequently tested:

Young healthy volunteers

- 'If insulin is predominantly an endothelium-dependent vasodilator, acting via stimulation of nitric oxide synthase (NOS), then co-infusion of a substrate inhibitor of NOS will abolish insulin's vascular effect, while vasodilation should still be apparent during co-infusion with low-dose angiotensin II, which causes a similar degree of vasoconstriction but is not endothelium-dependent.' (chapter 3)

- 'If insulin is predominantly an endothelium-dependent vasodilator, and endothelial function is positively associated with whole-body insulin sensitivity, then insulin-mediated vasodilation should be associated with insulin sensitivity.' (chapter 4)

- 'If endothelial dysfunction causes relative insulin resistance, then inducing an artificial state of endothelial dysfunction by systemically infusing a substrate inhibitor of NOS should result in a decrease in whole-body insulin-mediated glucose uptake.' (chapter 5)

- 'Skeletal muscle fibre type should be related to whole-body insulin sensitivity while capillary density may be related to insulin-mediated vasodilation; since fibre type and capillary density are related, this may partly explain any relationship between insulin's metabolic and vascular actions.' (chapter 6)

Older subjects

- 'Endothelial function and insulin-mediated vasodilation should be blunted in patients with either type 2 diabetes or essential hypertension, in parallel with reductions in whole-body insulin sensitivity.' (chapter 7)

- 'Sensitivity of platelets to thrombin-induced aggregation should be increased in more insulin-resistant subjects' (chapter 8)

Chapter 2

Methods

2.0 Summary

This chapter provides a description of the general protocols for the clinical techniques used in the studies described in this thesis.

2.1 Healthy volunteers and patients

All the studies were performed in the Clinical Investigation and Research Unit (CIRU), Department of Medicine and Therapeutics, Western Infirmary.

Young male healthy volunteers and older non-diabetic normotensive control subjects were recruited by advertisement (medical and nursing students were excluded). Non-diabetic hypertensive patients were recruited from the Glasgow Blood Pressure Clinic at the Western Infirmary; patients with type 2 diabetes were recruited from the Diabetes Centre at Gartnavel General Hospital. The study protocols were individually approved by the Ethics Committee of the West Glasgow Hospitals University NHS Trust.

Young healthy volunteers (chapters 3-6) and older healthy control subjects (chapter 7) were excluded if they were taking any form of regular medication, were smokers, were consuming more than 20 units of alcohol per week or had elevated fasting cholesterol levels (> 6mmol/l). They underwent routine medical screening at the

Clinical Investigation and Research Unit and were deemed to be healthy on the basis of past medical history, physical examination, routine biochemical and haematological indices, hepatitis screen and electrocardiography. All subjects gave written informed consent for their participation.

Hypertensive subjects were often newly-diagnosed with mild-to-moderate hypertension. Patients already on medication were asked to discontinue their drugs for at least three weeks after which time sitting blood pressure was re-measured in triplicate. The 'window' for recruitment was systolic 140-180mmHg and diastolic 90-110mmHg. Exclusion criteria were as follows:

- smoker
- alcohol intake > 20 units/week
- BMI > 30
- fasting hyperlipidaemia (cholesterol > 8mmol/l;triglycerides > 4mmol/l)
- co-existing peripheral vascular disease or cerebrovascular disease
- co-existing active ischaemic heart disease
- co-existing microalbuminuria
- co-existing type 2 diabetes or impaired glucose tolerance
- other conditions, for example urinary frequency and chronic low back pain, which would complicate being able to lie still for a number of hours

Type 2 diabetes was defined according to WHO criteria (World Health Organisation, 1980). Newly-diagnosed patients were stabilised on diet for three

months prior to study entry. Patients already attending the Return Clinic were recruited if glycaemic control was stable on either diet alone or a small dose of gliclazide (< 80 mg/day). Exclusion criteria were as follows:

- smoker
- alcohol intake > 20 units/week
- BMI > 30
- fasting hyperlipidaemia (cholesterol > 8mmol/l; triglycerides > 4mmol/l)
- co-existing peripheral vascular disease or cerebrovascular disease
- co-existing active ischaemic heart disease
- co-existing microalbuminuria
- co-existing hypertension
- other conditions, for example urinary frequency and chronic low back pain, which would complicate being able to lie still for a number of hours

2.2 General clinical protocol

Before study entry, all volunteers and patients underwent a health questionnaire and full clinical screening including physical examination, routine biochemistry, haematology, dip-stick urinalysis and an electrocardiogram as a screening test for significant cardiovascular disease or end-organ damage. In addition, random urine drug screens were carried out in a subset of volunteers to detect traces of any illicit drugs. Written informed consent was obtained form all subjects.

On each study day, subjects were transported by taxi to the CIRU at 0830hrs after an overnight fast from 2200hrs. All subjects were asked to avoid alcohol, caffeine and vigorous exercise in the 24 hours prior to an assessment. Following each study, a light meal was provided for subjects prior to taxi transport home.

2.3 Clinical and morphometric measurements

2.3.1 Body mass index

Body weight and height were measured with subjects in light clothes and without shoes to the nearest 0.5 kg of weight and to the nearest 0.5 cm of height. The same equipment was used throughout the studies, and the calibration of the weighing scales (Seca, Germany) was checked regularly. Body mass index (BMI, kg/m^2) was calculated as:

$$BMI = \frac{\text{body weight (kg)}}{(\text{height (m)})^2}$$

2.3.2 Blood pressure and heart rate

During all the clinical studies the technique of blood pressure and heart rate measurement was uniform. Systolic and diastolic blood pressure and heart rate were measured, after 10 minutes supine rest, by an oscillometric technique using a Dinamap Critikon (Johnson and Johnson Professional Products Ltd., U.K.) semi-automatic sphygmomanometer, maintained and calibrated at regular intervals by the

Department of Clinical Physics, Western Infirmary. Hypertension was defined as a mean supine diastolic BP of $\geq$ 95 mmHg, or systolic $\geq$ 160 mmHg, on three readings after 10 minutes supine rest (Sever et al, 1993).

2.4 Hyperinsulinaemic clamp technique

Insulin sensitivity was assessed using a modification of the hyperinsulinaemic clamp described by DeFronzo et al (1979). Rather than the 120 minute clamps described by DeFronzo, a duration of 180 minutes was chosen, except when hepatic glucose production was being measured concurrently, in which case the clamp was shortened to 150 minutes (to optimise total duration of protocol for subjects).

2.4.1 General clinical procedure

Subjects attended the CIRU according to the usual protocol (section 2.2). Two 18-gauge intravenous cannulae (Venflon, Helsinborg, Sweden) were inserted: the first antegradely into the left antecubital fossa for administration of infusions, and the second retrogradely into the right dorsal hand vein for blood sampling. Three-way taps enabled easy sampling and simultaneous infusion of insulin and glucose.

The infusion of soluble human insulin (Actrapid, NovoNordisk A/S, DK2880 Bagsvaerd, Denmark) was prepared in 45mls saline + 5mls (10% vol/vol) of each patient's own blood in order to minimise adsorption of insulin to the plastic surfaces of syringes and infusion lines. It was administered using a Braun Perfusor pump as a

primed, constant rate infusion for 180 minutes with the aim of achieving a steady-state serum insulin concentration approximately 120μU/ml above the fasting level (high physiological range). The priming regime was as follows:

0-4 minutes	4.5 mU/kg/min
4-7 minutes	3.0 mU/kg/min
7-180 minutes	1.5 mU/kg/min

After placement of cannulae, 20 minutes' supine rest was allowed during which baseline blood samples were withdrawn and blood pressure and heart rate were measured; subsequently, insulin infusion commenced at time (t) = 0 mins. A variable rate infusion of 20% glucose (Baxter Healthcare, Norfolk, U.K.) was administered via an IMED infusion system (IMED, Abingdon, U.K.) from t = 2-180 mins. Serum glucose concentrations were maintained at euglycaemia (target serum glucose 5.2 mmol/l) in subjects who did not have type 2 diabetes. In subjects with diabetes, serum glucose concentrations were maintained at isoglycaemia [target serum glucose = fasting glucose (Saad et al 1994)] in order to avoid the potentially confounding effect of inter-individual variations in fasting serum glucose concentrations. The infusion rate was adjusted for body weight for each individual and expressed as milligrams of glucose infused per kilogram of body weight per minute (mg/kg/min). For example, the infusion rate of 20% glucose equal to 1mg/kg/min for a 70kg man would be calculated as:

$$\frac{60 \text{ minutes}}{200\text{g glucose}} \times 70\text{kg} = 21 \text{ ml/hour}$$

At 5 minute intervals, 1.5 ml blood samples were withdrawn from the cannulated dorsal hand vein. Cannula patency was maintained using a slow infusion of 0.9% saline; a total of approximately 100 ml was administered during each procedure. After centrifugation at the bedside, the serum glucose concentration was determined (section 2.9.2), and the glucose infusion rate was manually adjusted to maintain target serum glucose concentration. The dorsal hand vein was surrounded by a heated box (55°C) with the aim of arterialising venous blood (section 2.6). Additional hourly blood samples were obtained for estimation of serum insulin and plasma potassium levels

2.4.2 Calculation of insulin sensitivity from the euglycaemic hyperinsulinaemic clamp

During hyperinsulinaemia with steady-state plasma glucose concentrations (usually during the last 40-60 minutes of the procedure), the rate of glucose infusion is equal to that of glucose removal from the glucose space (i.e. glucose metabolised, M). Assuming suppression of endogenous glucose production, the M-value is an estimate of total body glucose metabolism, and reflects the ability of insulin to enhance tissue glucose disposal.

In practice, the glucose infusion rate must be modified by two factors before it can be equated with M:

$$M = I - UC + SC$$

where: I = glucose infusion rate (mg/kg/min)

UC = correction for urinary glucose loss
(usually negligible during a clamp)

SC = 'space correction' (mg/kg/min)
(for inevitable deviations from euglycaemia)

The space correction is calculated as follows (DeFronzo et al 1979):

SC = (5.2 - G) x 17.86 x 0.095

where: G = ambient glucose concentration over last 40 minutes of clamp (mmol/l)

17.86 = unit conversion factor (mmol/l to mg/dl)

0.095 = glucose space constant

When the achieved ambient glucose concentration at steady-state is less than the desired value of 5.2 mmol/l (too little glucose has been infused) the space correction will be positive: M will therefore be greater than I; the converse also applies.

In some groups of subjects, particularly in those who are obese, use of a weight-adjusted insulin infusion protocol may result in higher steady-state serum insulin concentrations, and in this situation the M-value underestimates the degree of insulin resistance. In this situation, the insulin sensitivity index (S_{IP} x 10^4 dl/(min.kg) per mU/l) is calculated from the glucose infusion rate and ambient insulin and glucose concentrations at steady state (Bergman et al 1987):

$S_{IP} = \Delta R_d / (\Delta I \times G)$

where: ΔR_d = increment in glucose uptake (basal to steady state)

ΔI = increment in [insulin] (basal to steady state)

G = steady state [glucose]

When subjects are clamped at fasting glucose levels (for example, type 2 diabetic patients, chapter 7), further adjustment is required to take these differences into consideration, since a higher proportion of glucose disposal will be insulin-independent (mass action) in those subjects with higher fasting glucose levels (Greenfield et al, 1981). The best index is MCR (metabolic clearance rate) of glucose which equates to the M-value divided by the fasting glucose value; glucose levels should be converted to mg/dl from mmol/l (multiply by 18.019) so that MCR can be expressed as dl/kg/min (usually, this value is multiplied by 100 and expressed in ml/kg/min).

The reproducibility of M has previously been evaluated in the CIRU: between-day intra-subject coefficient of variation is 6% in healthy subjects (Morris et al 1997).

2.5 Measurement of endogenous (hepatic) glucose production using tracer infusates

Hepatic glucose production (both basal and during hyperinsulinaemia) was measured using tracer infusates (chapter 7).

2.5.1 Clinical procedure

The protocol was identical to that given above (section 2.4.1) except that the duration of the euglycaemic/isoglycaemic clamp was reduced to 150 minutes and

also for the following modifications. A primed continuous infusion of HPLC-purified [3-^{3}H] glucose was given during a 2 hour equilibration period (t = -120 to 0 mins) and continued throughout the procedure. The dose was halved (t = 20 to 40 mins) and then halved again (t = 40 to 150 mins). In order to avoid the underestimation of endogenous glucose production associated with inter-compartmental tracer fluxes, a variable rate infusion of exogenous 20% glucose pre-labelled with [3-^{3}H] glucose aiming to match basal plasma glucose specific activity was administered (t = 2 to 150 mins) (Finegood et al 1987, Levy et al 1989, Finegood et al 1990, Hother-Nielsen & Beck-Nielsen 1990, Hother-Nielsen et al 1992, Neely et al 1992).

2.5.2 Calculation of endogenous glucose production

The specific activity of glucose in plasma is measured in disintegrations per minute (dpm) per μmol of plasma glucose (section 2.9.3). At steady state, the total rate of glucose appearance (Tra, μmol / min) can be calculated by dividing the rate of tracer administration (dpm / min) by the glucose specific activity (SA = dpm / μmol) of plasma. Endogenous (hepatic) glucose production (EGP) is determined by subtracting the known rate of exogenous glucose infusion (I) [per kg of body weight] from the rate of glucose appearance (R_a) [per kg of body weight].

$$EGP = R_a - I$$

In practice, steady state assumptions may not be fully met, and the non-steady state equations of Steele et al (1956), as modified by DeBodo et al (1963) may be used as follows to determine rates of glucose appearance (Ra) and disappearance (Rd)

during t = -30 to 0 mins and t = 120 to 150 mins, assuming a pool fraction of 0.65 and an extracellular volume of 190 ml/kg.

The calculation of endogenous glucose production (EGP) can be summarised as follows:

i) Assuming steady state (ss):

$Tra_{(ss)}$ = F_{total}/SA (μmol/min)

where:

SA = glucose specific activity (dpm/min)

F_{total} = $(R_{const} \times F_{const}) + (R_{dex} \times F_{dex})$ (dpm/min)

F_{const} = specific activity of constant [3-^{3}H] glucose infusion

R_{const} = rate of constant [3-^{3}H] glucose infusion

F_{dex} = specific activity of variable [3-^{3}H] glucose infusion

R_{dex} = rate of variable [3-^{3}H] glucose infusion

ii) Adjusting for deviations from steady state:

$Tra_{(nss)}$ = $Tra_{(ss)}$ - error term

where:

Error term = (p.V.G.dSA/dt.weight)/SA (μmol/min)

and: p = pool fraction (0,65)

V = volume of distribution (190 ml/kg)

G = plasma glucose concentration (mmol/l)

SA = glucose specific activity of plasma (dpm/min)

weight = weight (kg)

iii) Final step:

EGP = $Ra_{(nss)}$ - GIR (μmol/kg/min)

where:

$Ra_{(nss)}$ = $Tra_{(nss)}$/weight (μmol/kg/min)

GIR = (R_{dex} x C_{dex})/weight (μmol/kg/min)

C_{dex} = 20% glucose concentration (μmol/ml)

2.6 Arterialisation of venous blood

In the original description of the euglycaemic clamp, the glucose infusion rate was adjusted according to glucose values in arterial blood (DeFronzo et al 1979). However, in order to avoid cannulation of systemic arteries, many investigators now adjust the glucose rate on the basis of 'arterialised' venous blood withdrawn from the retrogradely cannulated dorsal hand vein. To enable arterialisation of venous blood, the hand was placed in a cylindrical perspex heated-air box at the beginning of the period of supine rest prior to the start of the procedure (Liu et al 1992). Under thermostatic conditions, the heated box maintains the ambient temperature surrounding the hand at 55°C (University of Nottingham, Department of Physiology and Pharmacology, U.K.).

2.7 Forearm venous occlusion plethysmography

Forearm blood flow (FBF) was measured in the studies described in this thesis using forearm venous occlusion plethysmography (chapters 3,4,5 & 7). In this technique, the measurement of flow is derived from the rate of change in forearm volume during intermittent occlusion of venous return (with continued arterial inflow). The

original technique, dating from the late nineteenth century, required the use of cumbersome water jackets to measure changes in forearm volume (reviewed in Greenfield et al, 1963), but has been more readily applicable since the advent of mercury strain gauges together with a mathematical model for deriving changes in forearm volume (expressed in ml of blood flow / 100ml forearm / minute) from changes in circumference (Whitney 1953). The development of an electrical calibration technique for strain gauges (Hokanson et al, 1975), and the availability of computerised chart recorders, has led to increasing use of the technique to assess the effects on resistance vessel tone of agents infused directly into the brachial artery at subsystemic doses (Calver et al 1992; Benjamin et al 1995). An additional refinement is the simultaneous measurement of forearm blood flow in both arms: measurements in the experimental arm are adjusted for systemic changes unrelated to the local stimulus by expressing the two measurements as a ratio:

$$\frac{\dfrac{F(i)_d}{F(ni)_d} - \dfrac{F(i)_v}{F(ni)_v}}{\dfrac{F(i)_v}{F(ni)_v}} \times 100\%$$

where:

	F	=	flow
	i	=	infused arm
	ni	=	non-infused arm
	v	=	vehicle
	d	=	drug

A validation study was carried out in our department to investigate the reproducibility of forearm venous occlusion plethysmography (Petrie et al 1997). It

was demonstrated that the between-day intra-subject variation in unilateral FBF measurement was relatively high (coefficient of variation: 31-39%). However, the variability was less (coefficient of variation: 19%) when bilateral FBF measurement was made and responses were expressed as ratios (intervention : control arms).

2.7.1 General clinical procedure

These studies were carried out in a dedicated investigation room which was quiet and in which the lighting was dimmed. Room temperature was kept constant at around 24 - 25 °C. Subjects lay supine with arms supported on foam blocks at an angle of 30° to allow emptying of hand and forearm veins. Paediatric arterial occlusion cuffs (Hokanson SC5, PMS instruments, Maidenhead, Berkshire) were placed around the wrists and inflated to 200 mmHg for three minutes during each set of recordings. Hand circulation is occluded during the measurements of forearm blood flow because the physiology (especially the well-developed system of arteriovenous shunting) differs markedly from the forearm (mainly resistance arterioles in skeletal muscle vascular beds). Collecting cuffs (Hokanson SC10) were placed around the upper arms, and inflated and deflated (40 mmHg) in a 12 second cycle. Rapid cuff inflation was achieved using a commercially available air source (Hokanson AG101), coupled to rapid cuff inflators (Hokanson E20). On the first study day, the left forearm was measured at the largest circumference (usually around 24 - 28 cm), and a mercury-in-silicone elastomer (Silastic) strain gauge (Hokanson forearm set) 2 cm shorter was selected (in other words, the aim was to achieve roughly 10% resting stretch on the strain gauge which allows maximum sensitivity to any changes in circumference). The distance from the olecranon was

measured and recorded in order to standardise strain gauge position from day to day. The strain gauge was calibrated electrically on the arm to the chart recorder programme (MacLab, AD instruments, UK) and test readings were recorded.

In studies examining the effect of local intra-arterial infusions on forearm blood flow, a 27G unmounted steel needle (Cooper's Needleworks, Birmingham, UK) was inserted under local anaesthesia into the brachial artery of the non-dominant arm for drug infusion. Infusions were administered via an epidural giving set and needle patency was maintained using an infusion of 0.9% saline (1-2 ml/min). All solutions were prepared in 0.9% saline in the Pharmacy Sterile Productions Unit, Western Infirmary. When preparing an intra-arterial insulin solution, 4 ml of the volunteer's own blood was mixed with 45 ml of saline and 1ml of diluted soluble human insulin (Actrapid, Novo Nordisk, Denmark) (250 mU/ml for 5 mU/min). The insulin solution was prepared in the sterile unit of the hospital pharmacy using glass syringes and bottles and diluted in the research unit immediately before each study. With this method, the recovery of insulin was approximately 95% (S Ueda, unpublished data). When required, a venous cannula was introduced retrogradely into a cubital vein draining the deep venous supply of the forearm. Deep vein cannulation was confirmed when the tip of the catheter was not palpable, and oxygen saturation of samples was less than 50%.

Blood flow recordings began 45 seconds after wrist cuff inflation and continued for 135 seconds. Following insertion of the intra-brachial needle, subjects were allowed to acclimatise to inflation and deflation of the wrist and upper arm cuffs for 20

minutes before two baseline measurements 10 minutes apart: the mean of these was used as the baseline forearm blood flow.

2.7.2 Calculation of forearm blood flow

During venous occlusion, forearm circumference increases in proportion with the rate of arterial in-flow. Therefore, the gradient of the slope recorded on the plethysmographic traces is a surrogate measure of forearm blood flow - a steep gradient represents high blood flow, while a flatter gradient represents low blood flow. Data were acquired via a MacLab II chart recorder (AD instruments, Hampstead, London). Absolute values for forearm blood flow were derived according to the equation of Whitney (1953). Each blood flow measurement was the mean of five sequential recordings. Slopes were calculated from data points by acquiring co-ordinates using MacChart software (AD instruments), and pasting them into a customised spreadsheet (Microsoft Excel). Data from both infused and control arms were used to calculate the percentage change in forearm blood flow ratio, which is a more valid means of expressing blood flow changes (Petrie et al, 1998).

2.8 Percutaneous skeletal muscle biopsy

2.8.1 General clinical procedure

This procedure was performed during a routine weekly muscle biopsy list in the Western Infirmary (Dr W. Behan, Senior Lecturer in Pathology). Subjects attended

in a post-absorptive state and were positioned on a bed in order to achieve minimal postural contraction of thigh muscles. With an aseptic technique, 4ml of 2% lignocaine was injected into skin and subcutaneous tissue at a point on the lateral aspect of the thigh of the non-dominant leg, half-way between the knee and greater trochanter. An 8mm skin incision was then made with a disposable scalpel. A blunt-ended biopsy needle was then introduced through the fascial layer into vastus lateralis. Once in place, the central core of the needle was removed and a vacuum was applied using a 20ml syringe. Muscle tissue was sucked into the needle via a side-opening and removed using a side-cutting mechanism. This procedure is repeated twice at the same site in order to accumulate, on average, 200mg of tissue. The tissue is processed immediately by an experienced on-site technician. Firstly, the tissue is removed from the biopsy needle and placed onto dental wax resting on saline-soaked filter paper in a petri dish. Then the tissue is examined macroscopically to ensure that fat has not been biopsied. The tissue is then carefully organised to maintain the parallel nature of the muscle fibres; failure of this step results in poor-quality cross-sectional slides when cut for microscopy.

2.8.2 Preparation of frozen sections

The time from biopsy to freezing should be no longer than 30 seconds. The fresh sample is mounted upon a cork disc. The disc and tissue are then rapidly frozen by placing in isopentane. Once frozen, the sample is immersed and stored in liquid nitrogen (-70°C). When required, the biopsy block is cut into 10 μm sections using a carbon dioxide freezing stage and a cryostat. These sections are picked up by APES slides and stored at 4 °C prior to histochemical analysis.

2.8.3 Immunohistochemical analysis for fibre type and capillary density

Muscle fibre types consist of discrete myosins with different characteristics and expressed from different genes. Antibodies which differentiate fast and slow myosins are commercially available. Slow myosin antibody recognises an epitope within the heavy meromyosin portion of adult muscle slow myosin. Indirect visualisation of 'slow' type I fibres is therefore possible using the secondary antibody Rabbit anti-Mouse Peroxidase (RAM Po), which can subsequently be stained black using Vector Black. Fast myosin antibody recognises an epitope within skeletal muscle heavy chains. It cross-reacts with fast twitch fibres IIA and IIB and direct visualisation is possible after conjugation with Rabbit anti-Mouse Alkaline Phosphatase (RAM AP) allowing direct red staining.

There are several ways of quantifying capillary density within a given microscopic field, including number of capillaries per field, capillary number to fibre number ratio, capillary area to fibre area ratio. We chose to quantify an area ratio by staining endothelial cells with an antibody to CD45, an antigen which is selectively expressed on vascular endothelium. Direct visualisation is possible using RAM AP.

Tissue samples were taken from the freezer, labelled and air-dried for 10 minutes. Tissue was then fixed to the slides by immersing in acetone for 10 minutes. After fixation, slides were air-dried, sections were encircled with a grease pen and the blocker 5% NGS was applied for 10 minutes. The slides were tipped to drain off the blocker and the primary antibodies (slow myosin antibody or CD45 antibody) were

applied. The slides were left to incubate for 30 minutes in a humid environment before being washed off with PBS for 10 minutes. Secondary antibodies were then applied to the sections: RAM Po (30 minutes, for type I fibres) or RAM AP (60 minutes, for capillaries). To visualise type I fibres, the antibody is washed off and the commercial kit 'Vector SG' is applied for 5 minutes. The fibres immediately stain black. This reaction is stopped by applying water after which the sections are blocked again using 5% NGS. The process is repeated using fast myosin antibody as the primary antibody (incubated for 60 minutes) followed by RAM AP as the secondary antibody (incubated for 60 minutes). Both fast myosin and CD45 antibodies are visualised using the commercial kit alkaline phosphatase. This is applied to the slides for 15-20 minutes before the slides are mounted and red staining occurs. In addition, the intensity of staining varies between type IIB and type IIA fibres, the latter staining more weakly: this difference in staining intensity can be picked up by the image analysis system and the proportion of type IIA to IIB fibres can be determined.

Quantification of data was achieved using an image analysis system. This technique allows quantification of captured images from a microscope using a television camera. Two television screens are used: one showing the microscopic field under analysis, and the other showing details of the MCID version 3 programme being used. At random, 500 fibres were counted and the area of each was calculated by drawing around the perimeter of the structure using 'mouse' control. As far as possible, only fibres cut in cross-section were included and only the fibres in one biopsy section were analysed to prevent repetition. The edges of the section were

also avoided where fibres were often distorted. Results are expressed as percentages for both total number and total area of the three fibre types.

To measure vascularity, the proportion of endothelial cell area was measured. Two sections were analysed, large vessels were ignored and only capillaries cut in cross-section were evaluated. The program was set using the autoscan tool. This allows a chosen degree of colour to be detected within the screen and this was kept constant throughout. In addition, microscope illumination was also kept at a constant level. Results are expressed as endothelial cell area as a percentage of total area.

2.9 Laboratory methods

Venous blood samples for laboratory assay were withdrawn during clamp studies (chapters 4,5 & 7) from the right dorsal hand vein cannula and collected into plain (insulin, electolytes, lipids), lithium heparin (aldosterone) or potassium EDTA (renin) tubes. Samples for angiotensin II measurement were collected into tubes containing a mixture of enzyme inhibitors (25 mM o-phenanthroline, 125 mM EDTA, 2% ethanol, 0.2% neomycin sulphate, and 20 μM human renin inhibitor H142). All samples were immediately placed in crushed ice prior to centrifugation (3000 rpm, 4°C), decanting, and storage at -20°C, except those for insulin (which were allowed to clot at room temperature for 20 minutes). Samples for angiotensin II measurement were stored at -70°C. Assays were performed in batches in the laboratories of the Department of Medicine and Therapeutics.

2.9.1 Serum insulin concentrations

Serum insulin concentrations were determined at hourly intervals during clamp studies and at half-hourly intervals during plethysmography studies. They were measured by the Incstar radioimmunoassay (Stillwater, Minnesota, USA). This assay is non-specific for insulin with a molar cross-reactivity with intact proinsulin of 30%. Inter-assay coefficient of variation is 8 - 11%. Serum insulin concentrations were expressed in μU/ml.

2.9.2 Serum and plasma glucose concentrations

During the clamp studies, serum glucose was measured at 5 - 10 minute intervals at the bedside by the glucose oxidase method using a Beckman 2 glucose analyser (Beckman Instruments, Fullerton, CA, USA; inter-assay coefficient of variation = 2%). Results were expressed in mmol/l. For assessment of deep venous glucose levels during plethysmography studies, plasma samples were collected in fluoride-oxalate tubes and analysed in the hospital routine biochemistry laboratory.

2.9.3 Glucose specific activity

Plasma samples were collected in fluoride-oxalate tubes (15ml) at the following time points in relation to the euglycaemic hyperinsulinaemic clamp procedure (see chapter 7):

-30 mins, -20 mins, -10 mins, 0 mins, 120 mins, 130 mins, 140 mins, 150 mins

In addition, three control samples were prepared:

- 'background' - pre-infusate plasma collected in 6 fluoride-oxalate tubes

- 'syringe spike' - 10% solution of glucose syringe infusate in pre-infusate plasma
- 'bag spike' - 10% solution of variable-rate glucose bag infusion in pre-infusate plasma

Glucose specific activity was measured in triplicate for all timed samples and four times for each control. Tritiated glucose was extracted according to the following procedure:

- To deproteinise, add 5% barium hydroxide and 5% zinc sulphate. Vortex thoroughly until complete precipitation has occurred.
- Spin down precipitate at 1400 rpm for 10 mins at 4°C and transfer supernatant to numbered 20ml plastic scintillation vials.
- Resuspend precipitates in 1ml water and spin down at 2400 rpm for 10 mins at 4°C. Transfer second supernatants to corresponding vials.
- Freeze and dry samples.
- Reconstitute samples with 1ml water. Add scintillation fluid (8ml, Ultima Gold, Packard). Cap and shake vigorously.
- Count for 10 mins in scintillation counter.

Values obtained for glucose specific activity were entered into a spreadsheet (Microsoft Excel) and hepatic glucose production was calculated as explained above (section *2.5*).

2.9.4 Plasma aldosterone concentrations

Plasma aldosterone concentrations were measured in batches by a commercially available radioimmunoassay kit (Biodat, Milan) according to the technique described by MacKenzie & Clements (1974) (Chapter 3). Results were expressed in pmol/l. The intra- and inter-assay coefficients of variation were 5.0 and 5.4% respectively.

2.9.5 Plasma renin concentrations

Plasma renin concentrations were measured by an radioimmunoassay technique developed in the laboratories of the Department of Medicine and Therapeutics.

2.9.6 Plasma angiotensin II concentrations

Plasma angiotensin II was assayed after HPLC separation by direct radioimmunoassay according to a previously described technique (Ducsterdieck & McElwee, 1971). The intra- and inter-assay coefficients of variation were 6.4% and 8.8% respectively (Morton & Webb, 1985).

2.9.7 Serum potassium concentrations

Potassium (mmol/l) was measured in the hospital routine biochemistry laboratory by a diluting ion-selective electrode on an Olympus AU5200 auto-analyser.

2.9.8 Serum lipid concentrations

Lipid measurements were co-ordinated by Dr Naveed Sattar, Dept of Pathological Biochemistry, Glasgow Royal Infirmary. The following fasting measurements were

performed by a modification of the standard Lipid Research Clinics protocol (1975) on serum of patients and volunteers participating in the study described in chapter 7:

- total cholesterol
- triglycerides
- low density cholesterol (LDL)
- high density cholesterol (HDL)
- very low density cholesterol (VLDL)

2.9.9 Serum markers of endothelial function

Measurements of serum markers of endothelial function were organised by Dr Naveed Sattar, Dept of Pathological Biochemistry, Glasgow Royal Infirmary, in conjunction with Professor Gordon Lowe, Department of Medicine, Glasgow Royal Infirmary. The following fasting measurements were made on serum of patients and volunteers participating in the study described in chapter 7:

- Von Willebrand Factor (vWF) - ELISA; units: IU/dl
- Factor VIIc - one stage clotting assay; units: % activity
- Tissue Plasminogen Activator (tPA) - ELISA; units: ng/ml
- Fibrin D-dimer antigen - ELISA
- fibrinogen - units: g/l

2.10 Platelet aggregation studies

2.10.1 Preparation of human washed platelets

Peripheral venous blood (45ml) was obtained via a large bore cannula from subjects in the post-absorptive state who had not taken any aspirin-containing preparations

for at least 10 days. Washed platelets were then prepared according to the method of Radomski & Moncada (1983). Briefly, blood was collected into a trisodium citrate / prostacyclin mixture (0.315% / 2μg/ml) and centrifuged at 222x*g* at room temperature for 20 minutes. The resulting supernatant (platelet rich plasma) was carefully removed and centrifuged at 700x*g* at room temperature for 10 minutes in the presence of prostacyclin (0.3μg/ml). The platelet pellet was resuspended in Tyrode's solution including prostacyclin (0.3μg/ml), and centrifuged at 680x*g* at room temperature for 10 minutes. The pellet was washed three times with Tyrode's solution alone before resuspension. The platelet density was counted and adjusted to 2 - 2.5 x 10^8 cells/ml.

2.10.2 Measurement of platelet aggregation

Platelet aggregation was measured by a turbidimetric method in a Chrono-log dual channel whole blood lumi-aggregometer connected to a dual pen recorder (previously described by Whittle et al, 1978). Aliquots (240μl) of washed platelets were incubated at 37°C prior to the addition of one of 10 different doses of thrombin. Platelet samples were stirred with teflon coated magnetic stirrers at 1000 rev/min and the aggregation monitored for 6 minutes. Thrombin is known to induce a concentration-dependent aggregation of washed platelets; however, it cannot be used in platelet-rich plasma since it catalyses the formation of fibrin strands in the preparation, thus affecting turbidometric analysis. A dose-response curve of platelet aggregation to thrombin was constructed and the ED_{50} value (dose of agonist which causes 50% of maximum aggregation) was calculated.

2.11 Immunogold electon micoscopy studies

The main aim of this section was to produce a working protocol for the localisation of the glucose transporter GLUT-4 in skeletal muscle using electron microscopy immunohistochemistry. Since no experiments of this kind had been attempted in the EM Unit before, a number of technical questions had to be addressed:

- how best to fix skeletal muscle tissue in preparation for EM analysis
- whether ultrasections were best produced from frozen sectioning or resin processing
- for cryoultramicrotomy, which temperature was best for cutting sections
- which staining protocol gave the best resolution for gold-labelled GLUT-4 antibodies

In summary, what follows is a protocol which yielded the most promising results:

1. harvest and dissect rat hind leg muscle
2. fix at room temperature for 2 hours at 4°C, in 4% paraformaldehyde & 1% glutaraldehyde
3. wash in Sorensen phosphate buffer (SPB) containing 10% sucrose
4. transfer to 2.3M sucrose & SPB solution for cryoprotection (16 hours)
5. attach to stubs for ultra sectioning
6. freeze in liquid nitrogen
7. set up ultramicrotome temperatures: specimen -90 °C and glass knife -100 °C
8. take ultrasections (Reichter Jung Ultracut E with an FC 4D cryoattachment)
9. pick up sections with 2mm wire loop containing 2.3M sucrose
10. place on carbon coated formvar grids

11. place grids face down on a solution of Tris-HCl containing 1% BSA (allows sucrose to diffuse away) (3 x 2mins)
12. place grids onto 2% gelatin (reduces background staining) (10 mins)
13. place grids onto 0.02M glycine (quenches free aldehyde groups) (2 mins)
14. wash in Tris-HCl + 1% BSA (6 x 1min)
15. apply affinity-purified rabbit polyclonal GLUT-4 antibody: titre 1:200 (Tris-HCl + 1% BSA) (30 min at room temperature)
16. wash in Tris-HCl + 1% BSA (3 x 5 min)
17. apply colloidal (Protein A) gold conjugate: titre 1:10 (PBS + 1% BSA) (30 min at room temperature)
18. wash in Tris-HCl (15 x 2 min)
19. fix in 2% glutaradehyde (2 min)
20. wash in double distilled H_2O (10 x 1 min)
21. apply 10% uranyl acetate solution in a 9:1 solution of 1% methyl cellulose (20 min)
22. observation of grids in Phillips CM10 electron microscope (magnification approx: x38000)

Results, technical problems encountered, and plans for further experiments are outlined in chapter 9.

2.12 Statistical analysis

All data were checked for normality using the Shapiro-Wilks test (Minitab statistical package, Minitab Inc, Pennsylvania, USA). Normally-distributed data are

summarised as mean ± standard deviation (or standard error). Skewed data were analysed by non-parametric methods (summaries as median ± interquartile ranges; Wilcoxon signed rank test for correlation analysis) or logarithmically transformed (log_{10}) prior to multiple regression analysis.

Where possible, summary measures were calculated to prevent multiple comparisons. Details of specific statistical procedures used for each study are described in respective chapters.

Chapter 3

Physiological mechanisms of insulin-mediated vasodilation in man

3.0 Summary

In this clinical physiological study, the effects on insulin/glucose-mediated vasodilation of co-infusions of L-NMMA, angiotensin II, and ouabain were studied, in a double-blind, random-order, placebo-controlled manner using bilateral venous-occlusion strain-gauge plethysmography. It was concluded that insulin-mediated vasodilation may be dependent on endothelial nitric oxide production or stimulation of the Na^+K^+ATPase pump or both; however, these data cast doubt upon the widely-prevalent assumption that insulin-mediated vasodilation is specifically NO-dependent.

3.1 Introduction

There is increasing recognition that insulin, in addition to its well-known metabolic actions, exerts significant vascular effects. Elucidation of mechanisms of insulin-mediated vasodilation is critical in the search for pathophysiological links between metabolic insulin resistance and vascular endothelial dysfunction. The current consensus of opinion is that insulin is an endothelium-dependent vasodilator via an increase in basal endothelial nitric oxide (NO) production. This conclusion is mainly based on two widely-cited studies which demonstrated in man that insulin-mediated vasodilation was blocked by co-infusion of the substrate inhibitor of NO synthase,

N^G-monomethyl-L-arginine (L-NMMA) (Scherrer et al, 1994a; Steinberg et al, 1994).

However, these results should be interpreted with caution since intra-arterial L-NMMA infusion caused vasoconstriction and insulin's ability to cause vasodilation may have been blunted simply by virtue of non-specific pre-constriction. If it is to be demonstrated unequivocally that the mechanism of insulin-mediated vasodilation is specifically via stimulation of endothelial NO production, then it should be inhibited by L-NMMA but not to the same extent by blockers of other enzymes determining basal blood flow, or by matched doses of vasoconstrictor agents. In addition, the extent of insulin-mediated vasodilation should be greatest in those subjects whose basal blood flow is most dependent on endothelial NO production. It is well recognised that there are a number of other putative mechanisms by which insulin could cause vasodilation independently of endothelial NO production, the most obvious being direct stimulation of the Na^+-K^+ ATPase pump in vascular smooth muscle cells with subsequent membrane hyperpolarisation and vasorelaxation.

In order to address these issues, the present study was performed, using a previously-validated human forearm model with brachial artery infusion (Benjamin et al, 1995; section *2.7.1*) The first hypothesis was that if insulin-mediated vasodilation is not specifically NO dependent, then infusion of a control vasoconstrictor agonist (angiotensin II) or an inhibitor of Na^+-K^+ ATPase (ouabain), designed to pre-constrict the forearm vascular bed to the same extent as NO synthase inhibition, would also be expected to abolish insulin-mediated vasodilation. The second

hypothesis was that if endothelial NO production was the most important mechanism mediating insulin's vascular effects, then the extent of insulin-mediated vasodilation on one study day would be positively associated with the extent of vasoconstriction in response to L-NMMA infusion (which is determined by basal endothelial NO production) on a separate study day.

3.2 Methods

3.2.1 Subjects

Nineteen healthy, normotensive, male volunteers with normal glucose tolerance participated in this study with the approval of the West Ethical Committee of the West Glasgow Hospitals University NHS Trust (protocol 1: n=9; protocol 2: n=10). None of the subjects was taking medication, and all subjects abstained from alcohol, tobacco and heavy physical exertion for 24 hours and from food and caffeine-containing drinks overnight before study days. At a screening visit, physical health was confirmed by history and physical examination and supine blood pressure was measured in triplicate (Dinamap Critikon, Johnson and Johnson Professional Products Ltd). Characteristics (mean $\pm$ SD) of the subjects were as follows: protocol 1: age 23$\pm$5.4 years, body mass index 22$\pm$1.8 kg/m^2, and blood pressure 125/69$\pm$13/8 mmHg; protocol 2: age 29$\pm$6.5 years, body mass index 24$\pm$2.8 kg/m^2, and blood pressure 131/68$\pm$14/9 mmHg. Six subjects were smokers and median weekly alcohol consumption was 70g (range 0-200g).

3.2.2 Clinical procedure

Forearm blood flow was measured by bilateral venous occlusion plethysmography as described in section *2.7.1.*

3.2.3 Study Protocols

The protocols were designed in a double-blind, placebo-controlled, random order manner with measurements of forearm blood flow during infusion of vasoactive substances on three occasions, at least one week apart in all subjects. On each day, after baseline readings had been obtained, subjects received an intra-arterial infusion of low-dose D-glucose (75μmol/min) for 120 minutes in order to maintain local venous euglycaemia in the forearm vascular bed, and which we have shown maximises insulin-mediated vasodilation (Ueda et al, 1998a). After 30 minutes, and for the remaining 90 minutes, soluble human insulin (Actrapid, Novo Nordisk, Denmark) was co-infused at a dose of 5mU/min. This solution was prepared in the sterile unit of the hospital pharmacy using glass syringes and bottles, and diluted (with saline and 4ml of the subject's blood, 8% vol/vol) at the bedside immediately before each study (mean recovery of insulin was 95% - data not shown). In addition, subjects received (figure 3.1) a 120 minute concominant intra-arterial infusion of either N^G-monomethyl-L-arginine (L-NMMA, Clinalfa AG) at a dose of 1mg/min (n=19), ouabain at a dose of 3.5μg/min (protocol 1, n=9), angiotensin II (Ang II, CIBA Pharmaceuticals) at a dose of 20pmol/min (protocol 2, n=10), or placebo (0.9% sodium chloride, Baxter Healthcare) at a rate of 1ml/min (n=19). The local Ethical Committee did not permit cannulation of the brachial artery on four

consecutive occasions in the same subject; for this reason, while all subjects (n=19) received placebo and L-NMMA, nine received ouabain and ten received Ang II. A uniform infusion rate (2ml/min) was used throughout the study.

3.2.4 Statistical Evaluation

Percentage change from basal values in the ratio of blood flow between infused and non-infused arms was calculated, using the blood flow in the non-infused arm as a concurrent control. In order to avoid multiple comparisons, two summary measures were calculated; firstly, the mean value at 20-30 minutes and, secondly, the mean value at 100-120 minutes. As these values were non-parametrically distributed, they were expressed as median values with interquartile ranges. The Wilcoxon Signed Rank Test was used to assess changes in blood flow over time. In addition, the summary measure for insulin-mediated vasodilation (placebo-day) for each subject was plotted against the degree of vasoconstriction induced by L-NMMA (n=19), Ang II (n=10) and ouabain (n=9) and the rank correlation coefficient was calculated in each case. Glucose, potassium and insulin levels were normally distributed; summary measures were calculated as mean$\pm$S.D. of the final three deep vein samples (90, 120 and 150 minutes) and results were compared by analysis of variance.

3.3 Results

The procedures were carried out without complication and were well tolerated by all subjects.

3.3.1 Forearm blood flow

D-glucose infusion alone for 30 minutes (placebo-day) resulted in no change in FBF [-5.0% (-16.0, 1.25)]; insulin and D-glucose infusion together caused significant vasodilation on the placebo day [35.9% (12.1, 76.0)] ($p<0.01$, n=19, figure 3.2a: raw data; figure 3.2b). Significant forearm vasoconstriction occurred with L-NMMA [-34.3% (-44.2,-19.2)], Ang II [-27.9% (-41.7,-20.8)] and ouabain [-36.3% (-46.5,-23.5)], [median (interquartile range)]. Co-administration of insulin had no significant effect on either L-NMMA-induced vasoconstriction [-33.0% (-48.7,-18.6), n=19, figure 3.3], Ang II-induced vasoconstriction [-26.2% (-35.2,-9.2), n=10, figure 3.4] or ouabain-induced vasoconstriction [-23.4% (-30.9,-9.6), n=9, figure 3.5].

3.3.2 Insulin-mediated vasodilation and responses to vasoconstrictors

There was no significant relationship seen between insulin-mediated vasodilation and L-NMMA (R_s=-0.21, figure 3.6a) or Ang II (R_s=0.37, figure 3.6b) responses. However, a significant positive association was demonstrated with ouabain-induced vasoconstriction (R_s=0.69, $p<0.05$, figure 3.6c).

3.3.3 Glucose, potassium and insulin levels

Fasting glucose levels were not significantly different on any of the study days: [4.89±0.8 placebo, 4.79±0.4 L-NMMA, 4.89±0.3 Ang II, 4.83±0.3 ouabain (mmol l^{-1})]. Steady-state deep venous euglycaemia (90-150 minutes) was achieved with co-infusion of insulin with D-glucose: [4.89±0.6 placebo, 5.01±0.6 L-NMMA, 5.20±0.7 Ang II, 5.00±0.6 ouabain (mmol l^{-1})].

Fasting potassium levels were not significantly different on any of the study days: [3.86±0.1 placebo, 3.98±0.3 L-NMMA, 3.81±0.2 Ang II, 3.90±0.1 ouabain (mmol l^{-1})]. Steady-state deep venous potassium levels (90-150 minutes) were slightly lower as expected on three days: [3.75±0.2 placebo, 3.70±0.3 L-NMMA, 3.73±0.2 Ang II (mmol l^{-1})]; however, they were significantly higher on the ouabain day (4.55±0.3 mmol l^{-1}, $p<0.0001$), indicating effective inhibition of Na^+-K^+ ATPase.

Fasting insulin levels were not significantly different on any of the study days [2.3 (0.0,5.3) placebo, 1.3 (0.0,5.3) L-NMMA, 4.0 (2.3,8.5) Ang II, 4.5 (1.8,7.3) ouabain (μU ml^{-1}) {median (interquartile range)}]. Steady-state deep venous hyperinsulinaemia (90-150 minutes) was achieved with no significant differences detectable among the study days: [106±51 placebo, 138±86 L-NMMA, 120±53 Ang II, 153±67 ouabain (μU ml^{-1})].

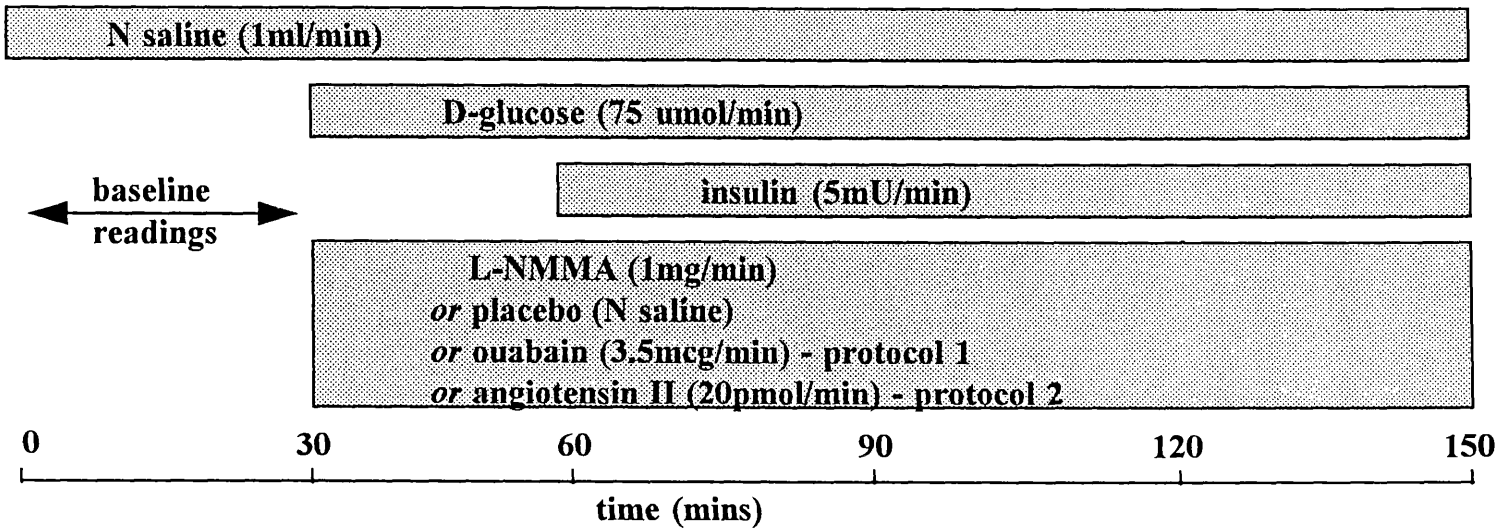


Figure 3.1
Protocol for intra-arterial infusions.

Figure 3.2
(a) Time course of individual values for local insulin/glucose-mediated vasoreactivity (n=10, protocol 2). (b) Time course of local insulin/glucose-mediated vasoreactivity (median ± IQ) (n=19).

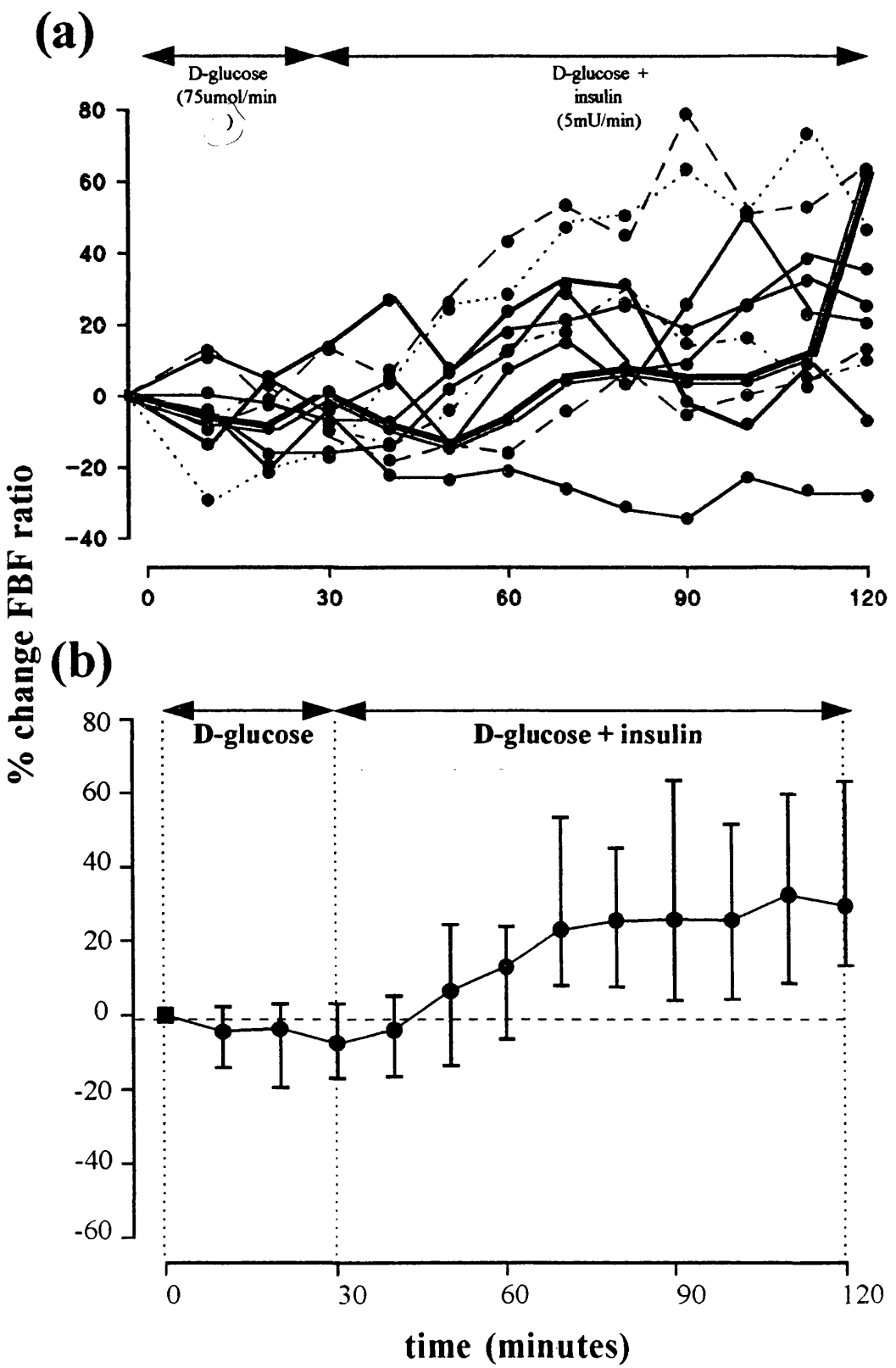


Figure 3.3
Time course of local insulin/glucose-mediated vasoreactivity (median ± IQ) in 19 healthy male volunteers, with and without co-infusion of L-NMMA. Double-blind, random order study design.

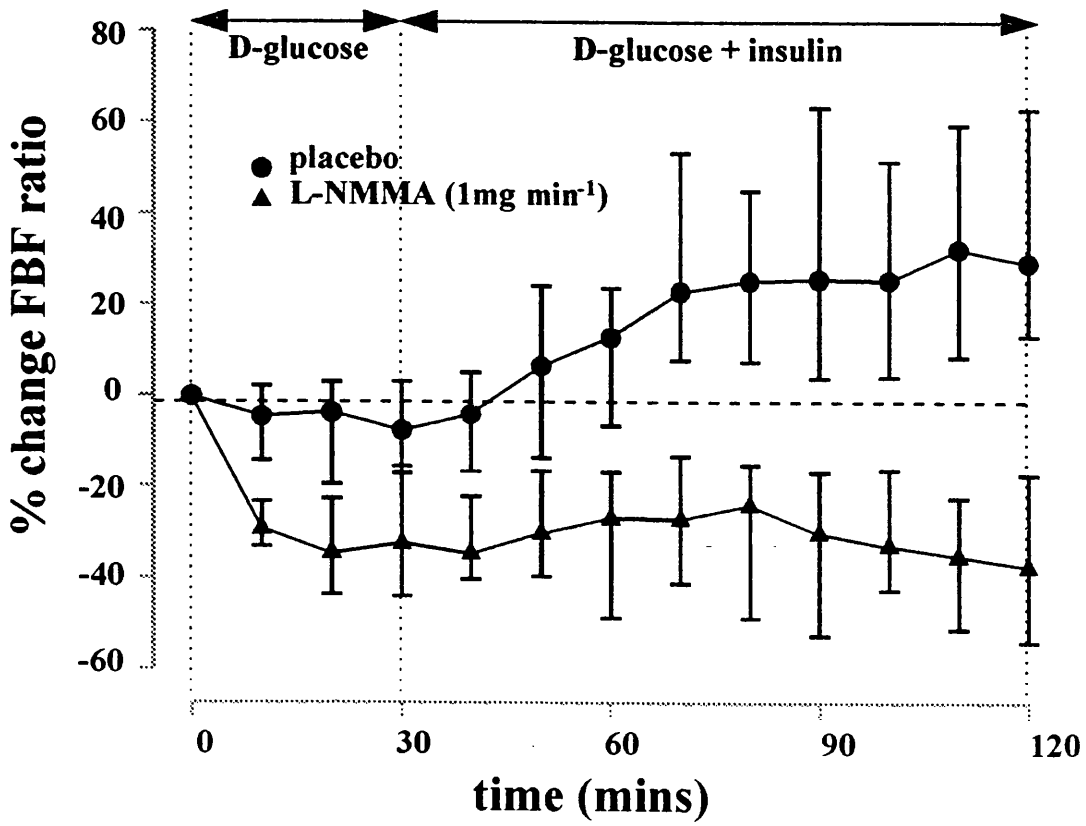


Figure 3.4
Time course of local insulin/glucose-mediated vasoreactivity (median ± IQ) with and without co-infusion of angiotensin II (n=10).

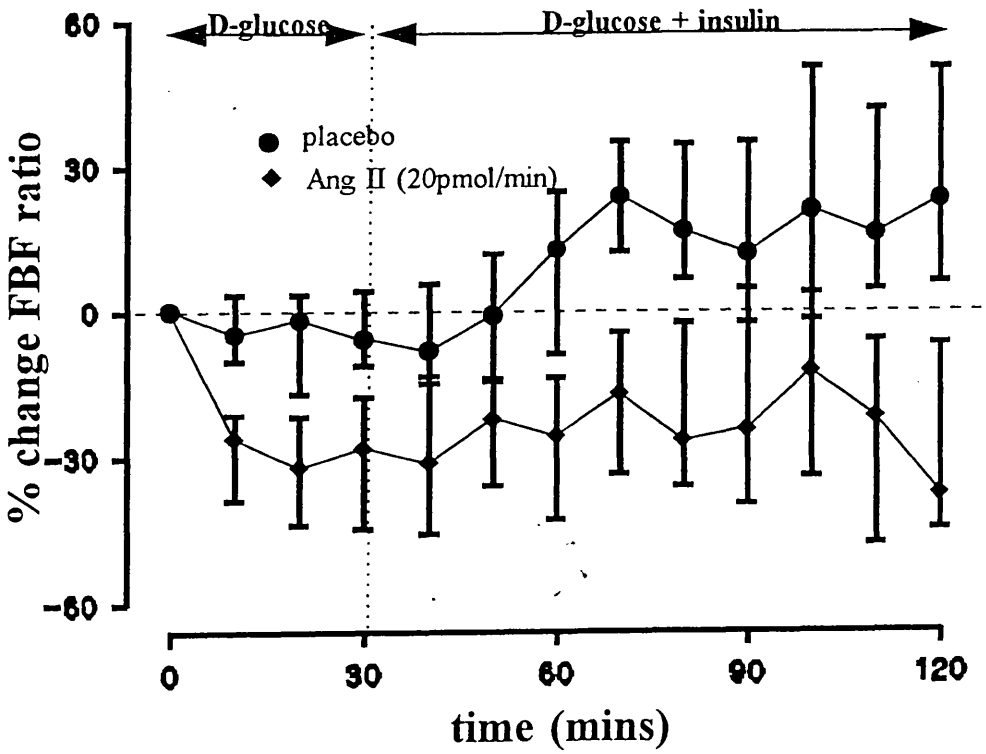


Figure 3.5
Time course of local insulin/glucose-mediated vasoreactivity (median ± IQ) with and without co-infusion of ouabain (n=9).

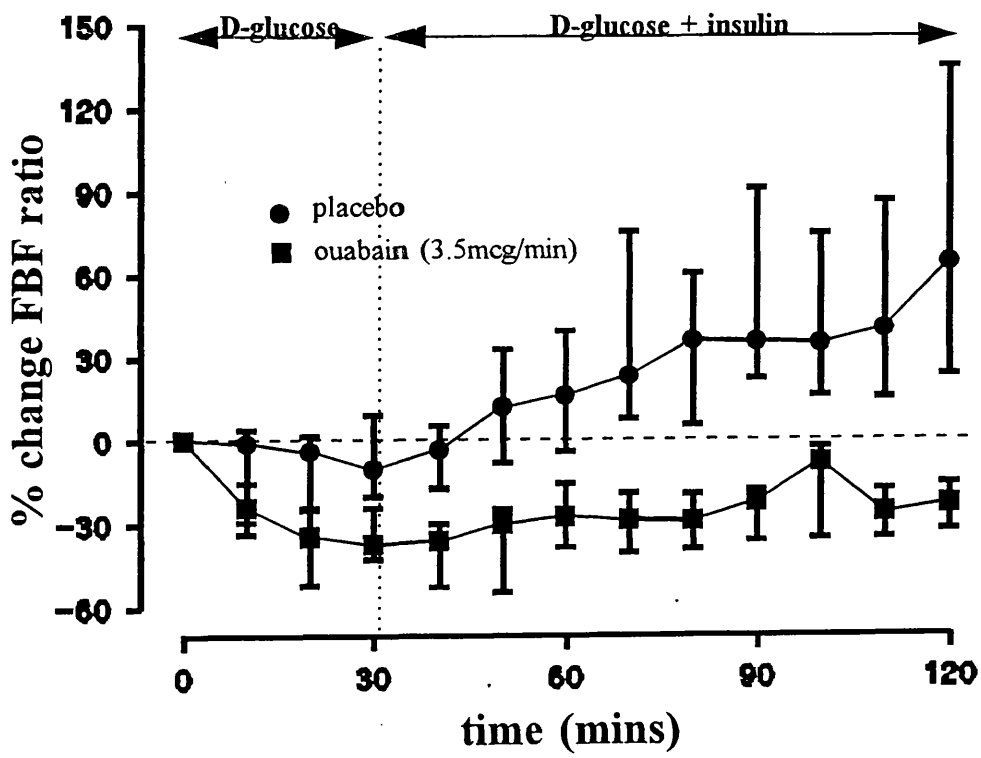
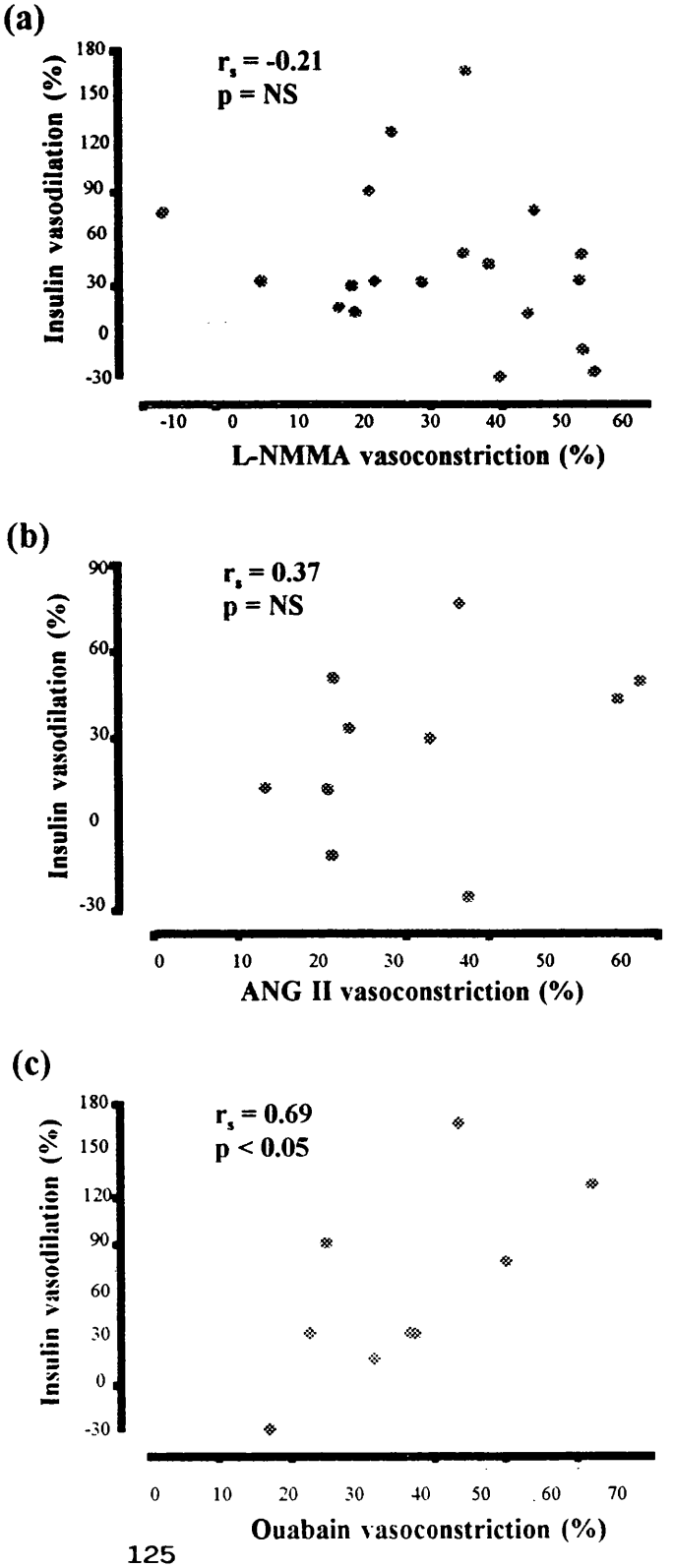


Figure 3.6
Local insulin/glucose-mediated vasodilation (placebo day) plotted against percentage forearm vasoconstriction in response to intra-arterial infusion of (a) L-NMMA (n=19), (b) angiotensin II (n=10), (c) ouabain (n=9).



3.4 Discussion

In this study a validated physiological model of local hyperinsulinaemia with venous euglycaemia in the human forearm vascular bed has been used to explore mechanisms of insulin-mediated vasodilation. By infusing insulin via the brachial artery, any confounding systemic haemodynamic effects, such as stimulation of the sympathetic nervous system, have been avoided. Infusion of insulin and D-glucose caused a significant (36%) vasodilator response after 90 minutes (consistent with our previous data [Ueda et al, 1998a]) but did not reverse vasoconstriction (34%) induced by a 30-minute intra-arterial infusion of L-NMMA, a substrate inhibitor of endothelial NO synthesis. One interpretation of these findings is that insulin exerts its vasodilator effect via stimulation of endothelial NO production, and this conclusion would be consistent with previous reports (Haylor et al, 1991; Wu H-Y et al, 1994; Scherrer et al, 1994a; Steinberg et al, 1994;)

However, after inducing a similar degree of vasoconstriction (28%) in the forearm vascular bed, using a 30-minute intra-arterial control infusion of Ang II, insulin's vasodilator action was also abolished. These data weaken the aforementioned conclusion i.e. insulin may lose its vasodilator action simply as a result of induction of non-specific background vasoconstriction.

Inhibition of vascular Na^+-K^+ ATPase activity by co-infusion of ouabain achieved a similar degree of vasoconstriction (36%). Again, insulin's vasodilator action was abolished, a result which is consistent with previous data (Tack et al, 1996). There are two ways of interpreting this result. Firstly, as outlined above, there is the

possibility that non-specific background vasoconstriction was responsible for abolition of insulin-mediated vasodilation. Secondly, there is a substantial body of evidence to suggest that insulin directly stimulates Na^+-K^+ ATPase activity (Ewart & Klip, 1995; Gupta et al, 1996), either by up-regulating gene expression (Tirupattur et al. 1993) or by causing translocation of the enzyme from intracellular stores to the plasma membrane surface (Omatsu-Kanbe & Kitasato, 1990) or as a secondary consequence of activation of Na^+-H^+ exchange (Sowers, 1996). In vascular smooth muscle the consequences include hyperpolarisation of the cell membrane leading to closure of voltage-gated Ca^{++} channels, consequent decrease in intracellular calcium concentration, and subsequent vascular relaxation. If insulin were to cause vasorelaxation by stimulation of Na^+-K^+ ATPase activity, inhibition of this pump would be predicted to abolish this action.

Additionally, the possibility remains that endothelial NO production may still be the predominant mechanism for insulin-mediated vasodilation but that the Na^+-K^+ ATPase pump may be important either in mediating NO effects or in the production of NO - therefore, inhibiting Na^+-K^+ ATPase activity would still block insulin's vascular action. There is evidence in support of an interaction between NO and Na^+-K^+ ATPase: for example, it has been reported that insulin may stimulate vascular smooth muscle Na^+-K^+ ATPase activity indirectly via its action to increase endothelial-derived NO synthesis/release, a mechanism which appears to be independent of cGMP production (Gupta et al, 1994). It has also been proposed that the mechanism by which insulin stimulates endothelial NO production may be via its action on endothelial Na^+-K^+ ATPase (Elliott & Schilling, 1992; Meharg et al, 1993):

since there are no voltage-gated Ca^{++} channels in endothelial cells, hyperpolarisation leads to an increase in intracellular [Ca^{++}] (Luckhoff & Busse, 1990) which may activate the Ca^{++}-dependent enzyme eNOS.

Therefore, it is clear that there are major difficulties in the interpretation of forearm blood flow studies with respect to drawing conclusions about underlying mechanisms of insulin-mediated vasodilation. It cannot be concluded from the present data that insulin-mediated vasodilation is specifically NO dependent, given the complexities and numerous interactions of mechanisms. Further insight into the question of which of the candidate mechanisms might be most closely coupled with insulin action was gained by use of the considerable inter-individual variation observed in vascular reactivity both to the three vasoconstrictors and to insulin, even in this relatively homogeneous group of fit, young, healthy subjects. The hypothesis was that, if endothelial NO production was the most important mechanism mediating insulin's vascular effects, then the extent of insulin-mediated vasodilation on one study day should be positively associated with the extent of vasoconstriction in response to L-NMMA infusion measured on a separate study day. However, contrary to the hypothesis, there was not a positive relationship between these variables - if anything there was a trend towards a negative relationship (figure 3.6a). In addition, there was no association between the extent of control Ang II vasoconstriction and insulin-mediated vasodilation (figure 3.6b).

However, a significant positive correlation between insulin-mediated vasodilation and ouabain-induced vasoconstriction was demonstrated (figure 3.6c). Since it is

reasonable to suggest that vasoconstriction in response to ouabain is a measure of basal Na^+-K^+ ATPase pump activity, it is proposed that insulin's vascular action may be closely coupled with Na^+-K^+ ATPase activity. Thus, while endothelial NO production may still be an important mechanism for insulin-mediated vasodilation, it is speculated that stimulation of Na^+-K^+ ATPase activity may be the most important primary determinant. This may reflect the dual actions of insulin on this pump at both the endothelium and vascular smooth muscle. In this regard, the relative delay and glucose enhancement of insulin-mediated vasodilation are of interest (Ueda et al, 1998a). Most endothelium-dependent vasodilators (eg acetylcholine) act acutely to release NO; in contrast, the maximal dilator response to insulin takes at least 50-80 minutes to develop. Additionally, the response is maximised by co-administration of D-glucose (Ueda et al, 1998a). As Na^+-K^+ ATPase activity is ATP-dependent, and is maximised by aerobic glycolysis, increased intracellular availability of glucose may be the initial determinant of insulin-mediated vasodilation. According to this hypothesis, insulin-stimulated glucose uptake and metabolism generates ATP which activates Na^+-K^+ ATPase: this, either by an endothelial effect to generate NO or a direct action to reduce intracellular calcium concentration in vascular smooth muscle cells, will result in vasodilation. This provides a unifying hypothesis which may account for the reported linkage between reduced insulin-mediated glucose uptake and metabolism and attenuated insulin vasorelaxation (Laakso et al, 1992; Feldman et al, 1995; see chapter 4).

Of course, it must be borne in mind that the 'control' vasoconstrictor used in this study, Ang II, may not be a 'neutral' vasoconstrictor, in the sense that there may be

a degree of 'cross-talk' between post-receptor signalling pathways of insulin and Ang II. There is some evidence from in vitro work to support this notion; for example, Ang II receptor (type 1) activation stimulates tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1) but inhibits activation of phosphatidylinositol 3-kinase (PI3-kinase) (Saad et al, 1995; Du et al, 1996; Velloso et al, 1996), which suggests that the octapeptide may modify insulin action, although it is unclear whether there is any functional association between the metabolic and vascular effects. Additionally, recent evidence suggests that Ang II increases vascular superoxide production, an effect which has been shown to inhibit relaxation to acetylcholine (but not norepinephrine) (Rajagopatan et al, 1996); if this mechanism occurs in man, insulin-mediated NO-dependent vasodilation might be similarly inhibited by Ang II.

In summary, it has been demonstrated in this study that local infusion of physiological doses of insulin and glucose causes 30-40% vasodilation in the human forearm vascular bed. This action is abolished by co-infusion not only of a NO synthase inhibitor (L-NMMA), but also of angiotensin II and ouabain (at doses calculated to result in a similar state of prior vasoconstriction to that induced by L-NMMA). One conclusion from these data is that the mechanism of insulin-mediated vasodilation involves stimulation of endothelial NO production, but stimulation of the Na^+K^+ATPase pump, or a combination of mechanisms can also be supported. This study highlights the pitfalls of interpreting data of this nature, since an alternative conclusion is that insulin-mediated vasodilation is inhibited in a non-specific manner simply as a result of induction of background vasoconstriction.

Correlation analysis of our data revealed that insulin-mediated vasodilation appears to be most closely coupled with the activity of the Na^+K^+ATPase pump, possibly due to the dual action of insulin on this pump at both an endothelial and vascular smooth muscle level.

Chapter 4

Insulin-mediated vasodilation and glucose uptake are functionally linked in man

4.0 Summary

This clinical physiology study was designed to test the hypothesis that insulin-mediated vasodilation and insulin sensitivity are functionally related. Eighteen healthy volunteers attended on two separate occasions for measurement of whole-body insulin sensitivity and forearm vasodilation in response to an intra-arterial infusion of insulin and glucose. In multiple regression analysis, insulin-mediated vasodilation was, indeed, a significant independent predictor of insulin sensitivity.

4.1 Introduction

Epidemiological studies have demonstrated a link between metabolic disorders (such as obesity and type 2 diabetes, and cardiovascular diseases such as essential hypertension, congestive cardiac failure and atherosclerosis (DeFronzo & Ferrannini, 1991). These disorders share two important pathophysiological features, namely relative resistance to insulin-mediated glucose uptake (Yki-Jarvinen & Koivisto, 1983; DeFronzo et al, 1992; Ferrannini et al, 1987; Swan et al, 1994) and vascular endothelial dysfunction, characterised by reduced basal and stimulated endothelial nitric oxide production (Williams et al, 1996; Calver et al, 1992; Panza et al, 1993; Drexler et al, 1993). However, the underlying mechanism(s) and significance of this association remain unclear.

Our group has previously reported a significant, positive relationship, in healthy volunteers, between insulin sensitivity and basal endothelial nitric oxide production (Petrie et al, 1996a) consistent with a functional link between insulin-mediated glucose uptake and endothelial function. However, in addition to its metabolic effects, insulin is also a directly acting vasodilator, an action which has been demonstrated to be largely dependent on endothelial nitric oxide production (Scherrer et al, 1994a; Steinberg et al, 1994). The importance of this phenomenon in cardiovascular regulation and the nature of the relationship between insulin-stimulated blood flow and glucose uptake are uncertain. However, in view of the evidence that both insulin sensitivity and insulin-mediated vasodilation are directly linked to endothelial function, the hypothesis was generated that insulin-mediated vasodilation would be directly related to insulin sensitivity. Previous studies reporting such a relationship in man have been subject to potentially confounding factors, including sympathetic nervous system activation, as they have examined vasodilation during systemic insulin infusions (Baron et al, 1995; Utriainen et al, 1997). There is, however, data to suggest a positive relationship, albeit using the rather unphysiological model of infusing insulin into preconstricted dorsal hand veins (Feldman et al, 1995). In the absence of published data adequately testing the hypothesis of a relationship between whole-body insulin sensitivity and vasodilation secondary to local intra-arterial infusion of insulin, the current study was designed to investigate this issue in a group of healthy male volunteers.

4.2 Methods

4.2.1 Subjects

Eighteen healthy, normotensive male volunteers aged 18-37 years participated in this study which was approved by the Ethics Committee of the West Glasgow Hospitals University NHS Trust. No subjects were taking medication, and all abstained from alcohol, tobacco and strenuous physical activity for 24 hours and from food and caffeine-containing drinks overnight before the two study days, which were at least one week apart. At a screening visit, physical health was confirmed by history and physical examination and supine blood pressure was measured in triplicate (Dinamap Critikon, Johnson and Johnson Professional Products Ltd). Subject characteristics included age 26±5.4 years, mean arterial pressure (MAP) 89±8.8 mmHg (mean±SD) and body mass index (BMI) 23.2 (21.9, 26.3) kg/m^2 (median, interquartile range); 2 subjects were smokers; median alcohol consumption was 20g/week.

4.2.2 Clinical procedures

Subjects attended for measurement of whole-body insulin sensitivity (M) using a 180-minute hyperinsulinaemic euglycaemic clamp as described in section *2.4.1.* On a separate study day, subjects attended for measurement of forearm blood flow by bilateral venous occlusion plethysmography, as described in section *2.7.1.* After baseline readings had been obtained, subjects received an intra-arterial infusion of low-dose D-glucose (75μmol/min) for 120 minutes in order to maintain local venous euglycaemia in the forearm vascular bed (Ueda et al, 1998a). After 30 minutes, and for the remaining 90 minutes, soluble human insulin (Actrapid, Novo Nordisk,

Denmark) was co-infused at a dose of 5mU/min. A uniform infusion rate (2ml/min) was used throughout the study.

4.2.3 Statistical Evaluation

For the forearm plethysmography data, percentage change from basal values in the ratio of blood flow between infused and non-infused arms was calculated, using the blood flow in the non-infused arm as a concurrent control (Benjamin et al, 1995). In order to avoid multiple comparisons, a summary measure was calculated which was the mean value of the last three readings (100-120 minutes) (Matthews et al, 1990).

Data were initially examined using simple correlation; multiple regression analysis (Minitab for Windows, Minitab Inc.) was performed in order to examine potential confounders. Insulin sensitivity, age and MAP data were normally distributed; insulin vasodilation and BMI data were non-parametrically distributed and were therefore log-transformed before undergoing multiple regression analysis. For simple correlation involving data which were non-parametrically distributed, the rank correlation coefficient was calculated.

4.3 Results

The procedures were carried out without complication and were well tolerated by all subjects.

4.3.1 Insulin levels

During the euglycaemic clamps, systemic insulin levels were raised to 86±13.9 μUml^{-1} during the last hour. During the local insulin infusions, insulin levels in the forearm vascular bed were raised to 109±39 μUml^{-1} during the last hour.

4.3.2 Whole-body insulin sensitivity

M-value (mean±S.D.) for the group was 10.0±2.2 mg kg^{-1} min^{-1} [range: 6.8 - 14.7].

4.3.3 Insulin-mediated vasodilation

Percentage change in forearm blood flow ratio (%FBFR) for the group (median, interquartile range) was 28.2% (13.6, 48.6).

4.3.4 Univariate analysis

Depending on the distribution of the data, simple (r) or rank correlation (r_s) analysis was performed. M-value was significantly correlated with %FBFR ($r_s = 0.60$, $p < 0.05$, figure 4.1), but not with BMI ($r_s = -0.42$, $p < 0.1$), age ($r = -0.39$, $p < 0.2$) or MAP ($r = 0.13$, $p > 0.2$) [figure 4.2]. %FBFR was not significantly correlated with BMI ($r_s = -0.15$), age ($r_s = 0.16$) or MAP ($r_s = 0.15$) [$p > 0.2$].

4.3.5 Multiple regression analysis

Table 4.1 displays adjusted R^2 and *t* for %FBFR as a predictor of M-value, with forward stepwise addition of BMI, age and MAP. %FBFR remains a significant

independent predictor of M-value after these potentially confounding variables are included in the model (R^2 (adj) = 0.48, t = 3.23, $p < 0.01$).

Figure 4.1
Whole-body insulin sensitivity (M) plotted against local insulin/glucose-mediated vasoreactivity in 18 healthy male volunteers.

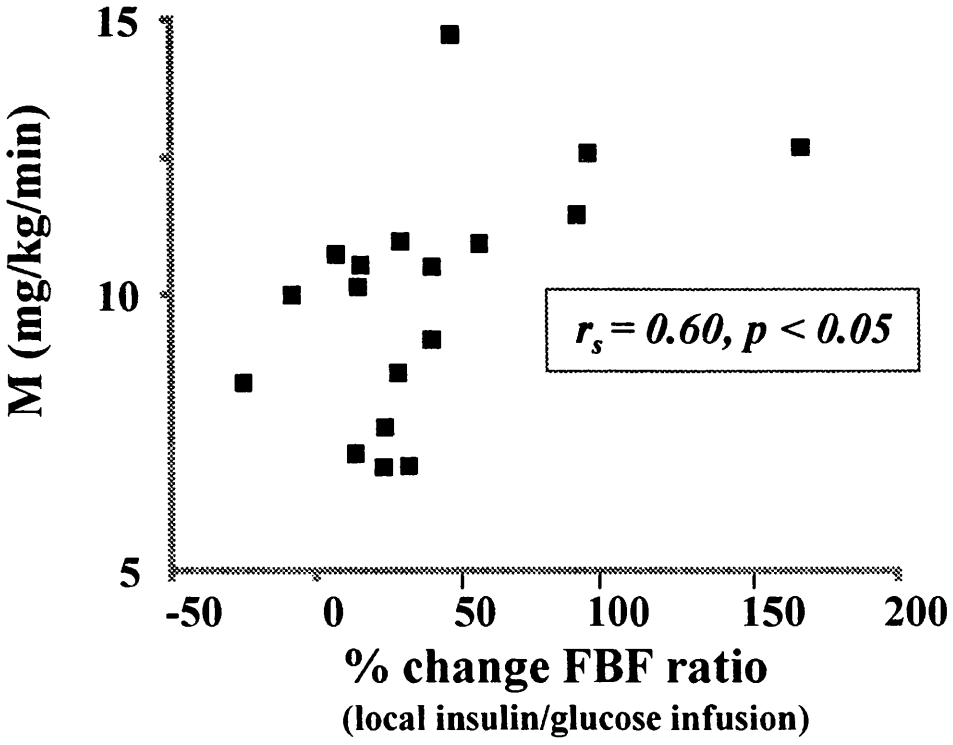


Figure 4.2
Whole-body insulin sensitivity (M) plotted against (a) age (b) body mass index (c) mean arterial pressure, in 18 healthy male volunteers.

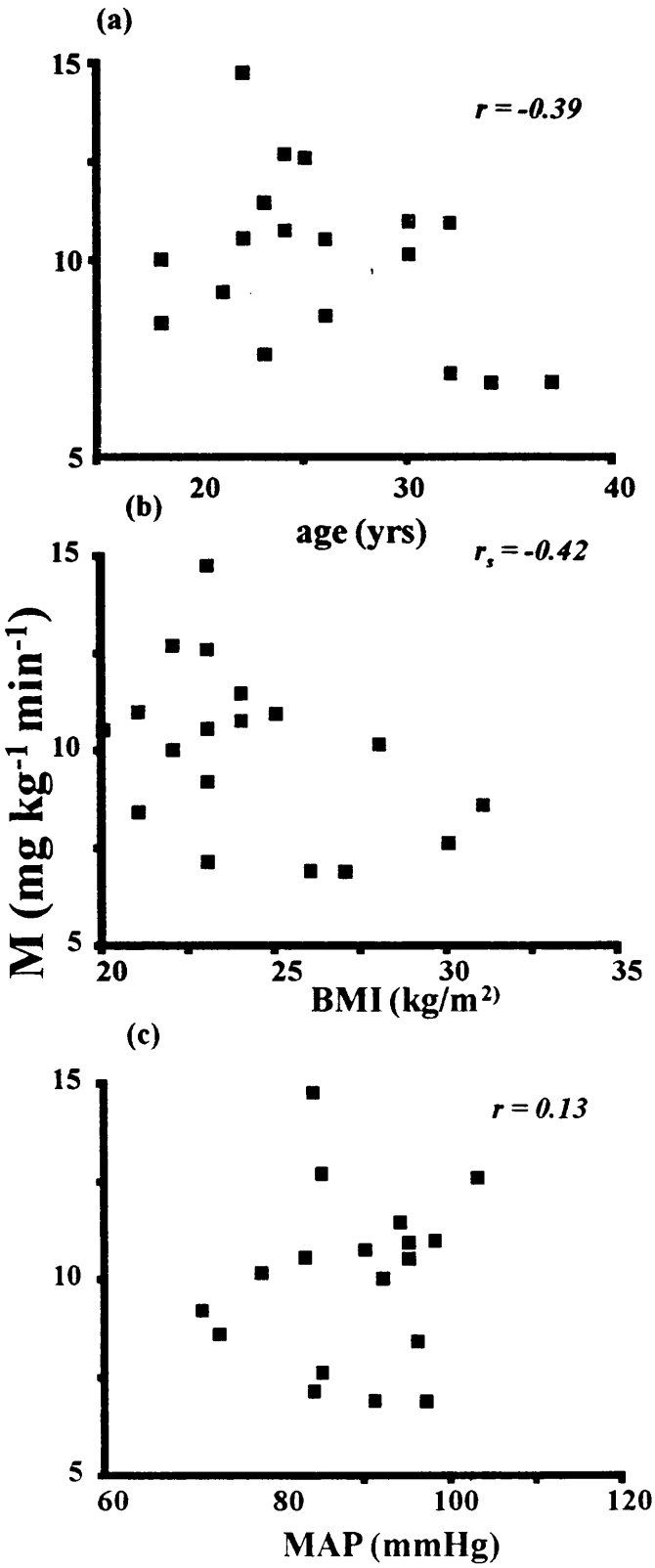


Table 4.1
Multiple regression models with insulin sensitivity (M) as dependent variable.

Model	insulin vasodilation	age	BMI	MAP	Adjusted R^2	*t* for insulin vasodilation	*p*
1	√				0.20	2.28	0.04
2	√	√			0.44	3.26	0.005
3	√	√	√		0.51	3.32	0.004
4	√	√	√	√	0.48	3.23	0.007

Prediction of insulin sensitivity (M) from percentage change in forearm blood flow ratio in response to local insulin infusion (insulin vasodilation), age, body mass index (BMI) and mean arterial pressure (MAP).Changes are shown as adjusted R^2 and *t* for insulin vasodilation, with addition of age, BMI and MAP into the model. Predictor variables: *model 1*, insulin vasodilation; *model 2*, insulin vasodilation +age; *model 3*, insulin vasodilation + age + BMI; *model 4*, insulin vasodilation + BMI + age + MAP.

4.4 Discussion

This study is the first to examine the relationship between whole-body insulin sensitivity and insulin-mediated vasodilation using a physiological model in the absence of potentially confounding haemodynamic variables such as stimulation of the sympathetic nervous system. The results demonstrate that vasodilation induced by local intra-arterial infusion of insulin/glucose was independently associated with whole-body insulin-mediated glucose uptake in healthy male volunteers. As expected there was a trend for insulin sensitivity to decrease with increasing age and body mass index, but these relationships were not significant due to the relatively narrow range of values in this group of young healthy volunteers.

There is currently a great deal of interest in the vascular effects of insulin, and especially whether its vasodilator action is coupled in a functional manner with its ability to stimulate cellular glucose uptake. Given that insulin is thought to be an endothelium-dependent vasodilator (Scherrer et al, 1994a; Steinberg et al, 1994), the mechanism of any functional coupling between insulin's vascular and metabolic actions may help to explain the observed association between endothelial dysfunction and insulin resistance in metabolic and cardiovascular disorders. Baron's group has repeatedly demonstrated a significant relationship between whole-body insulin sensitivity and clamp-induced leg vasodilation in healthy subjects (Baron et al, 1995). However, Yki-Jarvinen's group, while conceding that endothelial function is a significant determinant of insulin-mediated vasodilation (Utriainen et al, 1996a), has reported a lack of relationship between insulin sensitivity and forearm vasodilation in response to systemic hyperinsulinaemia (Utriainen et al, 1996a;

Utriainen et al, 1996b). In support of this conclusion, the same group used positron emission tomography (PET) and [^{18}F]fluoro-2-deoxy-D-glucose uptake to demonstrate a dissociation of insulin-stimulated blood flow and glucose uptake in thigh skeletal muscle; however, despite this finding, they demonstrated a significant relationship between the insulin-induced increase in whole leg blood flow and whole-body insulin sensitivity (Raitakari et al, 1996). In a more recent study, in which higher resolution methods were used, these investigators conceded that insulin-stimulated muscle blood flow co-localized with regional glucose uptake, consistent with redirection of flow by insulin to areas where it stimulates glucose uptake (Utriainen et al, 1997). The current data, which demonstrate a close correlation between direct insulin-induced arterial vasodilation and whole-body stimulated glucose uptake, support this hypothesis.

There are three possible explanations for this observation: firstly, that insulin-mediated vasodilation is an important determinant of insulin-mediated glucose uptake; secondly, that glucose uptake modulates the vascular response to insulin, possibly at an endothelial level; thirdly, that a third factor is responsible for determining both the metabolic and vascular actions of insulin.

If substrate delivery is the rate-limiting step for insulin-mediated glucose uptake (in other words, if skeletal muscle blood flow is a determinant of glucose uptake), then it can be argued that endothelial dysfunction, resulting in a relative inability of mediators, including insulin, to stimulate muscle blood flow, may be the underlying mechanism accounting for the association of atherosclerosis and other

cardiovascular disorders with insulin resistance (Baron, 1994). However, it has been suggested that substrate delivery is only likely to be rate-limiting for glucose disposal at supra-physiological insulin levels maintained for many hours and does not, therefore, explain the physiological association between endothelial function and insulin sensitivity (Yki-Jarvinen, 1995). One approach to resolve this issue is to manipulate limb blood flow during hyperinsulinaemia and assess whether insulin-mediated glucose uptake is affected. There is some evidence from this type of study that skeletal muscle blood flow may be a determinant of insulin-mediated glucose uptake; infusion of N^G - monomethyl-L-arginine (L-NMMA) into the femoral artery during a clamp reduces insulin-mediated stimulation of blood flow (via inhibition of endothelial nitric oxide production) resulting in a 25% decrease in limb glucose uptake (despite a 50% increase in arteriovenous fractional glucose extraction) (Baron et al, 1995). This observation is consistent with the notion that 20-30% of insulin-mediated glucose uptake is blood-flow dependent. In the same study, whole-body insulin sensitivity correlated significantly with the decrement in limb blood flow as a result of L-NMMA infusion (Baron et al, 1995). However, other investigations have failed to support the notion that skeletal muscle blood flow and tissue glucose uptake are functionally related. In one study, when human volunteers were fed a mixed physiological meal, arteriovenous glucose extraction was found to be the main determinant of limb glucose uptake; this rose by a factor of five, while blood flow increased by only 16% (Mijares & Jensen, 1995). In another study, no significant increases in forearm blood flow were observed during an oral glucose tolerance test (Kelly et al, 1988). Furthermore, dissociation of insulin-mediated glucose uptake from blood flow was observed in a study in which both limb blood

flow and muscle sympathetic nerve activity were stimulated to similar degrees at different systemic insulin concentrations, ranging from 100 to 400pmol/l (Anderson et al, 1991). Finally, again in man, forearm blood flow has been augmented by 100% using intra-arterial adenosine (Natali et al, 1994) and bradykinin (Nuutila et al, 1996) without a detectable effect on forearm glucose uptake, and it must be concluded that the case for skeletal muscle blood flow being a determinant of insulin sensitivity remains uncertain.

An alternative, and more tenable, hypothesis is that insulin-stimulated glucose uptake may determine muscle blood flow in response to the hormone by causing an increase in ATP production, as a consequence of aerobic glycolysis. This will provide energy for membrane ion pumps (for example Na^+-K^+ ATPase) resulting in hyperpolarisation and changes in Ca^{2+} fluxes in both endothelium and vascular smooth muscle cells (Sowers, 1996). It has been shown that insulin-mediated vasodilation can be inhibited by ouabain, an inhibitor of Na^+-K^+ ATPase (Tack et al, 1996; see chapter 3), which would support this proposal. Furthermore, if nitric oxide synthase activity is enhanced by stimulation of ATP-dependent ion pumps such as Na^+-K^+ ATPase, insulin-stimulated glucose uptake may result in increased nitric oxide production as a consequence of endothelial cell glucose metabolism; conversely, reduced glucose uptake would be predicted to lead to diminished nitric oxide production. In support of the notion that glucose uptake is necessary for insulin-mediated vasodilation is the finding that clamping glucose at 20 $mmol^{-1}$ during hyperinsulinaemia causes 50% augmentation of insulin-stimulated blood flow in man (Baron, 1993). In addition, a number of *in vitro* studies provide evidence that

glucose uptake may be important in determining vascular smooth muscle cell relaxation (Sobrevia et al, 1996; Graier et al, 1993; Kahn et al, 1995); of particular note is a recently published study demonstrating nitric oxide production secondary to insulin stimulation in endothelial cell culture and subsequent blocking of this in the presence of wortmannin, which inhibits phosphatidyl inositol 3-kinase, an essential enzyme involved in intracellular insulin-stimulated glucose transport and metabolism (Zeng & Quon, 1996). Furthermore, the delayed time course of insulin-mediated vasodilation would support this hypothesis, in that the vasodilator effect of the hormone is only evident after 30-40 minutes, consistent with primary effects to increase glucose uptake and metabolism. One implication of this hypothesis would be that a primary derangement of glucose uptake would lead to reduced insulin-mediated vasodilation (and, possibly, effects on other aspects of vascular endothelial function), so providing an explanation of the link between insulin resistance and vascular disease.

However, the current data, and most of the studies cited above, are based on studies performed in healthy subjects, and it is reasonable to examine whether similar relationships between insulin's metabolic and vascular effects are observed in disease. Previous studies in these circumstances have produced conflicting results. For example, it has been demonstrated in obese subjects (Laakso et al, 1990) and in patients with type 2 diabetes (Laakso et al, 1992) and insulin-dependent diabetes mellitus (Baron et al, 1991) that there is blunting of insulin-mediated vasodilation in parallel with reductions in whole-body insulin sensitivity, which would be consistent with the current data. However, the physiological relevance of some of these studies

has been questioned in view of the doses of insulin administered and the clamping of glucose values at the relatively hypoglycaemic value of 5mmol/l in type 2 diabetic subjects (Laakso et al, 1992). In another study, in which glucose levels were clamped at fasting levels in subjects with type 2 diabetes, blunting of the blood flow response to insulin was observed (Dela et al, 1995). Using PET scanning to measure forearm blood flow, Yki-Jarvinen *et al* recently demonstrated no defect in insulin-stimulated muscle blood flow in a group of patients with type 2 diabetes despite a significant reduction in muscle glucose uptake compared with controls, implying that insulin's metabolic function is impaired but its vascular action is preserved in type 2 diabetes (Raitakari et al, 1996). In a group of patients with essential hypertension, calf blood flow measured during systemic hyperinsulinaemia was not directly related to insulin sensitivity and there was no difference compared with a group of healthy matched controls (Hunter et al, 1997). More recently it was demonstrated in hypertensive patients that insulin sensitivity was weakly related to insulin-stimulated forearm blood flow, but not to stimulated endothelial function, although the lack of a control group reduces the ability to interpret these results (Natali et al, 1997). It should be borne in mind, however, that in all of the above experiments the measurements of limb blood flow were made during systemic administration of insulin, and may not give a true reflection of the direct vascular effects of the hormone: there is a need for investigations in patient groups to be re-evaluated in this regard (see chapter 7).

Finally, the possibility remains that insulin's metabolic and vascular actions are not causally related but are influenced by a third factor. The most obvious candidate is

skeletal muscle structure, fibre type and related capillarisation, which is largely determined by inheritance. It has been demonstrated that the proportion of type 1 fibres (oxidative, slow-twitch) is related to forearm and whole-body glucose uptake in healthy males, while the ratio of capillaries per fibre correlates with both basal and insulin-stimulated forearm blood flow (Utriainen et al, 1996c, but see chapter 6). Since type 1 fibres tend to be surrounded by a more dense capillary network, this raises the possibility that skeletal muscle structure is the common link between insulin's metabolic and vascular effects. In this context, it is of interest to note that similar structural characteristics (reduced capillary density, a predominance of type IIB glycolytic, fast-twitch fibres and a relative lack of type I fibres) are present in skeletal muscle of subjects not only with metabolic disorders, such as central obesity (Krotkiewski & Bjorntorp, 1986) and type 2 diabetes (Lilloja et al, 1987), but also with cardiovascular disorders, such as essential hypertension (Juhlin-Dannfelt et al, 1979). Therefore, underlying muscle structure could theoretically contribute to the observed insulin resistance and vascular dysfunction in these conditions (see chapter 6).

Finally, another possible confounding factor is obesity, which may affect insulin-mediated glucose uptake and vascular endothelial function by different mechanisms. It has been shown that obese insulin-resistant subjects are characterised by endothelial dysfunction and resistance to insulin's effect on enhancement of endothelium-dependent vasodilation, and that there is no difference in these variables in a group of patients with type 2 diabetes, matched for age and BMI (Steinberg et al, 1996). These results suggest that obesity may play a primary role in the link

between insulin resistance and endothelial dysfunction, and may help to explain why results obtained in obese diabetic and hypertensive patient groups cannot necessarily be extrapolated to patients of normal BMI. However, it should be noted that BMI was not a confounding variable in the current study.

In summary, in the current study, evidence is presented supporting a significant functional link between insulin sensitivity and local insulin-mediated vasodilation (which avoids the confounding haemodynamic effects of systemic hyperinsulinaemia) in young healthy male volunteers. The potential pathophysiological relevance and mechanisms remain unknown, although a more detailed understanding of the association may shed light on the underlying pathophysiology which leads to insulin resistance in a variety of disease states. Further studies in groups of patients with metabolic and cardiovascular disease are now required (see chapter 7).

Chapter 5

Systemic inhibition of endothelial nitric oxide synthesis does not impair insulin sensitivity in man.

5.0 Summary

In this clinical physiology study, an experimental state of systemic endothelial dysfunction was induced in healthy male volunteers and the effect on whole-body insulin sensitivity was assessed in a randomised double-blind, placebo-controlled manner. The results indicate that impairment of endothelial nitric oxide synthesis does not result in an impairment of whole-body insulin sensitivity, suggesting that substrate delivery to nutritive capillary beds is unlikely to be rate-limiting for insulin-mediated glucose uptake.

5.1 Introduction

Resistance to insulin-mediated glucose uptake and endothelial dysfunction have both been implicated in the pathogenesis of essential hypertension (Ferrannini et al, 1987; Lind et al, 1995; Panza et al, 1993a) and other forms of cardiovascular disease, including type 2 diabetes (DeFronzo et al, 1992; McVeigh et al, 1992). As stated previously, our group has demonstrated a relationship, in healthy subjects, between insulin sensitivity and basal endothelial nitric oxide production (Petrie et al, 1996a), and it was suggested that variations in endothelial function might account for variations in insulin sensitivity by decreasing skeletal muscle blood flow, which may be a determinant of insulin-mediated glucose uptake (Baron, 1994). However, it

remains unclear whether insulin sensitivity and endothelial function are causally related or whether both phenotypes are influenced by a common antecedent. In order to examine this hypothesis in the current study, an experimental state of impaired endothelial function has been induced in healthy male volunteers by systemic inhibition of endothelial nitric oxide synthesis, and the effect on insulin sensitivity has been assessed. In addition, the effects of N_G-monomethyl L-arginine (L-NMMA) were compared with angiotensin II (Ang II), which raises blood pressure predominantly by an endothelium-independent effect on vascular smooth muscle cells; in other words, Ang II acted as a pressor control infusate. Furthermore, since it has been proposed that altered blood flow to skeletal muscle is a determinant of insulin sensitivity, the effects of systemic L-NMMA on forearm blood flow were also assessed using venous occlusion plethysmography.

5.2 Methods

5.2.1 Subjects

Nine healthy, normotensive male volunteers aged 23-35 years participated in these randomised, double-blind, placebo-controlled studies which were approved by the Ethics Committee of the West Glasgow Hospitals University NHS Trust. No subjects were taking medication, and all abstained from alcohol, tobacco and strenuous physical activity for 24 hours and from food and caffeine-containing drinks overnight before study days. At a screening visit, physical health was confirmed by history and physical examination and supine blood pressure was measured in triplicate (Dinamap Critikon, Johnson and Johnson Professional Products Ltd). Subject characteristics (mean±SD) included body mass index (BMI)

25.5±2.4 kg/m^2, fasting glucose level 5.1±0.17 mmol/l, fasting cholesterol level 4.6±0.65 mmol/l, fasting triglyceride level 1.06±0.57 mmol/l and blood pressure 136±11/73±6 mmHg. Mean (±SD) age was 27.8±5 years; 2 subjects were smokers; median alcohol consumption was 50g/week. All subjects gave full informed consent prior to participation in the study.

5.2.2 Clinical procedures

Subjects attended on three study days, one week apart, for measurement of whole-body insulin sensitivity (M) using a 180-minute hyperinsulinaemic euglycaemic clamp (see section *2.4.1*). At 120 minutes, a further infusion was commenced of either: 1) L-NMMA (Clinalfa, 0.8 μmol/kg/min for 15 minutes, followed by vehicle for a further 45 minutes); 2) angiotensin II (ANG II, Ciba-Geigy, 5 pmol/kg/min for 60 minutes); or 3) vehicle (normal saline 1 ml/min for 60 minutes). These were administered in a randomised double-blind manner, using a double-dummy design because of the different infusion periods. L-NMMA was administered in this fashion to mimic a previous protocol in healthy volunteers in which the safety of systemic infusion was established (Haynes et al, 1993); its physiological effect is known to be long-lasting (hours) and therefore it was assumed that endothelial dysfunction would be induced throughout the whole of the third hour of the clamp procedure.

Blood pressure and heart rate were measured every 10 minutes throughout the procedure (Dinamap; Critikon Inc., Tampa, Florida, USA).

In addition blood samples were taken at 0, 60, 120 and 180 minutes for measurement of insulin, potassium, cholesterol, triglycerides (serum), plasma renin concentration and aldosterone (plasma). Blood was taken at 0 and 180 minutes for measurement of plasma angiotensin II concentration. Assay methods are described in section *2.9*.

Six subjects from the original nine attended on a fourth study day designed to assess the haemodynamic effects of a systemic L-NMMA infusion in the absence of background hyperinsulinaemia. The protocol was the same as for the previous study days except that a saline infusion was substituted for insulin and 20% dextrose ('sham clamp'); the subjects were not aware of this difference. Heart rate and blood pressure were assessed at 10 minute intervals during the second hour and at 5 minute intervals during the third hour.

In order to confirm that a systemic infusion of L-NMMA at the test dose would result in a change in forearm blood flow (FBF), FBF during systemic L-NMMA versus placebo was assessed in four volunteers of similar age and BMI to the original nine. Subjects attended on two occasions and, after a run-in period of 30 minutes to establish baseline values for limb blood flow, received a systemic infusion of either L-NMMA (12 μmol/kg over 15 minutes) or placebo (saline for 15 minutes) in a random-order, double-blind manner. Forearm blood flow in the non-infused arm was measured using venous occlusion plethysmography (see section *2.7.1.*).

5.2.3 Statistical analysis

All data were assessed for normality using the Shapiro-Wilks test. Mean blood pressure, heart rate, and insulin sensitivity (M) values in the final hour were calculated as summary measures (Matthews et al, 1990) and any differences were assessed using paired t-tests with Bonferroni correction.

Forearm blood flow and haemodynamic data as well as serum concentrations of insulin, potassium, cholesterol, triglycerides, and plasma concentrations of aldosterone, renin and angiotensin II on the different study days were compared using repeated-measures three-way ANOVA. The statistical significance of differences at individual time-points were assessed using a t-test with Bonferroni correction. All data are expressed as mean $\pm$ standard deviation.

5.3 Results

The euglycaemic clamps and systemic L-NMMA infusions were well tolerated in all subjects with no significant adverse effects.

5.3.1 Insulin sensitivity

M-value (mean±SD) was 9.9±2.52, 10.9±2.23 & 10.4±2.10 mg $kg^{-1}min^{-1}$ after placebo, Ang II or L-NMMA respectively (figure 5.1: raw data). L-NMMA infusion did not cause a significant change in M when compared with either placebo (p=0.48) or the pressor control Ang II (p=0.17). Ang II infusion tended to increase insulin

sensitivity compared with placebo (p=0.04), but this was not significant (significance level: <0.01, when corrected for multiple comparisons).

5.3.2 BP and HR

There were small but statistically significant increases in blood pressure with both L-NMMA and Ang II. Diastolic BP rose by 3.2±2.2 mmHg during L-NMMA ($p<0.01$) and by 6.0±3.8 during Ang II ($p<0.01$). Systolic BP did not change significantly with L-NMMA (+0.43±4.2) but rose by 4.6±5.8 with Ang II ($p<0.05$). MAP rose by 2.3±2.4 mmHg during L-NMMA ($p<0.05$) and by 5.6±4.2mmHg during Ang II ($p<0.01$). HR fell by 4.4±3.6 bpm during L-NMMA ($p<0.01$) and by 2.0±7.4 bpm during Ang II ($p<0.05$).

During the L-NMMA / 'sham clamp' study (n=6), MAP rose by 3.5±1.9 mmHg ($p<0.005$) which was not significantly different from the rise in MAP during the clamps (2.3±2.4 mmHg). HR fell by 3.7±3.6 bpm (p=0.05) during the 'sham clamp' study. The pattern and the extent of the haemodynamic changes in response to L-NMMA were similar when fasting euglycaemia and hyperinsulinaemic euglycaemia were compared (figure 5.2).

5.3.3 Forearm blood flow

Systemic L-NMMA infusion caused significant forearm vasoconstriction when compared with placebo at 40, 50 and 60 minutes after commencing the infusion (figure 5.3).

5.3.4 Serum insulin, potassium, cholesterol and triglycerides

Values obtained on the three study days were not significantly different at any of the time-points (figure 5.4). Insulin results [time-points 0, 60, 120 and 180 minutes (μUml^{-1}): 10.2±4.4, 116.2±22.1, 125.1±27.1, 136.3±26.2 (placebo); 13.0±13.7, 107.8±20.8, 112.2±11.2, 127.4±21.5 (Ang II), 8.4±4.4, 119.4±23.1, 120.6±18.5, 137.0±18.0 (L-NMMA) (figure 5.5).

5.3.5 Plasma aldosterone, angiotensin II, and renin concentrations

As expected, values at 180 minutes from the days on which subjects received Ang II infusions were significantly higher for Ang II concentration and aldosterone concentration ($p<0.001$); there was a decrease in plasma renin concentration after Ang II, which failed to reach statistical significance. L-NMMA had no significant effect on concentrations of these hormones (table 5.1).

Figure 5.1
Individual values for whole-body insulin sensitivity (M) in nine healthy male volunteers on three separate occasions, who received a systemic infusion in the final hour of (a) placebo (b) angiotensin II (c) L-NMMA.

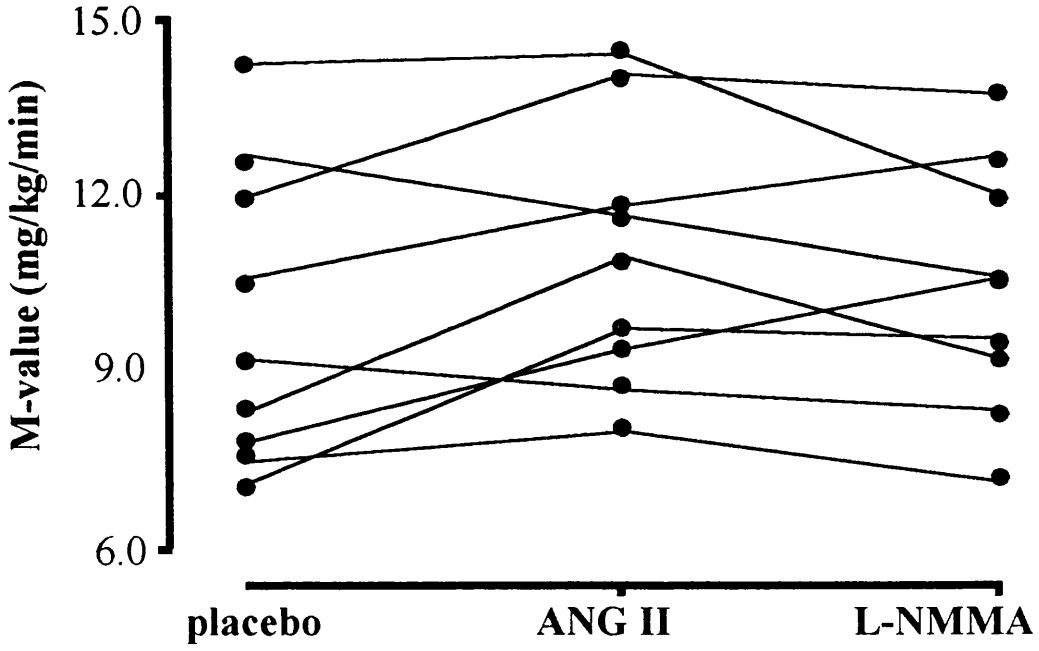


Figure 5.2
Time course of blood pressure and heart rate responses to systemic L-NMMA infusion (mean ± S.D.) in six healthy male volunteers (a) during clamp (b) fasting conditions.

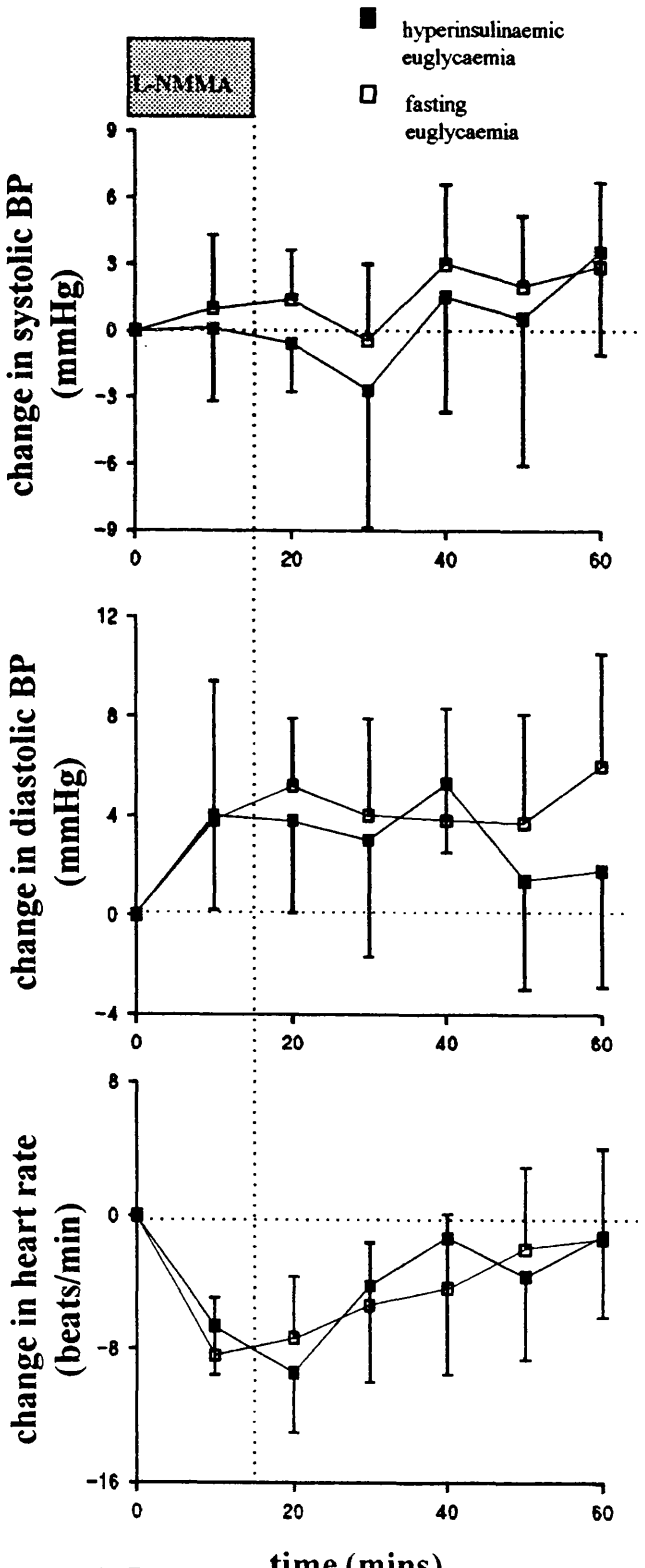
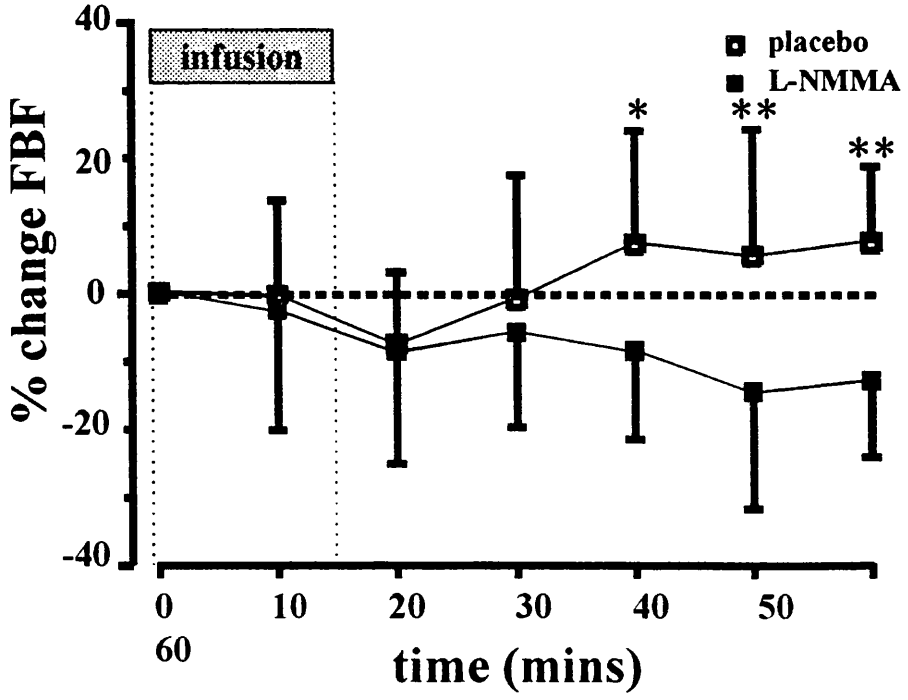


Figure 5.3
Time course of changes in absolute forearm blood flow (mean ± S.D.) in response to 15 minute systemic infusions of either placebo or L-NMMA (3mg/kg). * $p < 0.05$; ** $p < 0.01$



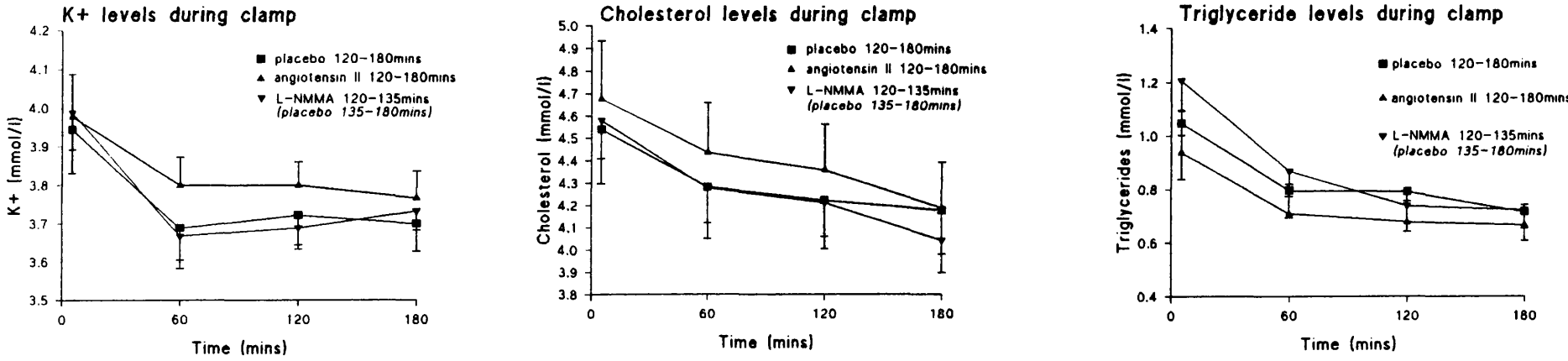
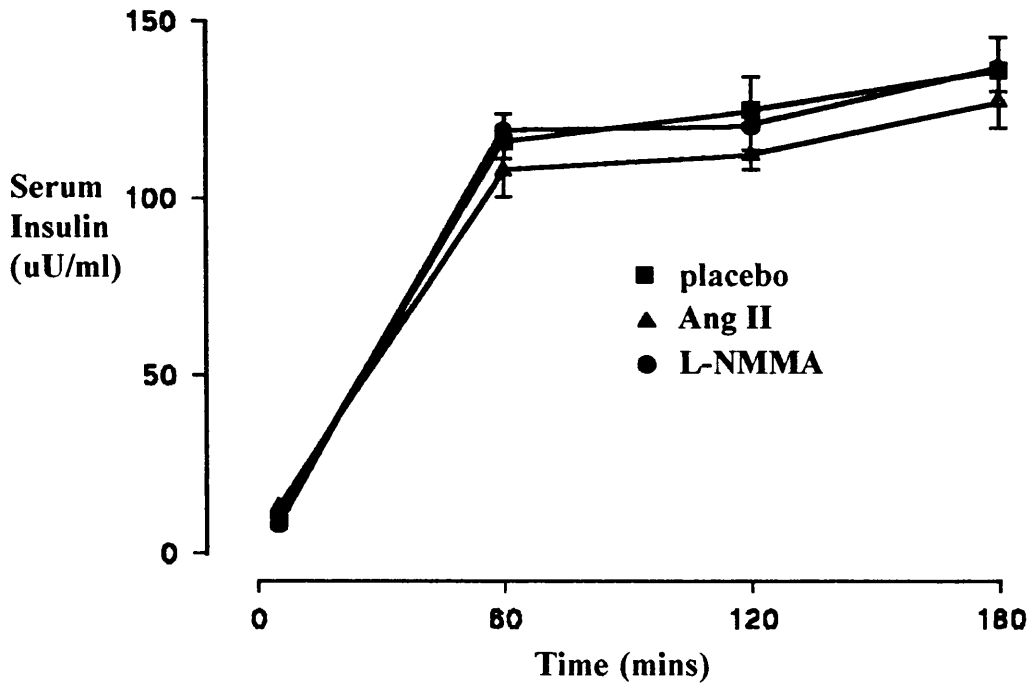


Figure 5.4
Time course of potassium, cholesterol and triglyceride levels (mean ± S.D.) during clamps in nine healthy volunteers. Systemic infusions of placebo, angiotensin II or L-NMMA were administered during the final hour.

Figure 5.5
Serum insulin levels (mean ± S.D.) during 180 minute euglycaemic clamps in nine healthy male volunteers. During the final hour systemic infusions were given of (a) placebo (b) angiotensin II (c) L-NMMA.



Variables	Time (mins) 0	60	120	180
Angiotensin II (pmol/l)				
Placebo	11.8±9.6			16.8±11.5
L-NMMA	10.5±10.1			14.4±10.9
ANG II	12.5±14.4			90.4±33.9*
Aldosterone (pmol/l)				
Placebo	372±118	211±61	233±158	216±118
L-NMMA	445±179	233±92	226±81	327±191
ANG II	405±118	251±167	246±86	765±178*
Renin (uU/ml)				
Placebo	34.0±19.1	32.8±14.7	33.5±15.8	29.6±10.2
L-NMMA	26.4±8.4	29.5±7.9	32.4±11.8	27.2±8.2
ANG II	20.7±11.7	23.7±10.0	26.0±5.3	13.4±5.5

Values given are Mean±S.D. *Indicates a significant change in these variables following ANG II infusion (by analysis of variance). Infusion of L-NMMA had no significant effect on these variables.

Table 5.1 Plasma aldosterone, angiotensin II and renin concentrations dur euglycaemic clamps.

5.4 Discussion

These results demonstrate that systemic inhibition of endothelial nitric oxide synthesis by acute infusion of L-NMMA (12 umol/kg over 15 mins) in healthy subjects is not associated with a reduction in whole-body insulin sensitivity as determined by the euglycaemic clamp technique. A systemic infusion of angiotensin II in a dose sufficient to cause a similar rise in blood pressure resulted in a small but non-significant increase in insulin sensitivity, consistent with previous results from our group in healthy volunteers (Morris et al, 1993). Furthermore, when the M-values are compared for L-NMMA and Ang II, there is no significant difference. Thus, despite attempting to control for haemodynamic influences on insulin sensitivity, it was not possible to demonstrate that systemic inhibition of endothelial nitric oxide synthesis, which is confirmed by a rise in blood pressure, impairs insulin sensitivity. In addition, it has been shown that systemic L-NMMA infusion causes similar changes in blood pressure and heart rate under hyperinsulinaemic and basal conditions, and that the increase in blood pressure is associated with significant forearm vasoconstriction. These haemodynamic changes during the infusion of L-NMMA are consistent with previously published results (Haynes et al, 1993). It was decided not to combine measurement of forearm blood flow with the clamp procedure since previous work from our group showed that hand-warming (required for 'arterialisation' of venous blood during the clamp) can, in itself, affect contralateral forearm blood flow (Petrie et al, 1996b). Furthermore, there were ethical constraints on using higher doses of L-NMMA in volunteers. Although it has not been confirmed that this dose of systemic L-NMMA induces significant endothelial dysfunction in man (for example, by demonstrating blunting of

acetylcholine-induced forearm vasodilation), it was assumed that significant endothelial dysfunction was induced as indicated by the significant increase in diastolic blood pressure.

On the basis of previous results from our group demonstrating, in a group of healthy subjects, a positive correlation between insulin sensitivity (assessed by a euglycaemic hyperinsulinaemic clamp) and basal endothelial nitric oxide production (assessed by the local vasoconstrictor response to L-NMMA in the forearm vascular bed) (Petrie et al, 1996a), the suggestion has been made that there may be a causal link between insulin resistance and endothelial dysfunction in the pathogenesis of cardiovascular disease. In the current study, it was decided to test the hypothesis that acute disruption of endothelial function would decrease insulin sensitivity. Ang II was used as a control pressure infusate in a dose designed to raise blood pressure to a similar extent without affecting vascular endothelial function. It is acknowledged that the haemodynamic effects of Ang II differed from those of L-NMMA, thus weakening its value as a pressor control infusate. However, our group had already demonstrated in a previous study that this dose of Ang II had significant haemodynamic effects without significantly affecting whole-body insulin sensitivity (Morris et al, 1993) and, therefore, this seemed a reasonable choice of agent to control for any direct effects of blood pressure changes *per se* on insulin sensitivity.

There are several areas of evidence to link insulin action with vascular endothelial nitric oxide production. Firstly, systemic hyperinsulinaemia causes peripheral vasodilation and there is evidence that this is dependent on nitric oxide production in

both the leg and forearm vascular beds (Scherrer et al, 1994a; Steinberg et al, 1994). Secondly, vasodilator responses to pharmacological doses of insulin in the human leg vascular bed are blunted in insulin-resistant states such as obesity (Laakso et al, 1990) and type 2 diabetes (Laakso et al, 1992); leg vasodilation secondary to systemic hyperinsulinaemia correlates well with whole-body insulin sensitivity (Baron et al, 1995; see chapter 4). Thirdly, there are data showing that insulin vasodilation is positively related to basal endothelial nitric oxide production (Utriainen et al, 1996a). These lines of evidence have led to the suggestion that decreased insulin sensitivity occurs as a result of decreased basal endothelial nitric oxide production, and hence decreased insulin-mediated vasodilation, with a consequent reduction in delivery of insulin and glucose to target tissues. Alternatively, insulin may, in some way, determine endothelial function, so that altered insulin sensitivity accounts for variations in endothelium-dependent vasodilation.

There is independent support for the hypothesis that endothelial dysfunction causes insulin resistance. Firstly, systemic infusion of L-NMMA induced insulin resistance in rats (Keech et al, 1993) although this was not confirmed by a separate study (Kohlman et al, 1995). Secondly, superimposing an infusion of L-NMMA into the femoral artery of healthy human subjects during a hyperinsulinaemic euglycaemic clamp resulted in reversal of insulin-mediated vasodilation and a 25% reduction in leg glucose uptake, despite a 50% increase in arterio-venous glucose extraction (Baron et al, 1995). In this latter study a significant inverse relationship was reported between whole-body insulin sensitivity and the decrease in leg glucose uptake during

intra-arterial L-NMMA, suggesting that inter-individual variation in insulin sensitivity may be partly explained by variation in insulin's ability to increase skeletal muscle blood flow (Baron et al, 1995). Thirdly, infusion of intra-arterial methacholine during hyperinsulinaemic euglycaemia increased limb blood flow and resulted in a significant increase in limb glucose uptake, leading to the conclusion that blood flow is a rate-limiting step for insulin-mediated glucose disposal (Baron et al, 1994).

However, evidence against the hypothesis that blood flow is a determinant of insulin sensitivity comes from studies in which limb blood flow during a clamp was increased by intra-arterial infusion of either adenosine (Natali et al, 1994) or bradykinin (Nuutila et al, 1996). In neither case did limb glucose uptake rise, despite a doubling of blood flow.

In the present study the hypothesis has been tested that induction of a state of relative endothelial dysfunction by systemic L-NMMA infusion would result in a decrease in insulin sensitivity. The results do not, therefore, support the notion that impairment of endothelial function is a direct cause of insulin resistance. Moreover, since it has been demonstrated that this dose of systemic L-NMMA causes significant limb vasoconstriction, it is concluded that skeletal muscle blood flow *per se* is unlikely to be a rate-limiting determinant of insulin-stimulated glucose uptake. Of course, it is conceded that using higher doses of L-NMMA for a longer period may have caused significant differences in insulin sensitivity, but ethical and safety constraints prevailed.

The discrepancy between these findings and those cited above, in which local administration of L-NMMA caused a decrease in limb glucose uptake, needs to be considered. Firstly, it cannot be assumed that infusing a vasoactive compound systemically will induce the same changes in peripheral blood flow as a local intra-arterial infusion. This has been demonstrated with Ang II: local infusion causes vasoconstriction while systemic infusion is reported to cause peripheral (limb) vasodilation (Buchanan et al, 1993). This may be due to differential effects in regional vascular beds causing re-distribution of blood flow from renal and splanchnic beds to the periphery. In addition, there is evidence from studies in rats that a bolus dose of systemic L-NMMA (12 μmol/kg) causes transient limb vasodilation, while higher doses (40 - 120 μmol/kg) cause limb vasoconstriction (Gardiner et al, 1990). However, the current study confirms in man, in the fasting state, that systemic L-NMMA (12 μmol/kg) causes significant forearm vasoconstriction. Since the pattern of blood pressure and heart rate responses during hyperinsulinaemia was identical, it was assumed that systemic L-NMMA also caused peripheral vasoconstriction in the clamp study (it was decided not to combine the two techniques for reasons stated above (Petrie et al, 1996b)). A second potential confounding factor is the effect of L-NMMA on renal haemodynamics: if L-NMMA caused significant renal vasoconstriction, reduced insulin clearance might increase insulin levels and consequent glucose disposal. However, in the current study there were no differences in insulin levels among the three study days.

Finally, central to the original hypothesis that systemic L-NMMA would decrease insulin-mediated glucose uptake was the assumption that the only effect of L-NMMA would be to attenuate insulin's stimulation of skeletal muscle blood flow via the endothelial nitric oxide pathway. There are very few published data on the metabolic effects of nitric oxide and substrate inhibitors of nitric oxide synthase. Therefore, when interpreting the results of this study, a possible non-haemodynamic role for nitric oxide in modifying insulin-mediated glucose uptake needs to be taken into account.

In summary, this study in healthy male volunteers demonstrated that whole-body insulin-mediated glucose uptake is not reduced during systemic inhibition of nitric oxide synthesis. In addition, systemic L-NMMA infusion caused significant forearm vasoconstriction. These data do not support the hypothesis that impairment of endothelial function results in insulin resistance.

Chapter 6

Skeletal muscle capillary density is unrelated to insulin's vasodilator action

6.0 Summary

Skeletal muscle fibre type is known to be associated with insulin sensitivity. Since fibre type is also related to capillary density, this study set out to test the hypothesis that insulin's vasodilator action is linked with muscle capillary density, which would support the notion that skeletal muscle structure and function may be a significant 'third factor' accounting for the observed association between insulin's metabolic and vascular actions. Following immunohistochemical staining of sections of muscle biopsy samples from 13 healthy volunteers in whom insulin sensitivity and local insulin/glucose-mediated vasodilation had already been measured, it was concluded that the quantity of capillary endothelium in muscle is unlikely to be a significant determinant of insulin's vascular action.

6.1 Introduction

Associations have been noted between skeletal muscle fibre types and both metabolic and cardiovascular disorders. Subjects with obesity (Lilloja et al, 1987; Wade et al, 1990; Simoneau & Bouchard, 1993), central obesity (Lilloja et al, 1987; Krotkiewski et al, 1986), hypertension (Juhlin-Dannfelt et al, 1979; Frisk-Holmberg et al, 1983) and type 2 diabetes (Lilloja et al, 1987) have a predominance of type IIB glycolytic, fast-twitch fibres and a relative lack of type I oxidative, slow-twitch fibres, which is associated with reduced capillary density. Given that there appears to

be increased basal blood flow in skeletal muscle comprising predominantly type I fibres (Frisk-Holmberg et al, 1981), it may be possible that insulin-induced changes in muscle blood flow are determined by capillary density and that muscle histology may be an underlying 'third factor' explaining the association between insulin's metabolic and vascular actions.

Using the same subjects as in chapter 4 (n=18), in whom a significant association was demonstrated between whole-body insulin-mediated glucose uptake and local insulin/glucose-mediated vasodilation, this hypothesis was tested by obtaining samples of skeletal muscle for immunohistochemical processing.

6.2 Methods

6.2.1 Subjects

Eighteen healthy, normotensive male volunteers aged 18-37 years participated in this study which was approved by the Ethics Committee of the West Glasgow Hospitals University NHS Trust. No subjects were taking medication, and all abstained from alcohol, tobacco and strenuous physical activity for 24 hours and from food and caffeine-containing drinks overnight before attending for muscle biopsy. At a screening visit, physical health was confirmed by history and physical examination and supine blood pressure was measured in triplicate (Dinamap Critikon, Johnson and Johnson Professional Products Ltd).

6.2.2 Clinical procedures

Subjects had previously attended (chapter 4) for measurement of whole-body insulin sensitivity (M) using a 180-minute hyperinsulinaemic euglycaemic clamp (section *2.4.1*) and measurement of local insulin/glucose-mediated vasoreactivity by bilateral venous occlusion forearm plethysmography (section *2.7.1*).

The procedure for vastus lateralis muscle biopsy is described in detail in section *2.8.1*. It has been demonstrated that the proportions of fibre types in this muscle are similar to those found in muscles of the forearm (Johnson et al, 1973).

6.2.3 Measurement of fibre type and capillary density

Once obtained, muscle biopsy specimens were immediately frozen in liquid nitrogen to allow the process of frozen sectioning (section *2.8.2*). Muscle fibre type was established by use of immunohistochemical techniques, utilising commercially-available antibodies to fast and slow myosin (section *2.8.3*). Capillary density was quantified in terms of an area ratio by immunostaining for CD45, an antigen which is selectively expressed on vascular endothelium. Direct visualisation was possible using rabbit anti-mouse alkaline phosphatase (RAM AP) allowing direct red staining. Area ratio was calculated in two good-quality cross-sections by an image analysis camera as described in section *2.8.3*.

6.3 Results

The procedure of muscle biopsy was tolerated well with no serious adverse events. Quantification of both fibre type and capillary density was only possible in 13 subjects for technical reasons (mainly inadequate quality of sample obtained by biopsy). Subject characteristics were as follows: age 26.5±5.6 yrs, BMI 24.0±2.8 kg/m^2.

6.3.1 Muscle fibre type

Type I fibres made up 45.8±9.2% (mean±SD) [range 29-63%] of the cross-sectional area, which is consistent with previous studies (Lilloja et al, 1987; Utriainen et al, 1996) where different techniques were used.

Type IIb fibres accounted for 46.1±5.7% (range 37-56%), and type IIa for 8.1±5.6% (range 0-17%) of the cross-sectional area. These proportions differ from previous studies (IIb 27%, IIa 27% [Lilloja et al, 1987]; IIb 16%, IIa 32% [Utriainen et al, 1996c]). Whether the current results reflect differences in technique for fibre staining or differences in subject population is unclear. Type I fibre area, which appears to be the most robust muscle fibre parameter, was used in subsequent correlation analyses.

6.3.2 Capillary density

Percent cross-sectional area comprising vascular endothelial cells was 5.1±1.8% (range 3.0-9.6%).

6.3.3 Insulin sensitivity (M)

M-value (n=13) was 9.87±2.4 mg/kg/min.

6.3.4 Local insulin/glucose-mediated vasodilation (IGMV)

Percent change in forearm blood flow ratio (n=13) was 33.7% (13.4, 42.9) [median (IQ range)].

6.3.5 Univariate analysis

There was a trend for % type I area to be positively related to capillary density ($r = 0.52$, $p < 0.1$), and for an inverse relationship between % type IIb area and capillary density ($r = -0.49$, $p < 0.1$) which would be expected in light of previous results (figure 6.1).

Contrary to the primary hypothesis that IGMV would be positively related with capillary density, there was no association ($r = -0.04$, $p = 0.9$, figure 6.2).

Likewise, there were no significant associations between M and either % type I area ($r = -0.002$) or capillary density ($r = -0.01$) or between IGMV and % type I area ($r = -0.02$).

Figure 6.1
(a) Percentage area of type I fibres and (b) percentage area of type IIb fibres, plotted against capillary density (percentage area of endothelial cells), in skeletal muscle biopsy cross-sections from 13 healthy male volunteers.

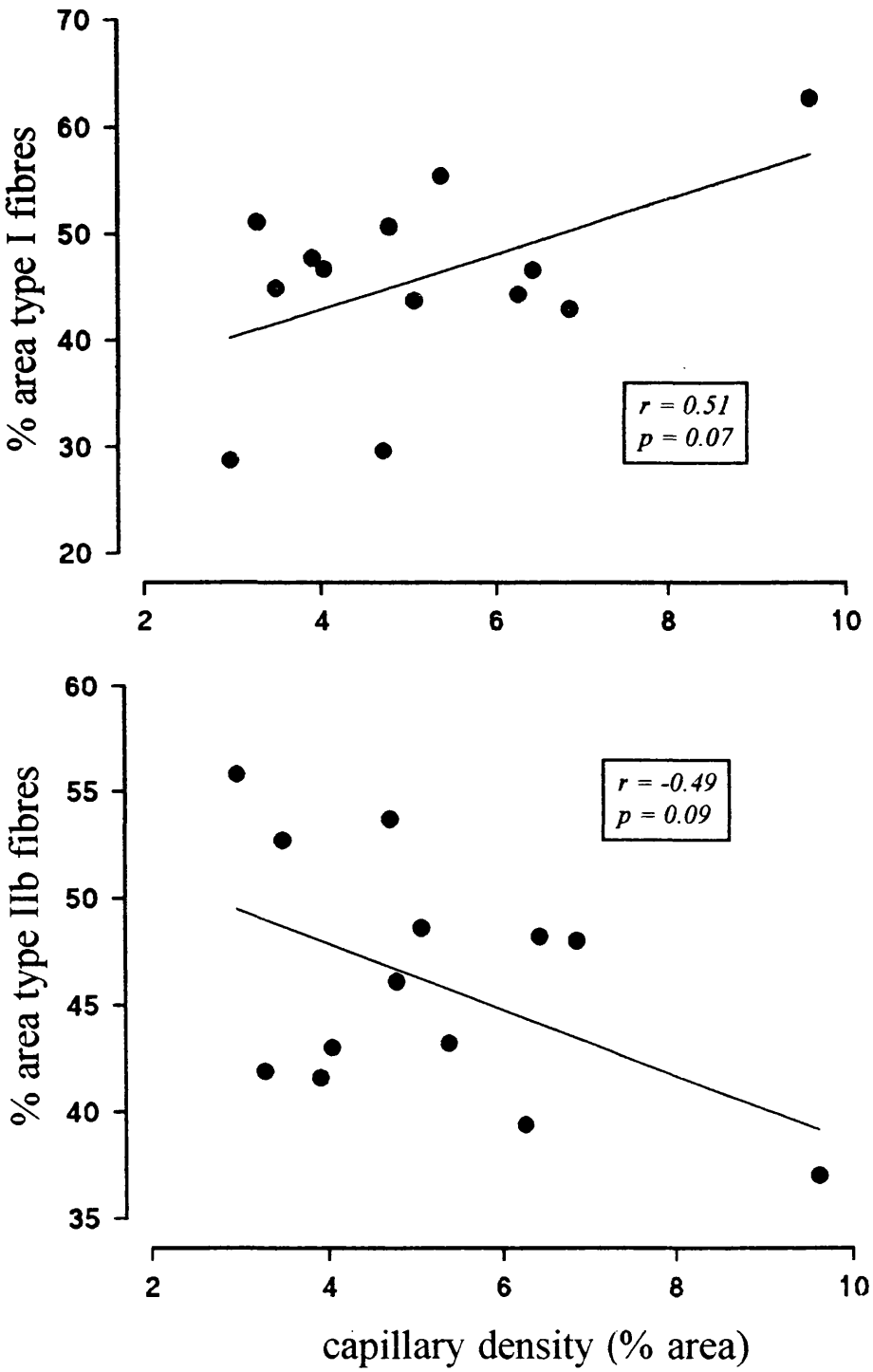
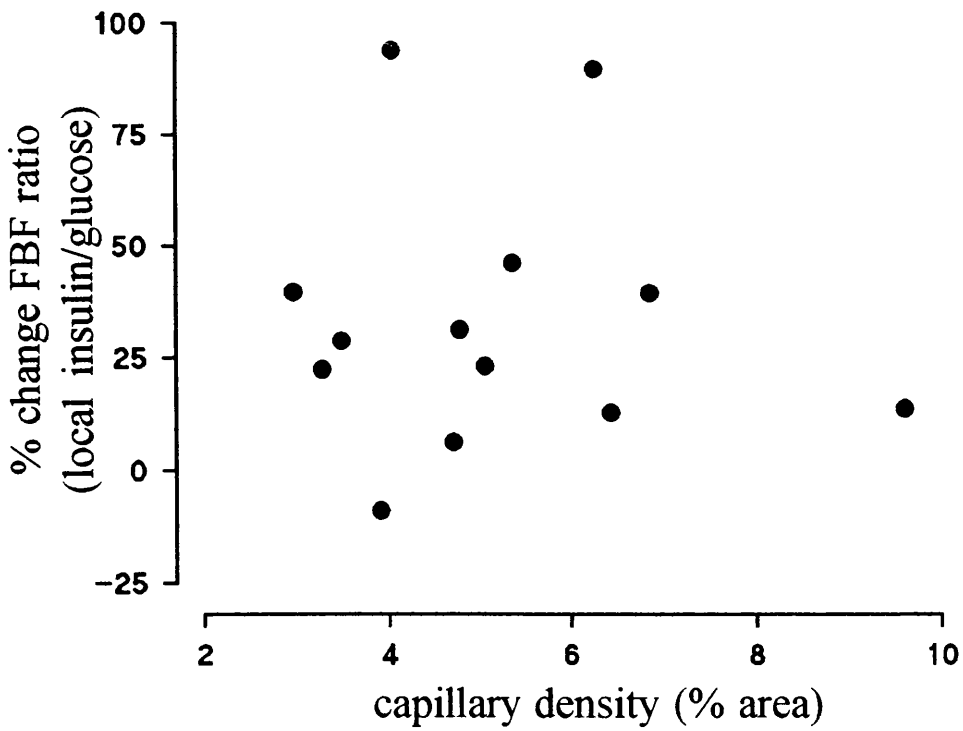


Figure 6.2
Local (forearm) insulin/glucose-mediated vasoreactivity plotted against capillary density (percentage area of endothelial cells) in skeletal muscle biopsy cross-sections from 13 healthy male volunteers.



6.4 Discussion

Insulin is, in large part, an endothelium-dependent vasodilator in skeletal muscle vascular beds (Steinberg et al, 1994; Scherrer et al, 1994) and has been shown to stimulate endothelial nitric oxide production in number of experimental studies (Wu H-Y et al, 1994; Chen & Messina, 1996; Zeng & Quon, 1996). However, even in healthy individuals there is a fivefold variation in insulin-mediated vasodilation (Utriainen et al, 1995; chapter 4) which is much greater than the expected variation in studies measuring either basal or stimulated endothelial function. This finding has led to interest in factors which might contribute to this variation and has generated the current study hypothesis - that quantitative differences in vascular endothelium in skeletal muscle might contribute to insulin's vasoactive effectiveness.

One previous study has addressed the issue of insulin's vascular action with reference to skeletal muscle histology (Utriainen et al, 1996c). The investigators studied sixteen healthy male volunteers and found that forearm fractional extraction of glucose in response to systemic hyperinsulinaemia was related directly to % type I fibres and inversely to % type IIb fibres. They also demonstrated that muscle capillary density was strongly related to forearm vasodilation induced by systemic hyperinsulinaemia. Taken together, these results imply that inherent properties of skeletal muscle may partly explain the observed association between insulin's metabolic and vascular actions (Feldman et al, 1995; Baron et al, 1995; Chapter 4 & 7). The results of the current study do not support these findings, although there are technical differences both in the method of insulin/glucose administration and of determining capillary density which may partly explain the different conclusions.

It has been established in a number of previous studies that conditions associated with insulin resistance (obesity, type 2 diabetes and hypertension) tend also to be associated with a certain muscle phenotype comprising predominance of type IIb glycolytic fibres and reduction in capillary density (Lilloja et al, 1987; Wade et al, 1990; Juhlin-Dannfelt et al, 1979; Frisk-Holmberg et al, 1983). The reasons for this are not clear. It has been suggested that a common factor might be lack of physical fitness since chronic aerobic exercise training has been shown to result in a shift from type IIB to type IIA (oxidative) fibres and also an increase in muscle capillary density (Anderson et al, 1977a; Anderson et al, 1977b; Krotkiewski et al, 1983), as well as improvements in insulin sensitivity (Erikkson et al, 1997) and blood pressure (Halbert et al, 1997). Alternatively, differences in muscle corticosteroid metabolism may be of relevance since excess of corticosteroids, both endogenous (Rebuffe-Scrive et al, 1988) and exogenous (Danneskjold-Samsoe & Grimby, 1986), has been shown to produce a similar histological picture in muscle, as well as being associated with components of the metabolic syndrome (Walker et al, 1996). Results from studies in rats have suggested that insulin itself may play a role in determining fibre type and capillary density (Holmang et al, 1993) - it is possible, therefore, that the muscle phenotype is a consequence of metabolic abnormalities.

In conclusion, the notion that quantitative differences in skeletal muscle vascular endothelium are important in determining vascular reactivity to insulin is not supported by the data in the current study. Thus, while differences in fibre type may, in part, determine insulin-mediated glucose uptake in muscle, it is more likely that

qualitative, not quantitative, differences in vascular endothelial function contribute to the large biological variability inherent in insulin's vasodilator action.

Chapter 7

Insulin action and endothelial function in type 2 diabetes and essential hypertension

7.0 Summary

Twenty seven men participated in this clinical physiology study (9 controls, 9 hypertensives and 9 type 2 diabetics) which was designed to characterise insulin action and basal endothelial function. There was a trend for reduced insulin-mediated vasodilation in type 2 diabetic patients although statistical significance was not achieved due to high biological variability. Pooled analysis revealed a significant association between (1) insulin's metabolic and vascular actions, (2) insulin sensitivity and basal endothelial NO production, and (3) insulin-mediated vasodilation and basal endothelial NO production. Multiple regression analysis with basal endothelial NO production as the dependent variable revealed that a model including insulin-mediated vasodilation and age accounted for over 40% of the variance. The fact that insulin sensitivity did not persist as an independent predictor is supportive of the hypothesis that insulin-stimulated endothelial NO production is a key intermediate mechanism linking insulin-mediated glucose uptake with basal endothelial function.

7.1 Introduction

Reduced insulin-mediated glucose uptake and endothelial dysfunction are shared features of both metabolic (type 2 diabetes and obesity) and cardiovascular (essential hypertension and heart failure) disorders (see introduction to chapter 4). As previously discussed, insulin's predominant vascular action is considered to be dependent on an increase in endothelial NO production (chapter 2) and it is possible that this, in turn, is dependent on insulin-mediated cellular glucose uptake (see section *1.4.1*) which may account for the observed physiological association between insulin's metabolic and vascular actions (chapter 4).

Based on this evidence, one would expect patients with disorders characterised by insulin resistance (such as type 2 diabetes or essential hypertension) to exhibit blunting of vascular responsiveness to insulin with associated reductions in basal endothelial NO production. Therefore, the primary objective of this study was to characterise insulin action and endothelial function in three groups of subjects - essential hypertensives, type 2 diabetics and healthy controls - carefully matched for obvious confounding factors such as age and body mass index.

The secondary objective of this study was to search for possible 'third factors' which might be contributing to defects in both insulin action and endothelial function. The most obvious candidates (as discussed in section 1.6.2) are lipid components and these were characterised in detail in the following study.

7.2 Methods

7.2.1 Subjects

Twenty seven male volunteers participated in this study which was approved by the Ethics Committee of the West Glasgow Hospitals University NHS Trust. No subjects were taking medication during the study, and all abstained from alcohol, tobacco and strenuous physical activity for 24 hours and from food and caffeine-containing drinks overnight before the three study days, which were at least one week apart.

Nine healthy control volunteers, recruited by advertisement, attended a screening visit at which physical health was confirmed by history, physical examination, routine biochemical and haematological blood tests and ECG. Nine patients with uncomplicated diet-controlled type 2 diabetes were recruited via the Diabetes Centre, Gartnavel General Hospital. Nine patients with essential hypertension were recruited from the Glasgow Blood Pressure Clinic, Western Infirmary. Patients were included according to the recruitment criteria detailed in section *2.1.* All subjects gave informed consent. Six subjects were smokers (3 diabetics, 2 hypertensives, 1 control). None of the subjects consumed more than the recommended weekly alcohol intake (280g). Details of age, BMI, BP and fasting glucose are displayed in figure 1.

7.2.2 Clinical procedures

Subjects attended for measurement of whole-body insulin sensitivity using a 180-minute hyperinsulinaemic isoglycaemic clamp as described in section *2.4.1.* with

concurrent measurement of hepatic glucose output using the isotope tracer technique (section *2.5.1*). To adjust for differences in fasting glucose concentrations, insulin sensitivity is expressed as the metabolic clearance ratio of glucose (MCR; ml/kg/min).

On two further study days, subjects attended for measurement of forearm blood flow by bilateral venous occlusion plethysmography, as described in section *2.7.1*. On one day, they received an intra-arterial infusion of insulin (5mU/min) and D-glucose (75μmol/min) for 96 mins, with concurrent infusion of L-NMMA (1mg/min) for the final 24 mins. Forearm blood flow was measured every 8 mins throughout; the mean of three readings (56-72 mins) was used as a summary measure for insulin vasodilation. On a separate day, they received an intra-arterial infusion of noradrenaline (50ng/min) for 24 mins followed by a 16 minute washout period and then L-NMMA (1mg/min) for a further 24 mins. Again, forearm blood flow was measured every 8 mins throughout, and the final measurement for each infusate was used in subsequent analysis.

In addition, fasting blood samples were processed for measurement of lipid profile and markers of endothelial activation, as described in section *2.9.8* and *2.9.9*.

7.2.3 Statistical Evaluation

To compare results among the three groups, summary measures were calculated where appropriate, and unpaired t-tests were used. Data from all three groups were pooled for correlation and multiple regression analyses, after ensuring that the

associations under examination were not influenced by either blood pressure or glucose levels which could result in clustering of patient groups and misleading results.

7.3 Results

The procedures were carried out without complication and were well tolerated by all subjects. All results are summarised as mean ± standard error unless otherwise stated.

7.3.1 Insulin sensitivity

MCR (ml/kg/min) was 7.22±0.99 (controls), 6.32±0.78 (hypertensives) and 5.06±0.53 (diabetics). This trend is as expected although there were no significant differences between either patient group and the control group due to the high inter-individual variability and relatively small numbers. Raw data are displayed in figure 7.2. Insulin levels achieved during the clamp studies were not different among groups, reflecting tight BMI matching (figure 7.3).

7.3.2 Hepatic glucose output during systemic hyperinsulinaemia

Data are available on 25 subjects (data not obtained on two for technical reasons). In general, results obtained are physiologically plausible, since estimates of basal hepatic glucose production are markedly suppressed during hyperinsulinaemia in the majority of cases (raw data displayed in figure 7.4). However, one would predict that insulin-mediated suppression of hepatic glucose output would be associated with whole-body insulin sensitivity, but this is not the case ($r = 0.12$, figure 7.5a). In

addition, one might expect estimates of basal hepatic glucose output to be related to fasting variables such as glucose, triglycerides and VLDL-cholesterol, but there are no associations, although clamp hepatic glucose output does correlate with fasting glucose in the diabetic sub-group ($r = 0.83$, $p = 0.01$, figure 7.5b). Originally, the intention was simply to add estimates of hepatic glucose production (during clamp) to whole-body insulin sensitivity to obtain estimates of peripheral insulin sensitivity, thought to be a more relevant parameter for comparison with insulin's vascular effects in skeletal muscle. However, in view of the uncertainty about the physiological meaning of the data, it was decided to use the metabolic clearance rate of glucose (section *2.4.2*) as the measurement of 'insulin sensitivity' for use in further analyses.

7.3.3 Local insulin/glucose-mediated vasodilation

Percentage change in forearm blood flow ratio was 17.1±5.6% (controls), 17.2±5.5% (hypertensives) and 12.3±6.4% (diabetics). Raw data for summary measures are displayed in figure 7.6. Again, due to high variability within each group and relatively small numbers, there were no significant differences between either patient group and the control group. The time-course of vasodilation is plotted in figure 7.7. Even if the area under the curve is calculated for controls and diabetics, there is no significant difference between groups ($p = 0.34$).

7.3.4 Insulin sensitivity *v* insulin vasodilation

When data from the three groups are pooled and plotted (figure 7.8a) there is no clustering of patient groups. This allows justification of pooled correlation analysis (figure 7.8b) which demonstrates a significant positive association ($r = 0.46$, $p < 0.05$) between insulin's metabolic and vascular effects which persists independently of either hyperglycaemia or high blood pressure. This relationship is in keeping with the results obtained in chapter 4 in younger, leaner healthy volunteers.

7.3.5 Vasoreactivity to L-NMMA and noradrenaline

Percent vasoconstriction to L-NMMA was 37.9±5.1% (controls), 37.5±2.3% (hypertensives) and 33.6±2.8% (diabetics). Raw data are displayed in figure 7.9, demonstrating the large biological variability, especially in the control group. There were no significant differences among groups for L-NMMA vasoconstriction.

Percent vasoconstriction to noradrenaline was 20.9±6.2% (controls), 21.9±5.6% (hypertensives) and 29.4±5.6% (diabetics). Again, there were no significant differences among groups.

7.3.6 L-NMMA vasoconstriction - fasting *v* hyperinsulinaemia

On a background of hyperinsulinaemia, percentage changes in FBF ratio in response to L-NMMA can be expressed in two ways: firstly, with reference to baseline (prior to insulin/glucose infusion): 25.3±6.0% (controls), 20.8±5.5% (hypertensives) and 26.6±8.4% (diabetics); secondly, with reference to achieved vasoreactivity to

insulin/glucose: 35.6±4.5% (controls), 30.1±3.1% (hypertensives) and 35.0±6.5% (diabetics). In neither case are there any significant differences among groups.

Taking the latter values and pooling data to look at the time-course of vasoconstriction, it is clear that the vascular response to L-NMMA is delayed and steady-state has still not been achieved at 24 mins, while it appears that L-NMMA infused in fasting conditions achieves near-steady-state vasoconstriction within the first 10 mins (figure 7.10).

7.3.7 Insulin sensitivity *v* L-NMMA vasoconstriction

Pooled correlation analysis (n=27) reveals that insulin sensitivity (MCR) is significantly positively related to % L-NMMA vasoconstriction (surrogate for basal endothelial NO production) ($r = 0.44$, $p < 0.05$) but not to % noradrenaline vasoconstriction ($r = -0.35$) (figure 7.11).

7.3.8 Insulin vasodilation *v* L-NMMA vasoconstriction

Pooled correlation analysis (n=27) reveals that insulin/glucose-mediated vasodilation is significantly positively related to % L-NMMA vasoconstriction (surrogate for basal endothelial NO production) ($r = 0.52$, $p < 0.01$) but not to % noradrenaline vasoconstriction ($r = -0.15$) (figure 7.12).

7.3.9 Fasting lipids

Figure 7.13 displays data for total cholesterol, triglycerides, LDL-cholesterol and HDL-cholesterol for all three groups. There were no significant differences among

groups for any of the measured lipid parameters. In addition, VLDL-cholesterol levels (mmol/l) were 0.73±0.15 (controls), 0.53±0.11 (hypertensives) and 0.85±0.18 (diabetics).

Of all the lipid parameters, HDL-cholesterol level was most closely related to both BMI ($r = -0.48$, $p < 0.05$) and insulin sensitivity ($r = 0.44$, $p < 0.05$) (figure 7.14).

7.3.10 Markers of endothelial activation

Pooled data: TPA 11.2±2.9 ng/ml; FVIIc 132.9±26.8 % activity; fibrinogen 3.16±0.8 g/l; vWF 121.7±44.5 IU/dl (mean±SD); D-dimers 41 [23, 68] (median & IQ range). There were no differences among the three groups for these variables (one-way ANOVA). Table 7.1 displays simple correlation co-efficient values for each marker against (1) L-NMMA vasoconstriction (2) MCR (3) insulin/glucose-mediated vasodilation. None of the correlations reached statistical significance.

7.3.11 Multiple regression analysis

Three dependent variables were studied separately: insulin sensitivity, insulin vasodilation and L-NMMA vasoconstriction. The following additional factors were entered into the initial models: age, BMI, diastolic BP, fasting glucose, triglycerides and vWF. Minitab's 'Best Subsets' analysis was used to identify the best model to predict each dependent variable.

With insulin sensitivity as dependent variable, a model including BMI, insulin vasodilation, triglycerides and age explained 59% (adj R^2) of the variance.

With insulin vasodilation as dependent variable, a model including L-NMMA vasoconstriction, insulin sensitivity and age explained 42% (adj R^2) of the variation.

With L-NMMA vasoconstriction as dependent variable, a model including insulin vasodilation and age explained 41% (adj R^2) of the variance.

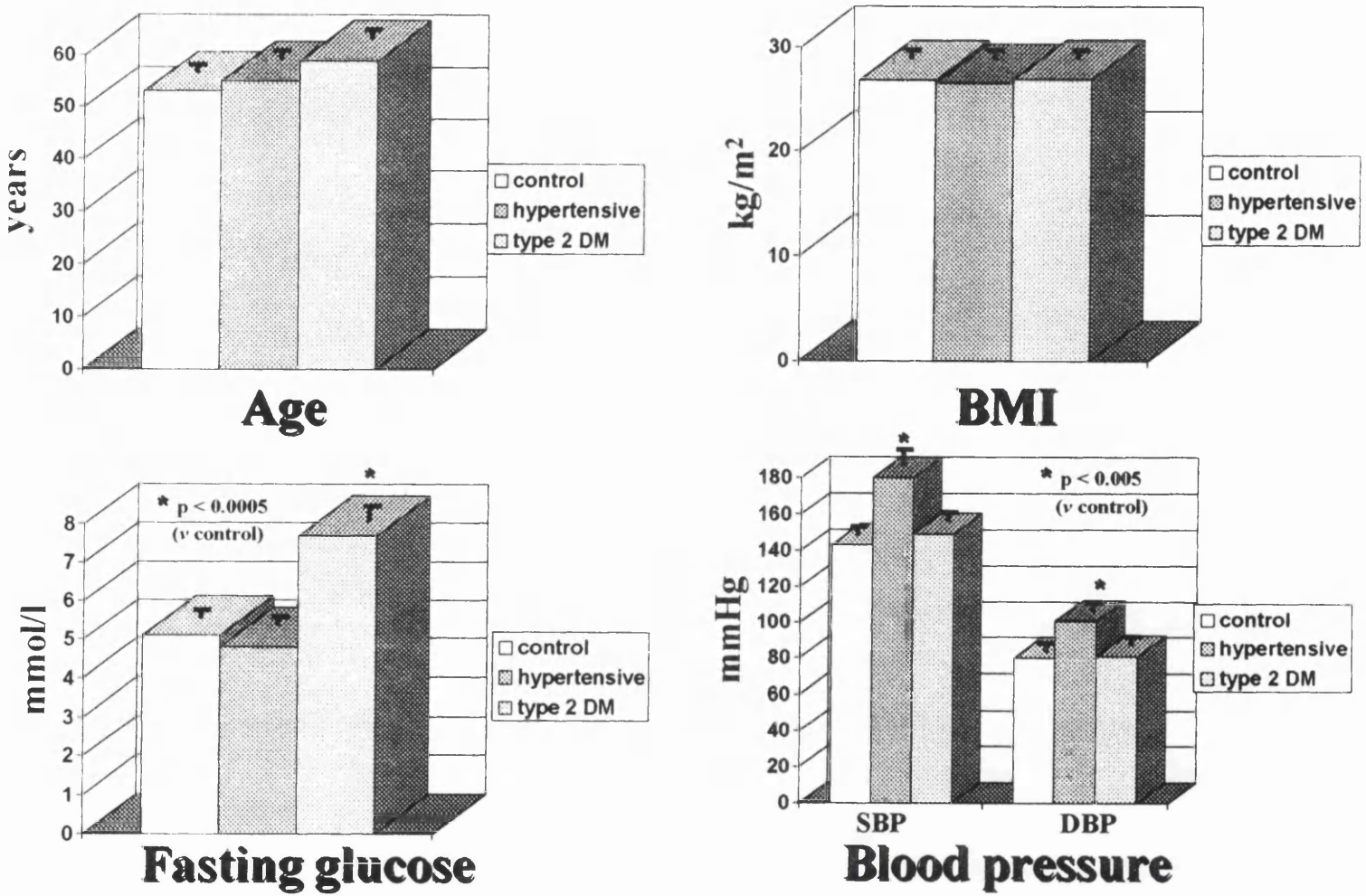


Figure 7.1
Subject characteristics (mean ± S.E.) in healthy controls (n=9), and patients with essential hypertension (n=9) or type 2 diabetes (n=9).

Figure 7.2
Individual values for whole-body insulin sensitivity (mean clearance rate of glucose: MCR) in healthy controls (n=9), and patients with essential hypertension (n=9) or type 2 diabetes (n=9). Group means also displayed.

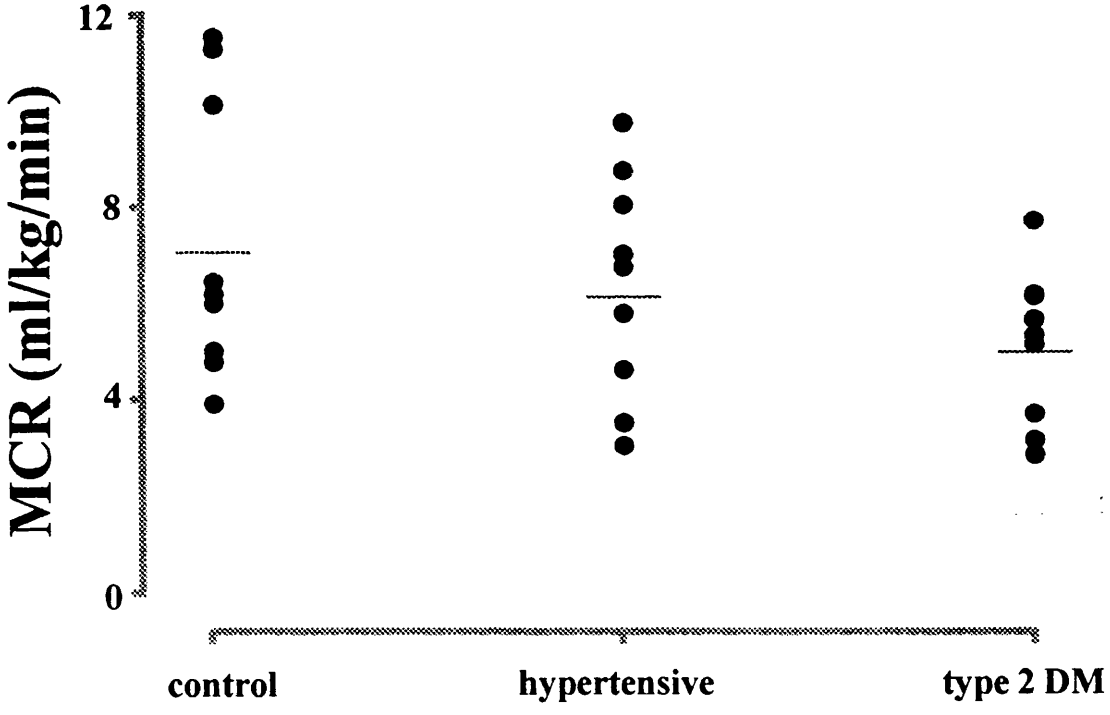
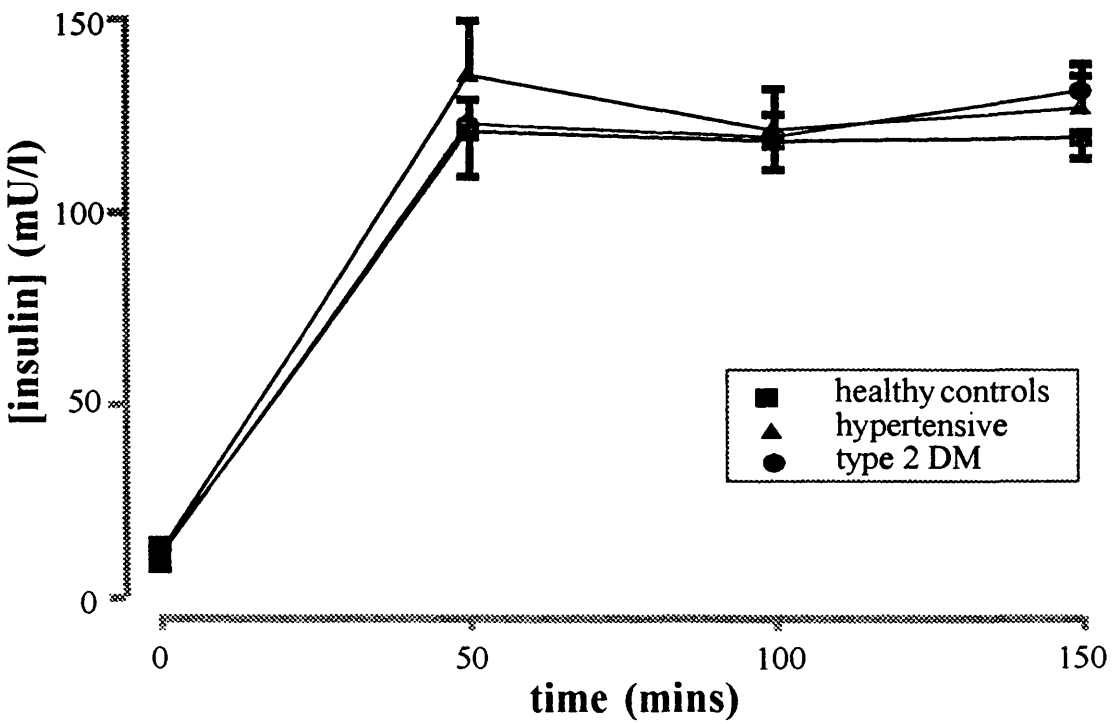


Figure 7.3
Serum insulin levels (mean ± S.E., mU/l) during 150-minute isoglycaemic clamp.



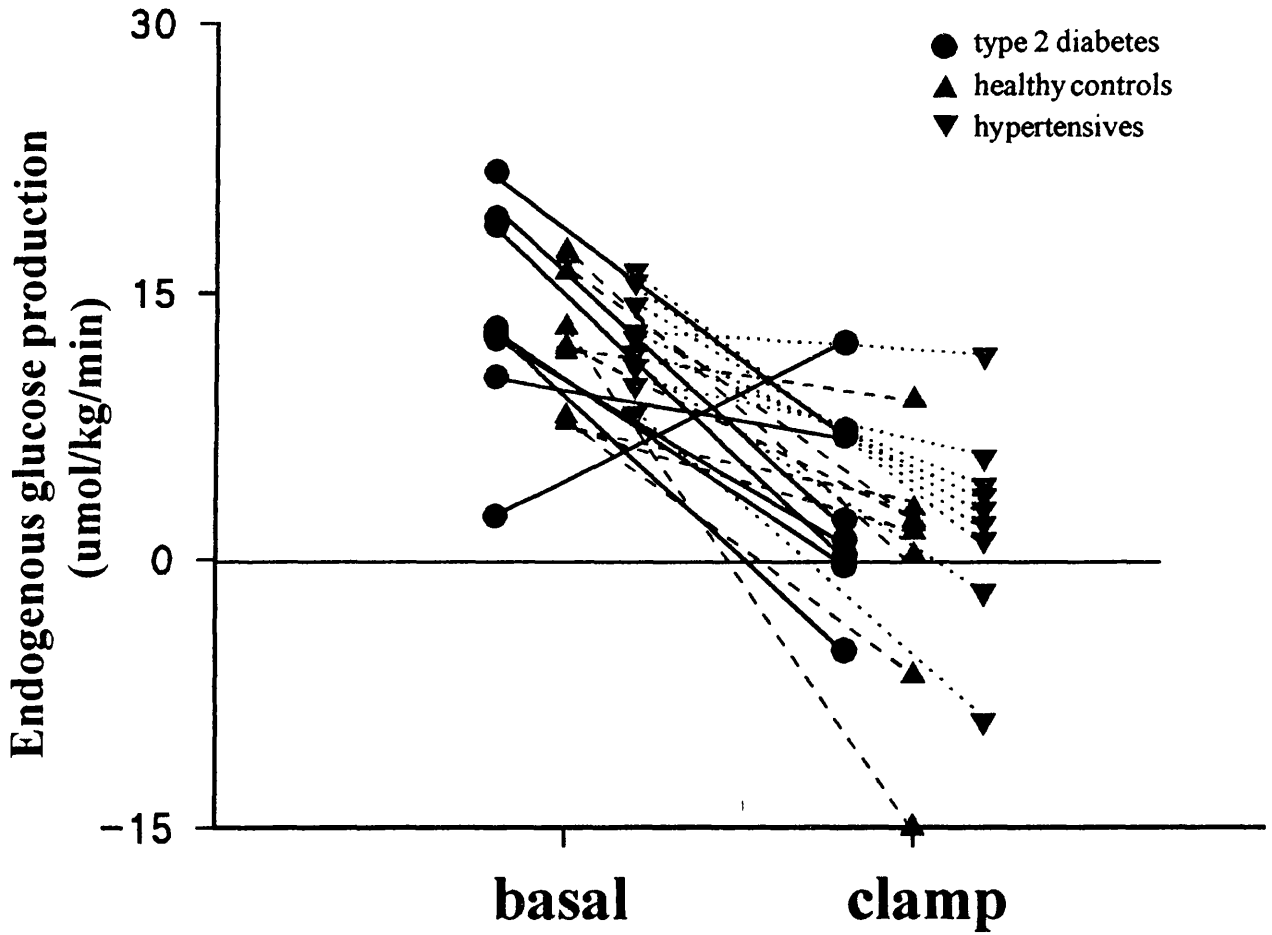


Figure 7.4
Individual values for hepatic glucose production (umol/kg/min) measured by the isotope tracer technique in both basal and hyperinsulinaemic conditions. Values are displayed for healthy control, hypertensive and type 2 diabetic subjects (n=25).

Figure 7.5
Endogenous (hepatic) glucose production (mg/kg/min) during hyperinsulinaemia plotted against (a) whole-body insulin sensitivity (M-value) in 25 subjects (b) fasting plasma [glucose] in eight patients with type 2 diabetes.

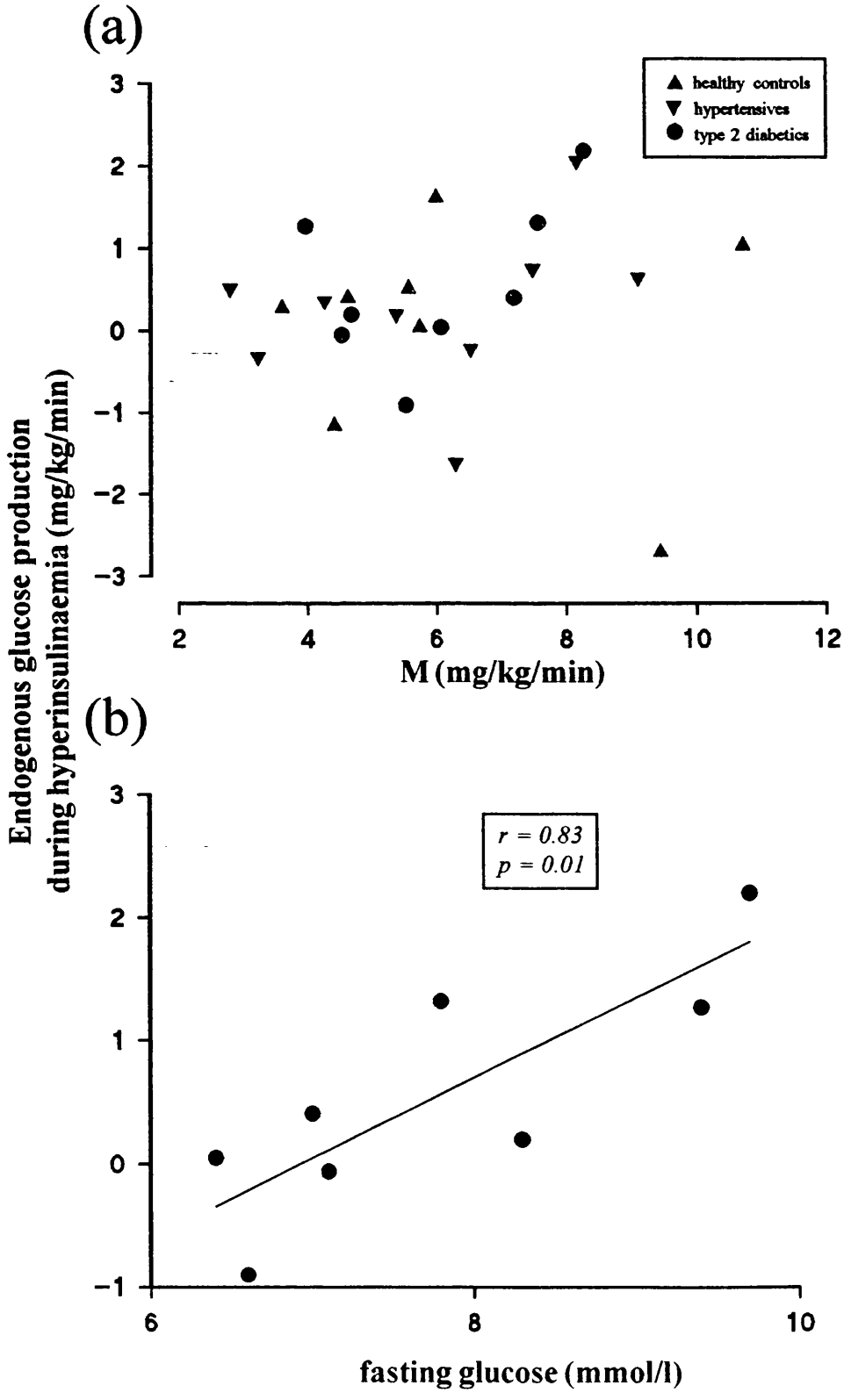


Figure 7.6
Individual values for percentage change in forearm blood flow ratio in response to intra-arterial infusion of insulin and D-glucose in healthy controls (n=9), and patients with essential hypertension (n=9) or type 2 diabetes (n=9). Group means also displayed.

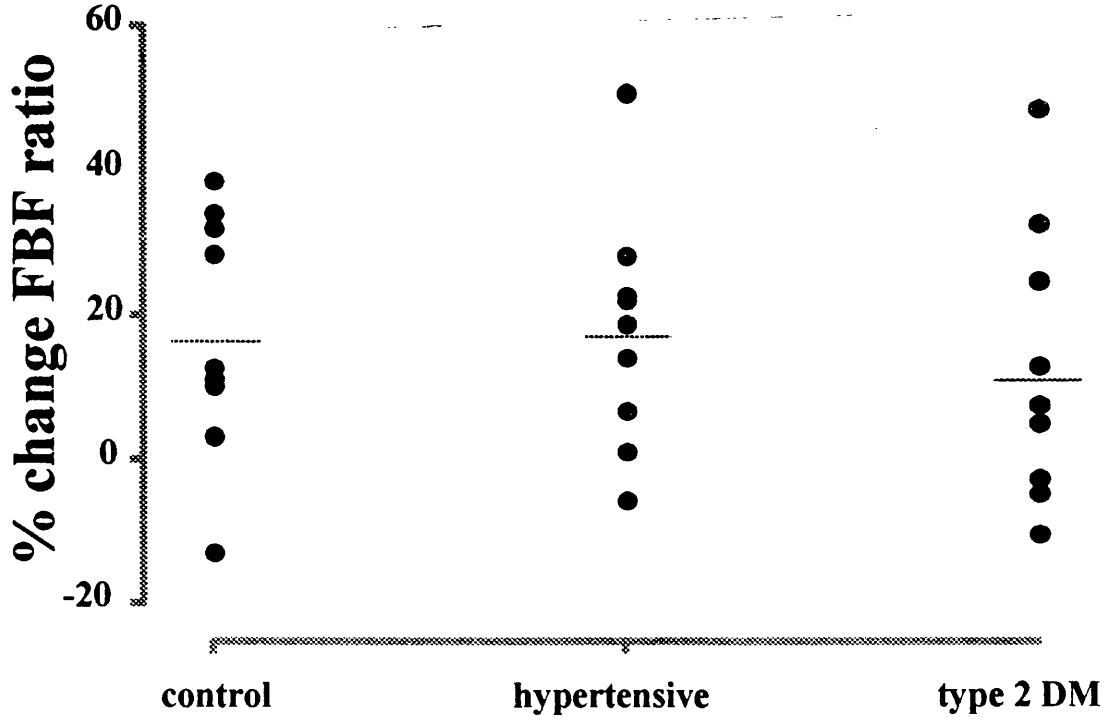


Figure 7.7
Time course for percentage change in forearm blood flow ratio in response to intra-arterial infusion of insulin and D-glucose (mean ± S.E.) in healthy controls (n=9), and patients with essential hypertension (n=9) or type 2 diabetes (n=9).

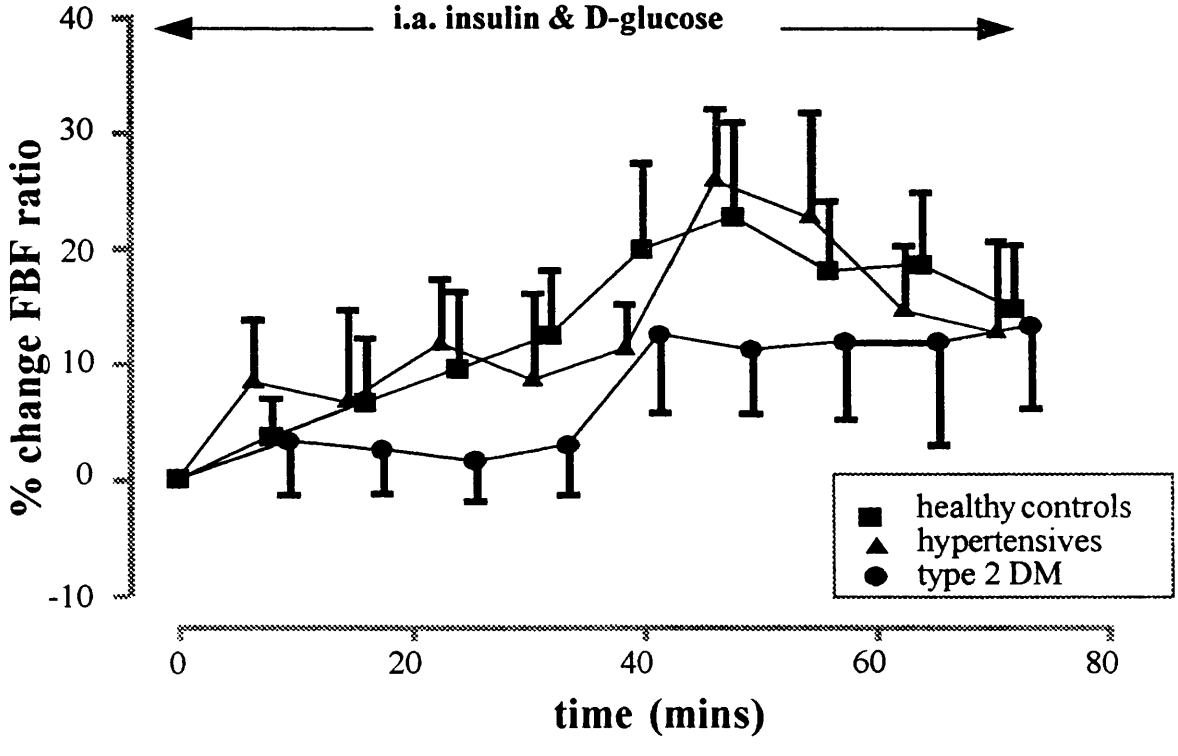


Figure 7.8
Insulin sensitivity (MCR) v insulin vasodilation (percentage change in FBF ratio): (a) data displayed for each sub-group (b) pooled correlation analysis (n=27).

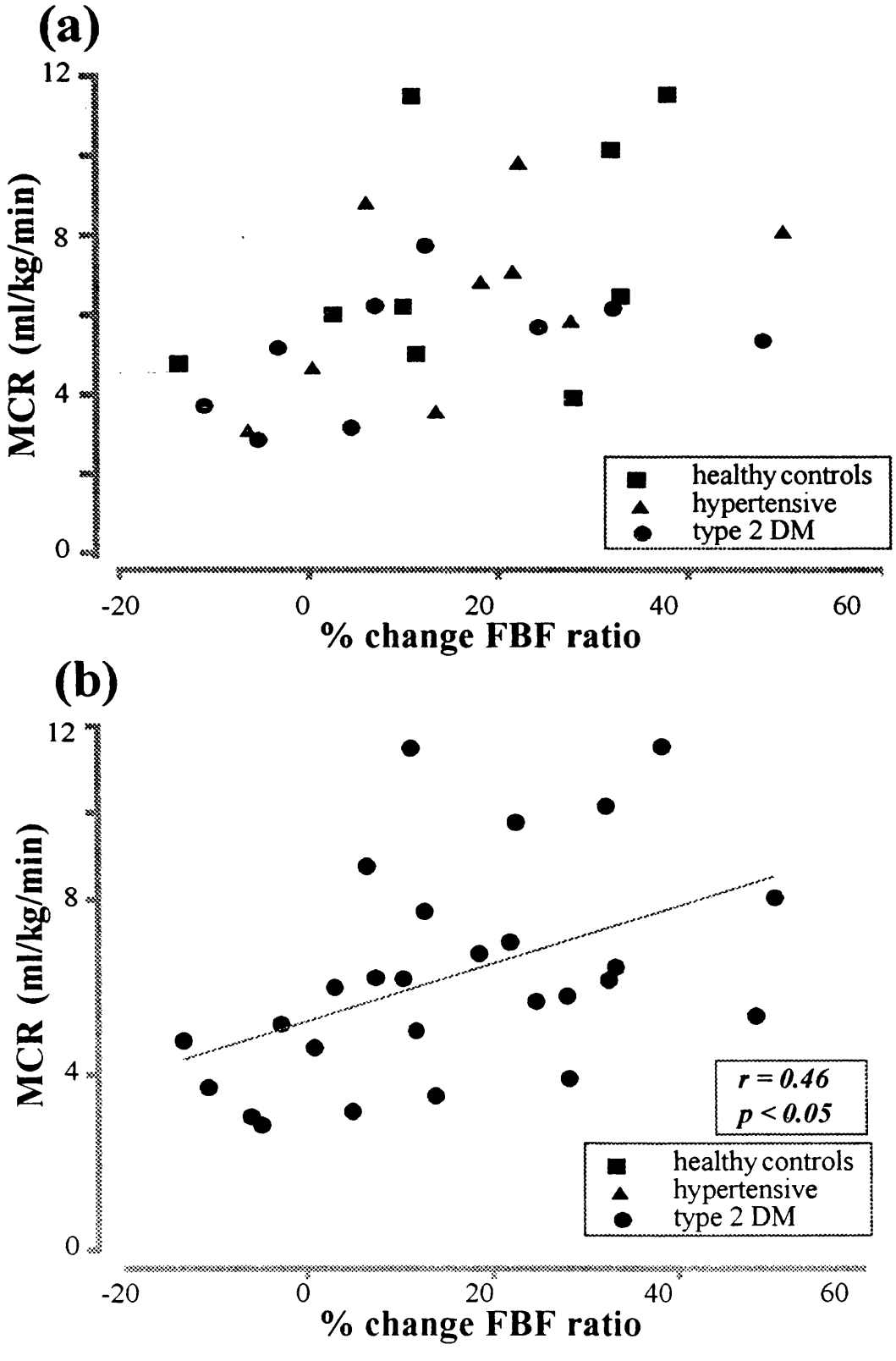


Figure 7.9
Individual values for percentage forearm vasoconstriction in response to intra-arterial infusion of L-NMMA (surrogate measurement of basal endothelial NO production) in healthy controls (n=9), and patients with essential hypertension (n=9) or type 2 diabetes (n=9). Group means also displayed.

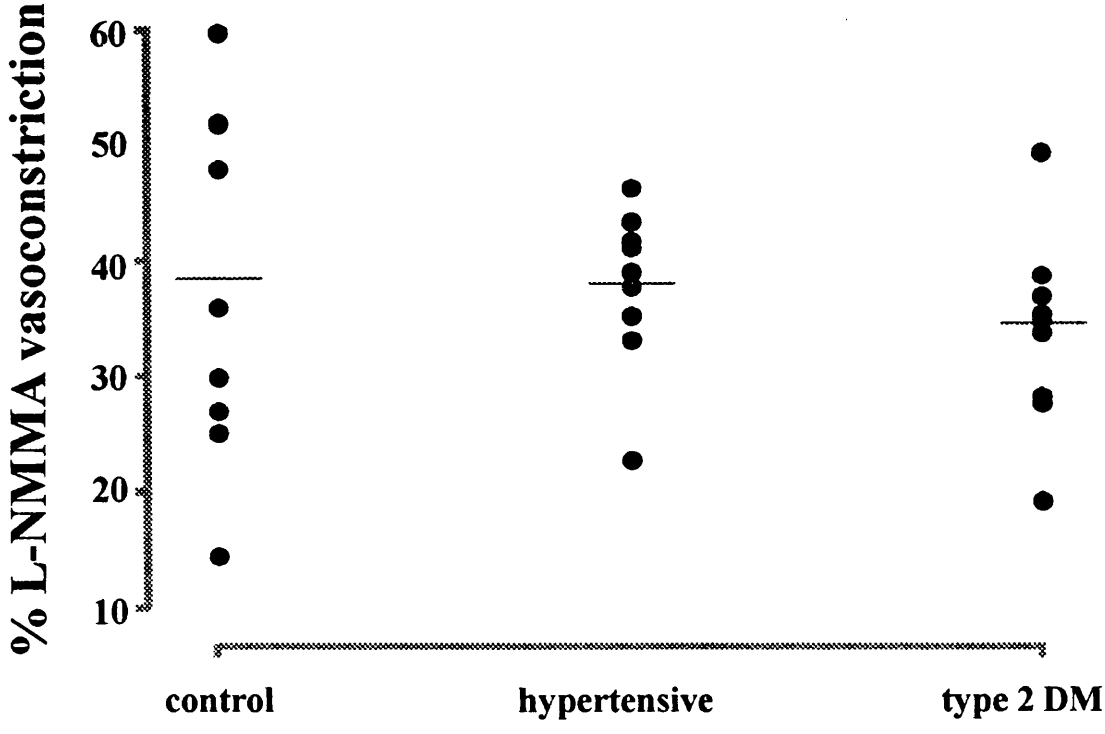


Figure 7.10
Comparison of the time course of percentage change in FBF ratio in response to intra-arterial L-NMMA (mean ± S.E.) between (a) basal conditions and (b) superimposed on a background of local hyperinsulinaemia. Pooled data (n=27)

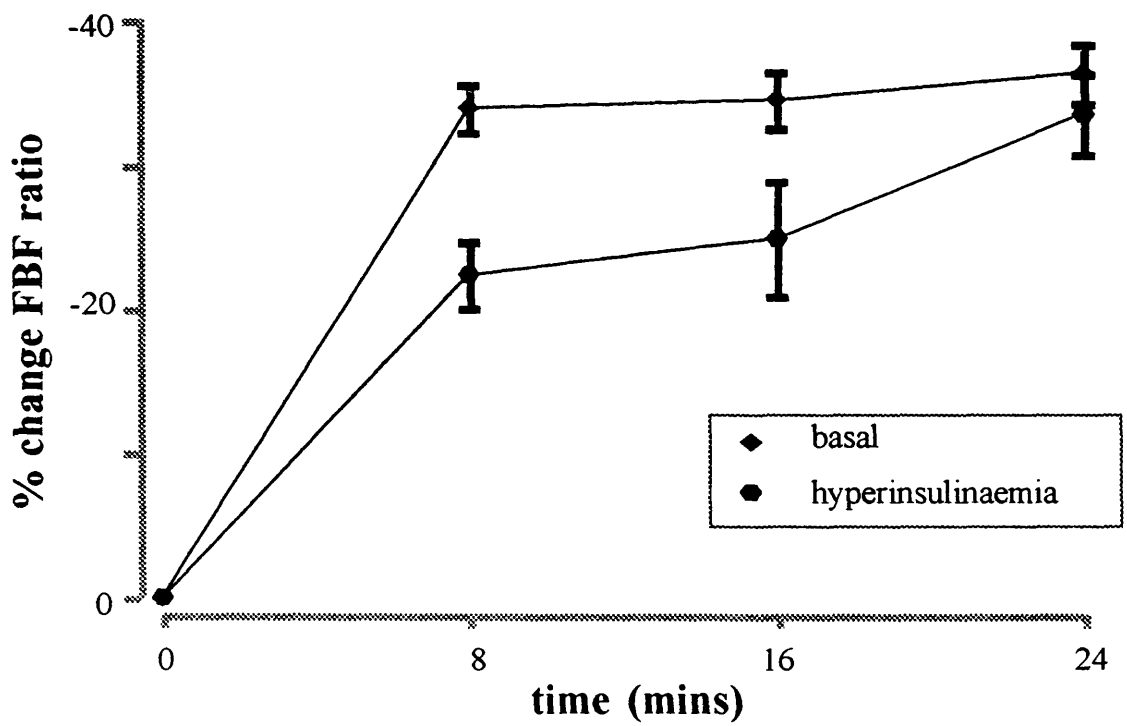


Figure 7.11
Percentage change in forearm blood flow ratio in response to intra-arterial infusions of L-NMMA and noradrenaline plotted against insulin sensitivity (MCR). Pooled correlation analysis (n=27).

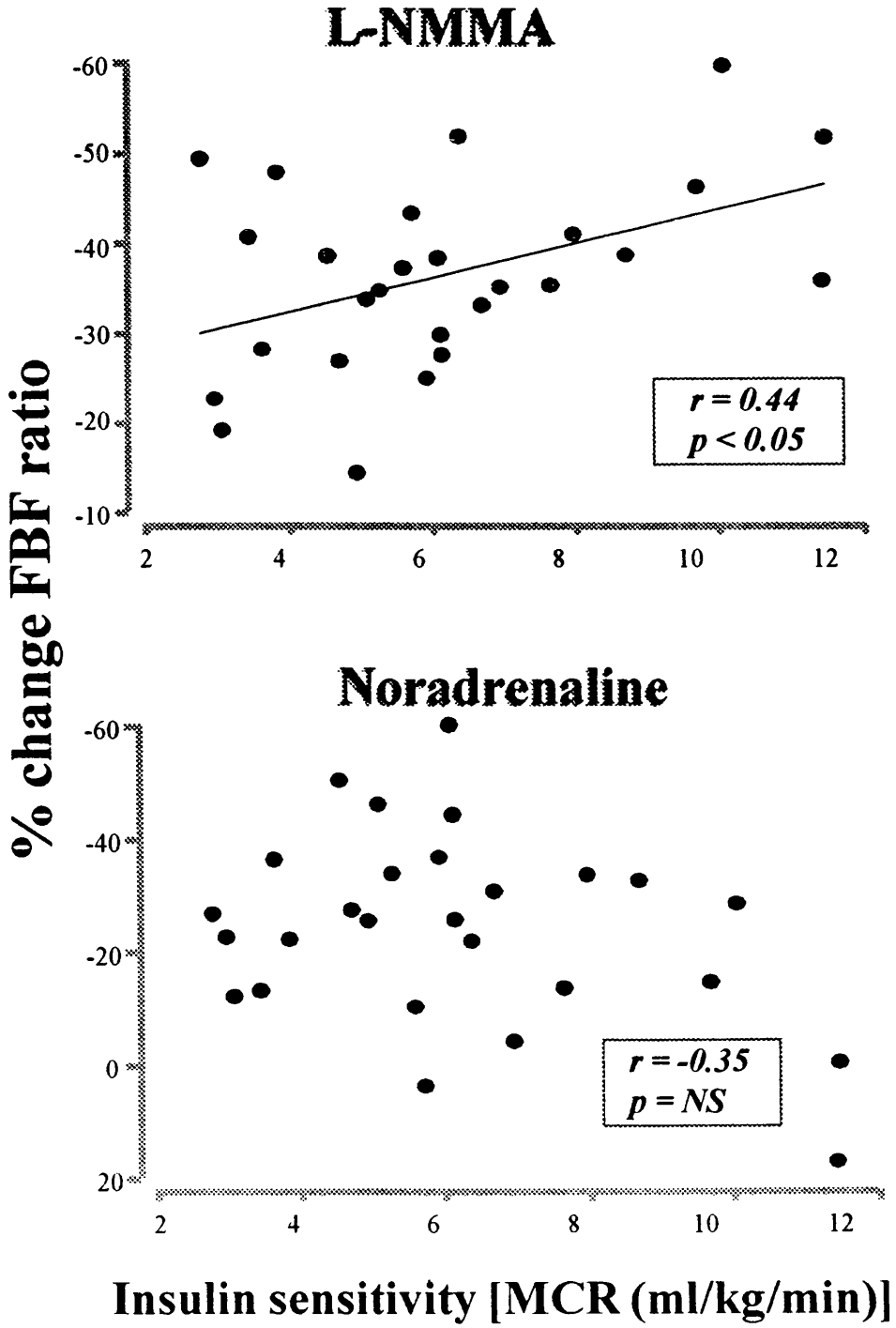
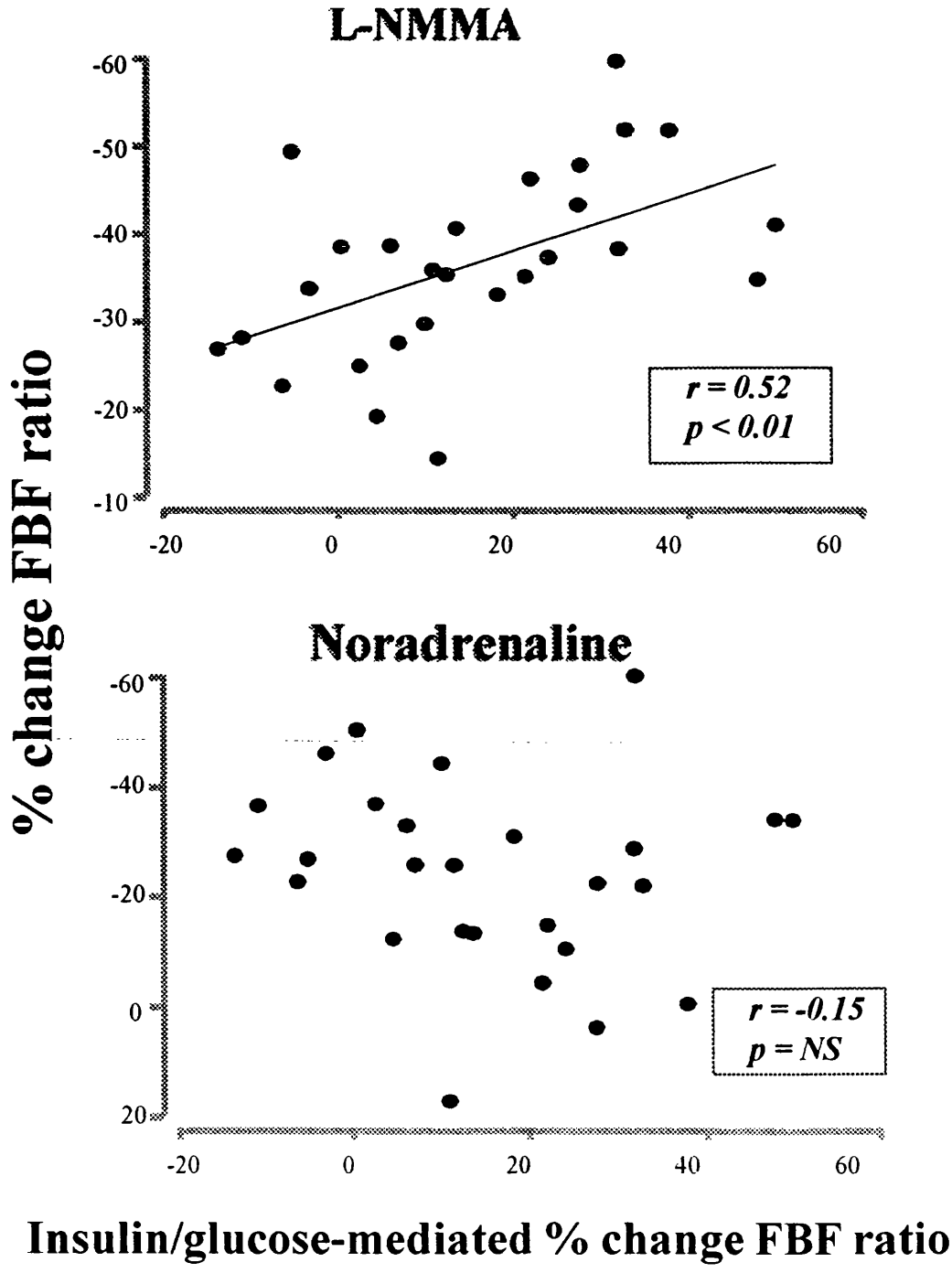


Figure 7.12
Percentage change in forearm blood flow ratio in response to intra-arterial infusions of L-NMMA and noradrenaline plotted against local insulin/glucose-mediated vasodilation (percentage change in FBF ratio). Pooled correlation analysis (n=27).



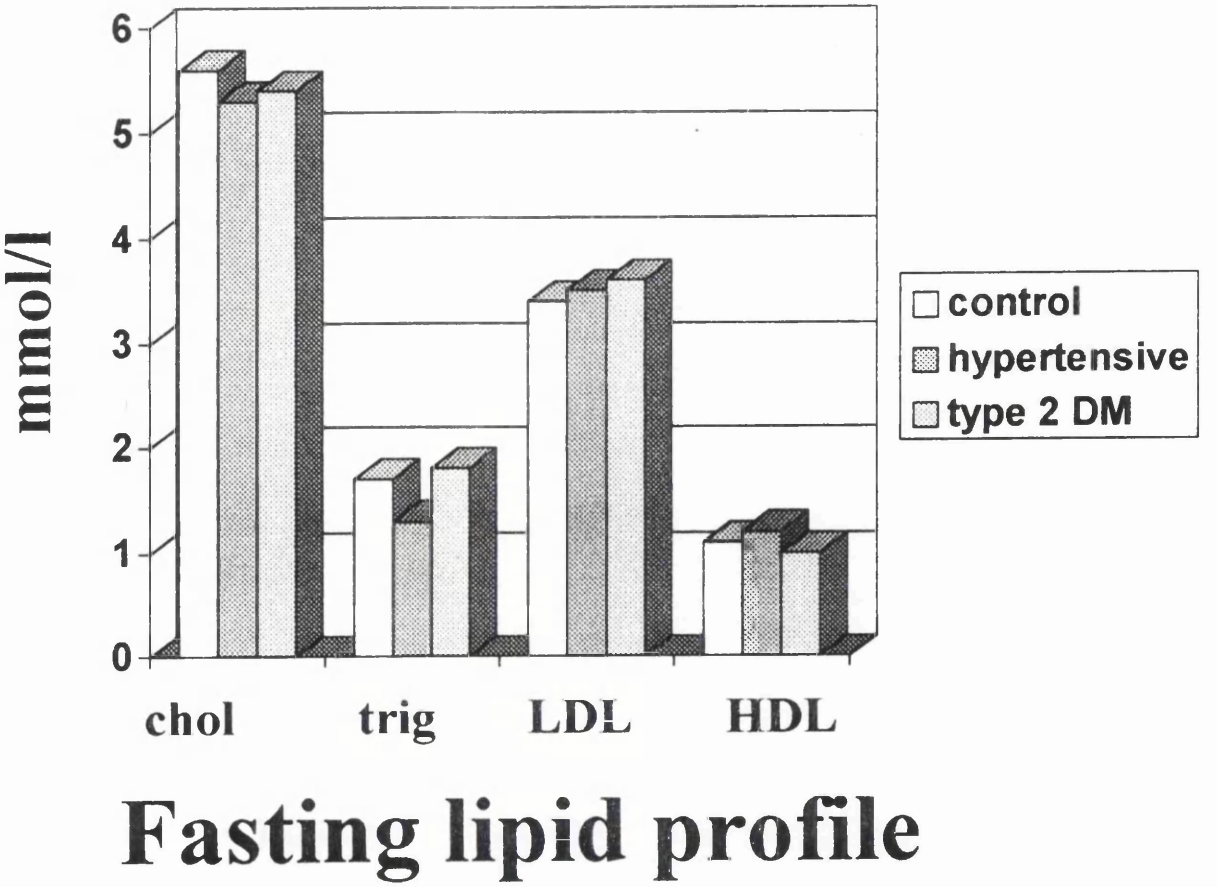


Figure 7.13
Fasting levels (mean ± S.E., mmol/l) of cholesterol, triglycerides, LDL-cholesterol and HDL-cholesterol in healthy controls (n=9) and patients with essential hypertension (n=9) or type 2 diabetes (n=9).

Figure 7.14
Pooled correlation analysis (n=27): HDL-cholesterol plotted against (a) body mass index and (b) insulin sensitivity (MCR glucose).

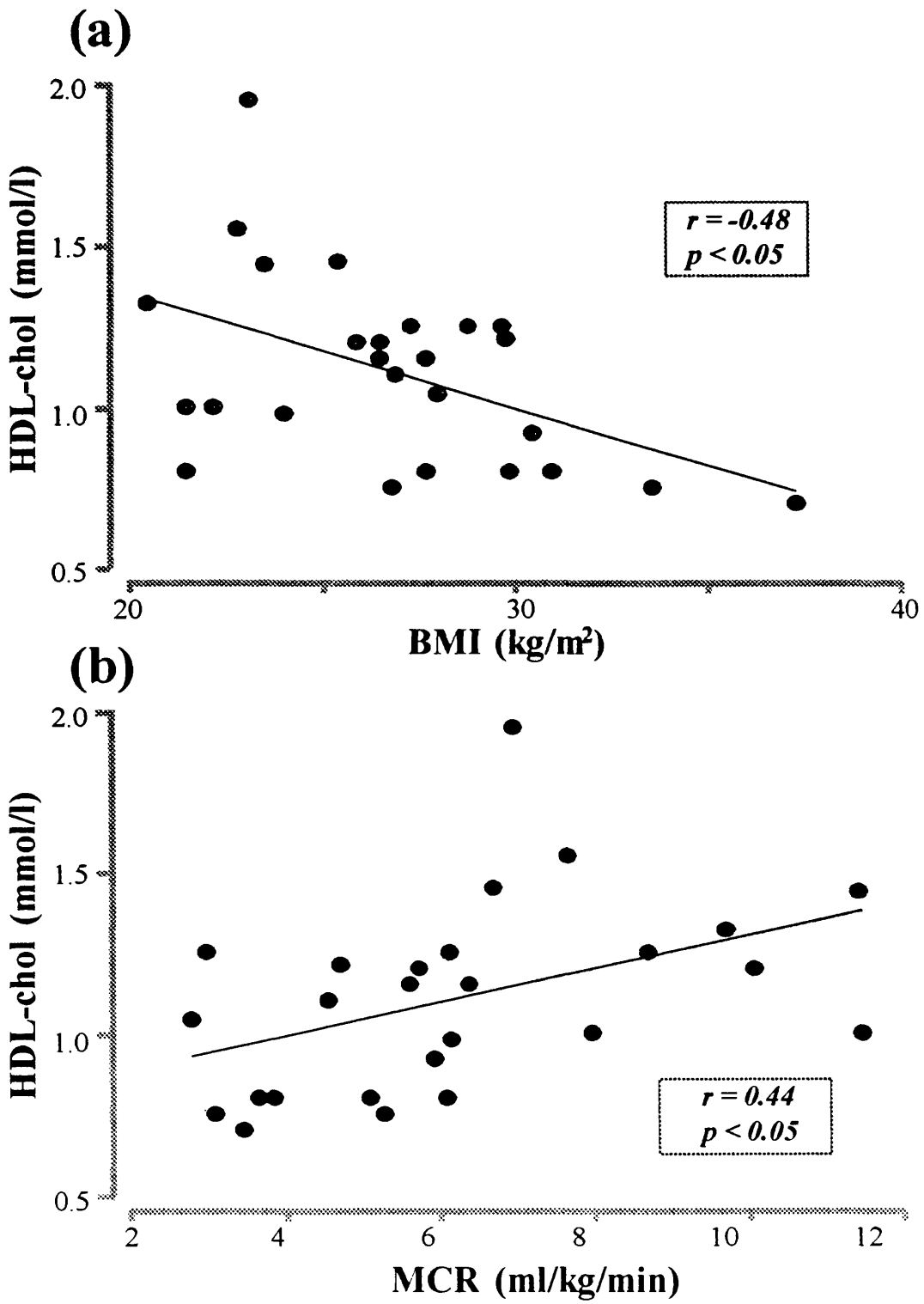


Table 7.1
Simple correlation co-efficients (r) for markers of endothelial activation plotted against (1) L-NMMA vasoconstriction (% change blood flow ratio) (2) Insulin sensitivity (MCR) (3) insulin/glucose-mediated vasodilation (% change blood flow ratio (n=27). 'TPA': tissue plasminogen activator (ng/ml); 'FVIIc': % activity of factor VIIc; 'fibrinogen' (g/l); 'vWF' von Willebrand factor (IU/dl); 'D-Dimers' D-Dimer antigen.

	L-NMMA VASOCONSTRICTION	INSULIN SENSITIVITY	INSULIN VASODILATION
TPA	-0.14	-0.30	-0.10
FVIIc	-0.06	0.09	0.10
Fibrinogen	-0.33	-0.34	0.13
vWF	-0.26	-0.20	0.05
D-Dimers	0.00	-0.03	-0.07

7.4 Discussion

The main purpose of this study was to characterise insulin action and endothelial function in a group of older subjects sub-grouped into healthy controls, essential hypertensives and type 2 diabetics. The primary hypothesis was that the more insulin-resistant groups (ie the patients) would exhibit blunting of vasoreactivity to insulin with associated reduction in basal endothelial function.

To ensure as 'clean' a study as possible, recruitment criteria were extremely tight and patients were free of other cardiovascular and metabolic complications as well as being of similar age and level of obesity. By definition, therefore, patients had very mild disease and this is reflected in the obvious overlap in measurement of insulin action and endothelial function in apparently 'healthy' volunteers. While the large range of biological variability in these parameters means that inter-group comparisons are unlikely to show differences without very large numbers of subjects, there is greater potential for interesting correlation analyses and multiple regression. It has been necessary to pool data from all three sub-groups to facilitate these analyses - this is valid on the premise that there is no clustering of the sub-groups which might produce misleading results. The presence of sub-group clustering has been carefully excluded in any correlation analysis presented in this study.

As in younger, leaner healthy volunteers, local insulin/glucose-mediated vasodilation was a feature in older healthy subjects, although the mean value (17%) is approximately half of that seen in the younger subjects (see chapter 4). The time-

course of insulin vasodilation is similar with a slow onset over the initial 40-50 mins with near-steady-state thereafter. This tends to imply that insulin is unlikely to be a physiologically-relevant vasodilator in the traditional sense, since insulin levels are only elevated temporarily to this extent in the post-prandial period. Rather, the implication is that insulin may play a role in modulating basal activity of eNOS, possibly by up-regulation of gene expression. Taken together with our previous results demonstrating the importance of cellular glucose uptake in determining insulin's vasoactive effect (Ueda et al, 1998a), this has led to the generation of a working hypothesis, presented in figure 7.15. The hypothesis states that the post-receptor pathway for insulin-stimulated endothelial nitric oxide production is common to the pathway involved in glucose uptake (via IRS-1, PI3-K etc) and, therefore, any reason for a reduction in efficacy of the metabolic pathway (ie cellular insulin resistance) will also result in blunted NO production and reduced vasodilation. If insulin does, indeed, exert a promoting influence on eNOS activity, this may account for observed associations between measurements of insulin-mediated glucose uptake and basal endothelial NO production in both experimental and clinical studies. Taking this hypothesis one step further, it may be that insulin plays a crucial role in the post-prandial period, especially after a high-fat meal. Recent studies have demonstrated that endothelial function is temporarily blunted post-prandially, probably due to increased oxidant stress caused by oxidised lipid particles (Vogel et al, 1997; Plotnick et al, 1997). Insulin may help to buffer this oxidant stress by stimulating endothelial NO production. This hypothesis, displayed in figure 7.16, is open for testing in future studies.

Figure 7.15
Hypothetical mechanism for insulin resistance to cause endothelial dysfunction via a reduction in endothelial nitric oxide production.

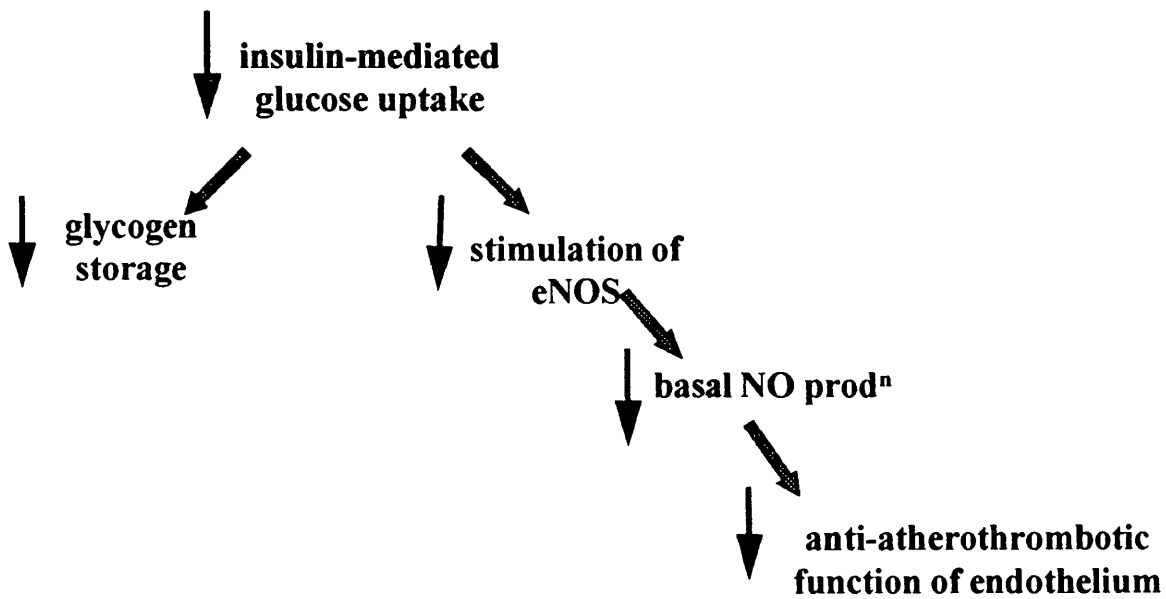
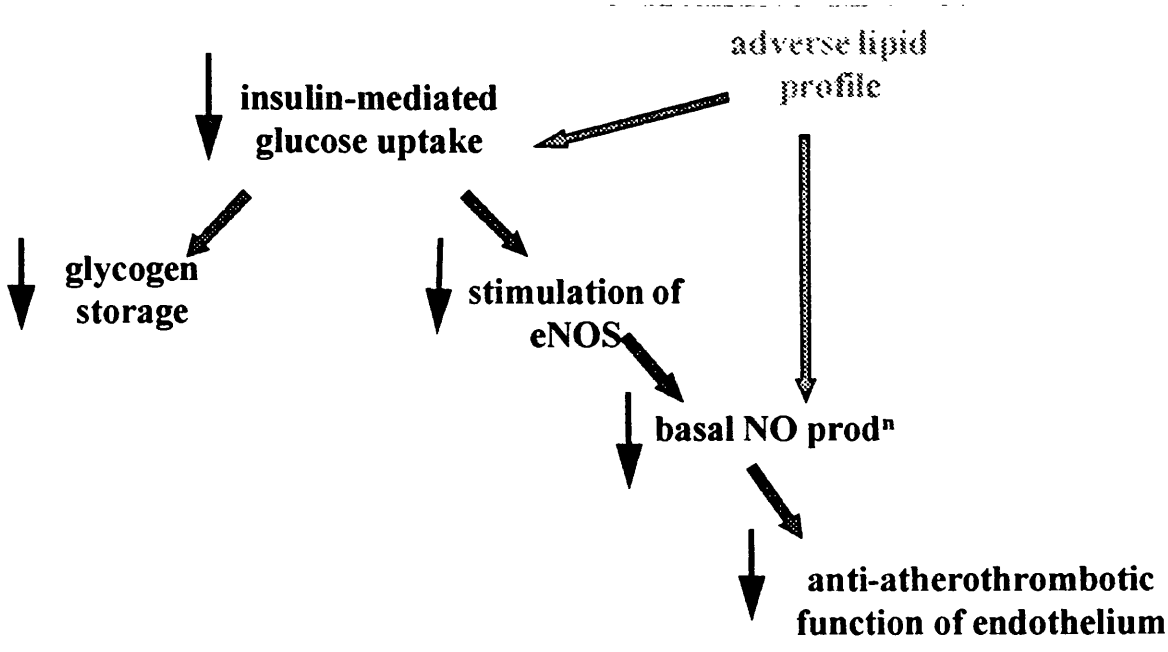


Figure 7.16
Hypothetical mechanism for an adverse lipid profile in the post-prandial period to cause endothelial dysfunction both directly via quenching of nitric oxide and indirectly via a reduction in insulin-mediated glucose uptake.



Do the results presented in the current study support the tenets of the above hypothesis? Firstly, one would predict that the metabolic and vascular actions of insulin are significantly associated, which appears to be the case in pooled correlation analysis (figure 7.8b). This relationship persists despite differences in BP and glycaemic level.

Secondly, one would predict a positive association between insulin's vascular action and basal endothelial NO production (assessed by degree of vasoconstriction in response to inhibition of eNOS). This proved to be a convincing relationship, contrary to the result obtained in younger volunteers (chapter 3) where, if anything, there was a trend for the opposite association. How can these two results be reconciled? It is possible that other mechanisms of insulin-mediated vasodilation are more important in younger (or leaner) men (eg stimulation of Na^+K^+-ATPase on vascular smooth muscle cells) and, with ageing, the predominant mechanism becomes stimulation of endothelial NO production.

Thirdly, one would expect similar results in the current study to those forming the platform for this thesis, demonstrating that insulin sensitivity correlates with L-NMMA vasoconstriction in young healthy men (Petrie et al, 1996a). This is indeed the case (figure 7.11) although it is interesting that this relationship does not persist in multiple regression analysis, with the best model to predict L-NMMA vasoconstriction including insulin vasodilation and age (adj R^2 = 41%). Although one must be cautious when proposing cause / effect mechanisms with this type of analysis, the data are consistent with the notion that insulin-stimulation of

endothelial NO production is a key intermediate mechanism accounting for the observed association between insulin-mediated glucose uptake and basal endothelial function (figure 7.15).

Further evidence to support the proposal that insulin stimulates endothelial NO production comes from the comparison of vascular response to L-NMMA in fasting or hyperinsulinaemic conditions (figure 7.10). The delayed time-course of effect on a background of hyperinsulinaemia implies that eNOS is more active. However, the extent of L-NMMA vasoconstriction after 24 mins is similar under both conditions, which is contrary to the findings of Steinberg et al (1994) and Scherrer et al (1994). The main difference in the current study is that vasoreactivity to insulin/glucose is relatively small due to differences in technique and study demographics.

Chapter 8

Sensitivity of washed platelets to thrombin-induced aggregation is not associated with whole-body insulin sensitivity

8.0 Summary

Previous studies have demonstrated that platelet aggregation is increased in association with insulin resistance. However, it remains unclear whether this feature is intrinsic to platelet function or secondary to influencing factors in plasma. In the present study, a washed platelet preparation was used to determine sensitivity of platelets to thrombin-induced aggregation in the absence of plasma. There was no association between platelet aggregation and either insulin sensitivity or insulin-mediated vasodilation. This negative result might be due to differences in technique from previous studies and supports the notion that plasma factors associated with insulin resistance exert influences on platelet function rather than there being intrinsic platelet defects secondary to insulin resistance.

8.1 Introduction

Platelet aggregability has been shown to be increased in conditions of insulin resistance and this may be a key factor in terms of predisposition to thrombotic vascular disease. However, there are a number of experimental methods in use for measurement of platelet aggregation and results may vary depending on the method

used. Previous published studies on insulin and platelet aggregation have tended to use either whole blood (eg Mandal et al, 1993) or platelet-rich plasma (eg Trovati et al, 1995). Conclusions reached from these studies are limited since it remains unclear whether increased sensitivity of platelets to agonist-induced aggregation in insulin resistance represents a defect in platelet function *per se* or an influencing factor(s) present in plasma. Use of the washed platelet model may help to differentiate these effects since experiments are conducted in a plasma-free environment.

Data are presented on 11 subjects (2 from study described in chapter 4, and 9 from study described in chapter 7) who exhibit a wide range of metabolic and vascular sensitivity to insulin *in vivo*.

8.2 Methods

8.2.1 Subjects

Eleven male subjects participated in this study. All subjects had been well characterised in terms of *in vivo* insulin action and basal endothelial function (chapters 4 and 7). Subject characteristics (mean $\pm$ S.D.) were as follows: age 53$\pm$18 years, BMI 24.7$\pm$3.2 kg/m^2, MAP 105$\pm$14 mmHg, fasting glucose (FG) 6.3$\pm$1.5 mmol/l, insulin sensitivity (MCR) 7.1$\pm$3.0 ml/kg/min, % change FBF to local insulin/glucose (IGMV) 13$\pm$20 %.

8.2.2 Preparation of washed platelets

Peripheral venous blood (45ml) was obtained via a large bore cannula from subjects in the post-absorptive state (prior to the clamp studies) who had not taken any aspirin-containing preparations for at least 10 days. Washed platelets were then prepared according to the method of Radomski and Moncada (1983) (section *2.10.1*).

8.2.3 Measurement of platelet aggregation

Platelet aggregation was measured by a turbidimetric method in a Chrono-log dual channel whole blood lumi-aggregometer as described in section *2.10.2*. A dose-response curve ('Hill plot': figure 8.1) of platelet aggregation to thrombin was constructed and the ED_{50} value (dose of agonist which causes 50% of maximum aggregation) was calculated.

8.3 Results

ED_{50} (log thrombin) was unrelated in univariate analysis to age, BMI, MAP or fasting glucose. In addition, there was no association between ED_{50} and MCR ($r = -0.06$, $p = 0.85$, figure 8.2) or IGMV ($r = 0.07$, $p = 0.82$, figure 8.3).

Figure 8.1
Example of Hill Plot: percentage platelet aggregation is measured in response to ten doses of thrombin. ED50 = estimated dose of thrombin required which causes 50% of maximal platelet aggregation (measurement of sensitivity of washed platelets to thrombin-induced aggregation).

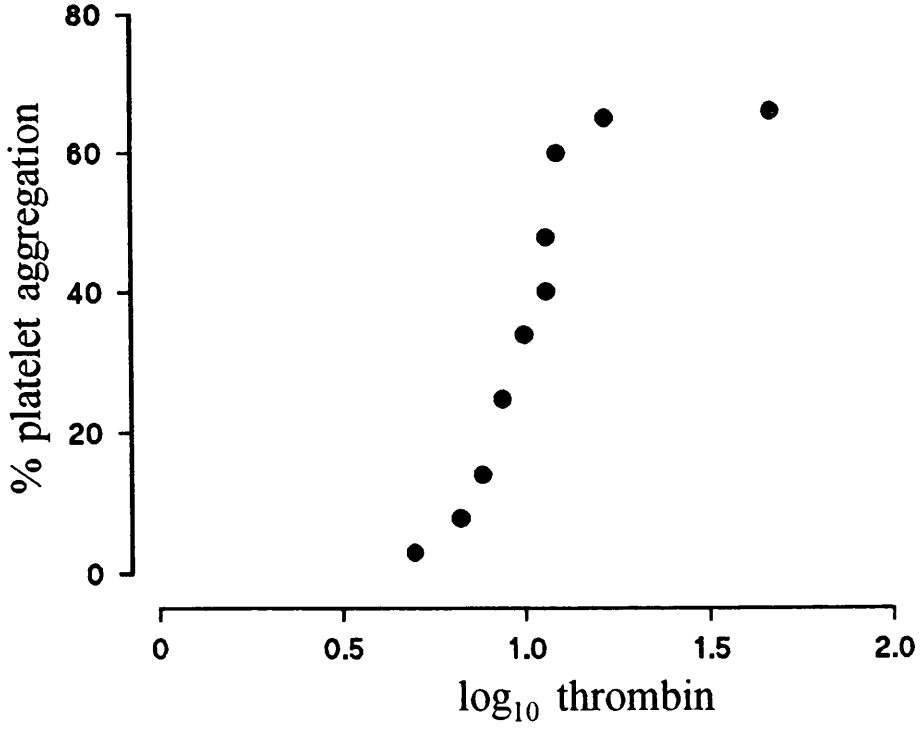


Figure 8.2
Insulin sensitivity (MCR glucose) in 11 subjects plotted against ED50 (measurement of sensitivity of washed platelets to thrombin-induced aggregation).

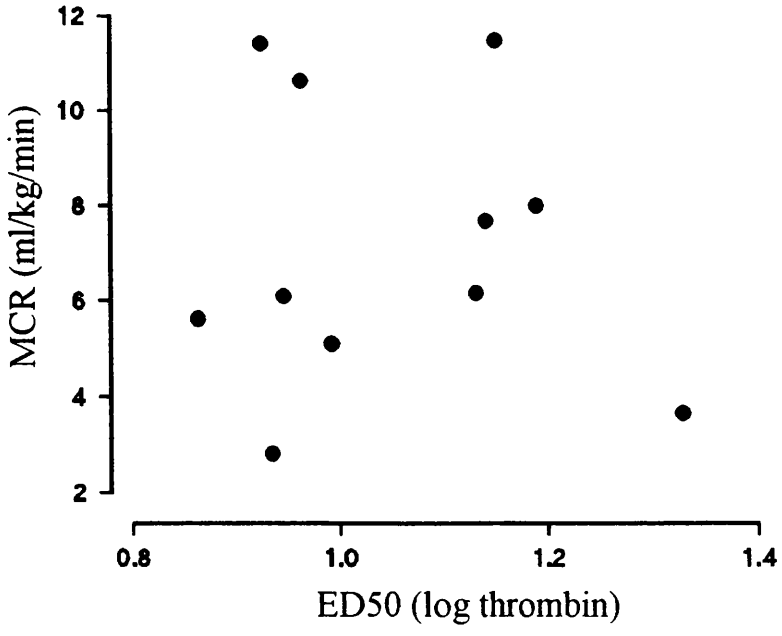
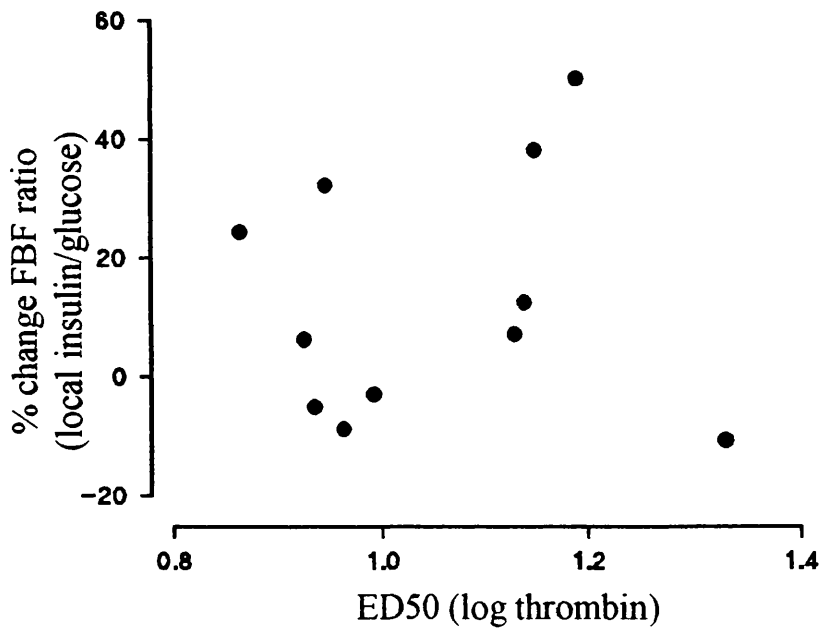


Figure 8.3
Forearm insulin/glucose-mediated vasoreactivity in 11 subjects plotted against ED50 (measurement of sensitivity of washed platelets to thrombin-induced aggregation).



8.4 Discussion

In newly-diagnosed NIDDM there appears to be significant platelet hyperaggregation in whole blood (Mandal et al, 1993; Menys et al, 1995). After metabolic control of blood glucose there is significant reduction in platelet aggregability, suggesting that both hyperinsulinaemia and hyperglycaemia may be influencing aggregation mechanisms (Mandal et al, 1993). In another study, platelet aggregation was measured in healthy volunteers before and after intravenous insulin: aggregation was significantly reduced after insulin suggesting that insulin may have important anti-aggregatory properties (Kahn et al, 1993).

One group has used platelet-rich plasma (PRP) to study the relationship between insulin and platelet aggregability: firstly, they demonstrated an anti-aggregatory effect of insulin to platelet stimulation by collagen or ADP in PRP form healthy volunteers (Trovati et al, 1994). They also measured cGMP production and concluded that insulin's anti-aggregatory effect was via a cGMP-dependent mechanism. They went on to show that this effect was significantly impaired in the insulin resistant states of obesity and type 2 diabetes (Trovati et al, 1995) and, recently, they demonstrated that lean patients with type 2 diabetes exhibited normal insulin anti-aggregation (Anfossi et al, 1998), suggesting that insulin resistance is the most important underlying mechanism for this effect rather than hyperglycaemia.

However, it remains unclear whether platelet function *per se* is defective in insulin resistance or whether platelets are influenced by as-yet-unknown plasma factors since the studies cited above are conducted in the presence of the subject's plasma. Using a washed platelet technique, it can be concluded that any association between platelet aggregation and insulin action is due to an intrinsic defect in platelet function rather than an adverse environmental effect. The lack of any relationship in the current study would tend to suggest that plasma factors play a key role in mediating increased platelet aggregation in insulin-resistant conditions. The negative result may also be a reflection of the agonist used. In studies cited above, collagen or ADP tends to be used rather than thrombin - it is possible that increased sensitivity to platelet aggregation in insulin resistance is agonist-specific. Clearly, there are a number of further studies required to clarify these issues, including a study comparing different platelet preparations and different agonists of aggregation in the same group of subjects.

The L-arginine/nitric oxide pathway has been known for some time to be an important mechanism regulating aggregation in platelets (Radomski et al, 1990), and it appears likely that insulin's anti-aggregatory effect is mediated via production of nitric oxide and consequent elevated levels of cGMP (Trovati et al, 1996; Trovati et al, 1997). Plans for further studies using the washed platelet technique include *in vitro* incubation with varying doses of insulin and concomitant manipulation of platelet NO synthase.

Finally, the role of insulin-mediated platelet glucose uptake in determining sensitivity to aggregation can be investigated using *in vitro* incubation with insulin and either D- or L-glucose in a similar way to limb infusion studies investigating mechanisms of insulin-mediated vasodilation (Ueda et al, 1998a). The presence of GLUT3, an insulin-regulatable glucose transporter, has been confirmed in platelets in our Department using immunoblotting and autoradiographic techniques (C. Clark, unpublished observations). It is not known whether platelet glucose uptake is reduced in insulin-resistant conditions, but if platelet metabolic function depends on adequate glucose uptake, this is a potential mechanism for platelet dysfunction secondary to insulin resistance.

Chapter 9

Direct visualisation of glucose transporter protein GLUT4 in skeletal muscle using immunogold electron microscopy

9.0 Summary

In this pilot study, a collaboration was initiated with experts in membrane biochemistry and cell ultrastructure with a view to developing the technique of immunogold electron microscopy for use in direct visualisation of key cellular proteins involved in the insulin signalling pathway. The primary aim of this ongoing work is to demonstrate and quantify GLUT4 translocation to myocyte t-tubules in response to insulin stimulation. Once this technique is established, it is planned to explore mechanisms of GLUT4 translocation to provide clues for possible defects which may underlie cellular insulin resistance.

9.1 Introduction

Insulin stimulates glucose uptake into adipocytes and muscle by virtue of the specific expression of an insulin-regulatable glucose transporter, GLUT4. The translocation of this transporter from an intracellular site to the plasma membrane in insulin-exposed cells is responsible for the large increase in glucose transport observed. Under basal conditions in both fat and muscle, >90% of GLUT4 is sequestered intracellularly (Slot et al, 1991; Slot et al, 1997). It has been suggested that the majority of the intracellular GLUT4 is housed in vesicular structures similar to small

synaptic vesicles observed in neuroendocrine cells (Slot et al, 1991; Slot et al, 1997; Martin et al, 1998).

Recent studies have established that GLUT4 translocation in muscle is sufficient to account for the effects of insulin on glucose transport (Lund et al, 1995; Zorzano et al, 1996; Lund et al, 1997). Skeletal muscle t-tubules possess insulin receptors, and thus may be a potential site for translocation of glucose transporters in response to insulin in addition to the sarcolemma (Zorzano et al, 1996). There is some debate regarding the relative contributions of translocation of GLUT4 to the sarcolemma and t-tubular regions. Biochemical and immuno-EM evidence has clearly demonstrated insulin-stimulated translocation of GLUT4 to both these sites in rat skeletal muscle (reviewed in Zorzano et al, 1996). The present consensus suggests that t-tubules contain more GLUT4 per unit membrane than the sarcolemma after insulin treatment, suggesting some heterogeneity in the translocation process to these different plasma membrane domains (reviewed in Zorzano et al, 1996).

Reductions in cellular glucose uptake in response to insulin may be secondary to defects in GLUT4 translocation. The technique of immunogold-EM allows direct vision of GLUT4 and its relationship with membrane structures such as sarcolemma, t-tubules and sarcoplasmic reticulum. This chapter describes the process of setting up this technique and the methodological problems encountered. Thus far, rat muscle has been studied in the basal state with the result that GLUT4 can be consistently demonstrated in relation to intracellular membrane structures. The next step in this ongoing work is to study the response of GLUT4 to insulin stimulation

and to attempt quantification of GLUT4 translocation. The eventual aim is to use this technique as a model to characterise the biochemical steps involved in GLUT4 translocation and possible defects in this process which may account for cellular insulin resistance.

9.2 Methods

A detailed description of methods is contained in section 2.11. Briefly, rat skeletal muscle was harvested, fixed and cryoprotected. After freezing the sample in liquid nitrogen, ultrasections were obtained and placed on grids. Polyclonal GLUT4 antisera was applied followed by colloidal (Protein A) gold conjugate (10nm). The sections were embedded with methyl cellulose and uranyl acetate was applied to optimise contrast. Grids were observed using a Phillips CM10 electron microscope (magnification approx: x38000).

9.3 Results

9.3.1 Modifications to technique

Embedding and Contrast

Cryo-ultrathin sections need support to withstand the surface tension produced by air-drying and the effects of the electron beam. Methyl cellulose acts as this support but a balance needs to be achieved to obtain the optimal protection and allow the beam to penetrate. Too thick a support layer and the electron beam has difficulty penetrating, resulting in reduced contrast; too thin a layer and the sections rupture. Methyl cellulose concentrations of 1 %, 2 %, and 3% were used to determine the optimal support film. Sections with 2 % and 3 % methyl cellulose, although stable

under the electron beam, had diminished contrast resulting in poor membrane visualisation. The best result was achieved using 1 % methyl cellulose as the support film. This resulted in good visualisation of membrane structures, while allowing for sufficient support.

Using high density colloidal gold allows for negative "staining" and the heavy metal solution helps to support the cell structures. With higher concentrations of uranyl acetate, increased negative staining is seen. Different concentrations of uranyl acetate (3, 6 and 10 % as a 1:1 and 1:9 solution with methyl cellulose) were used to establish optimal contrast of membrane structures within the muscle tissue sections. Superior contrast and support was achieved with the 9:1 methyl cellulose / 10 % uranyl acetate solution. Using methyl cellulose as a 1:1 solution with uranyl acetate did not provide enough support for the sections.

Background staining

The reduction in incubation times to 30 minutes (originally 1 hour) and increased washing decreased background staining. The introduction of Tris-HCL buffer at pH 8.2 assisted the removal of non-specific background staining. The original immunogold technique employed gelatine to block non-specific background. However, by a process of trial and error, it was found that gelatine actually introduced non-specific staining with the Protein A colloidal gold solution and consequently the GLUT4 antiserum staining appeared heavier than expected. After removal of gelatine from the protocol, the non-specific background staining diminished considerably (Figure 9.1). This reduction in background staining

permitted the increase in concentration of GLUT4 antiserum from 1:800 (with the use of gelatine) to 1:200 without gelatine (Figure 9.2).

9.3.2 Cryo-ultrastructural Immunocytochemistry

The GLUT4 antiserum showed positive localisation with the immunogold technique. Labelling of the GLUT4 antigenic sites in rat muscle tissue in fasting conditions was observed in the I band, near the A – I band junction of the myofibrils and close to the triad junctions, and also associated with the sarcoplasmic reticulum (SR) (Figure 9.3). Immunolabelling is also observed in close proximity to t-tubules and at the sarcolemma (Figure 9.4).

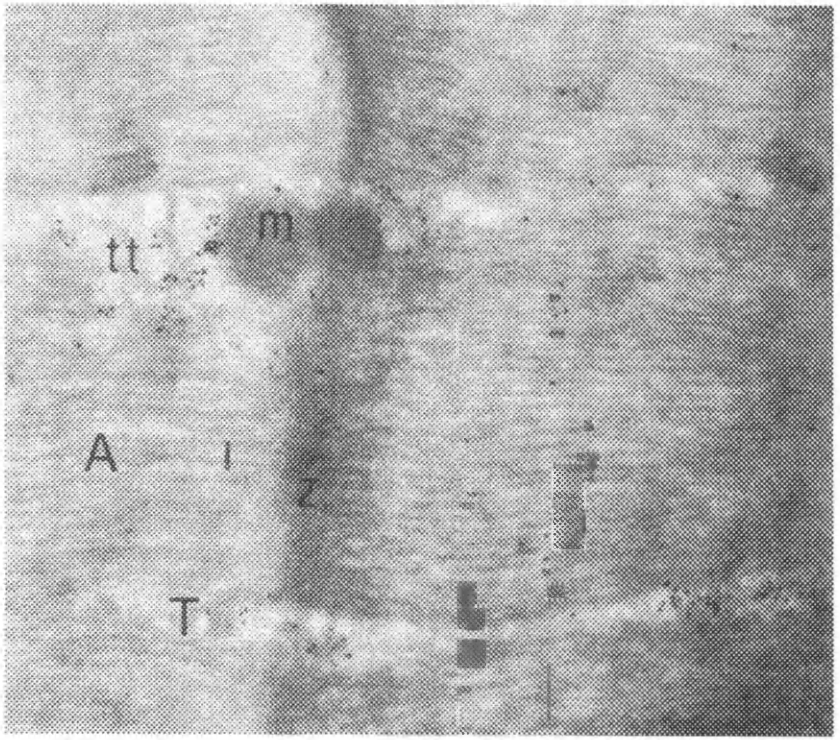


Figure 9.1 No gelatine (magnification x 47,250)
Cryo-ultrathin section of rat skeletal muscle without gelatine as a blocking agent for non-specific background staining. The removal of gelatine resulted in discrete localisation at the t-tubule, triad and between the sarcomere repeat units (GLUT4 antisera 1:200, Protein A (10nm) 1:10).
Key:

Z - Z band	m - mitochondria
I - I band	tt - t-tubule
A - A band	T - muscle triad

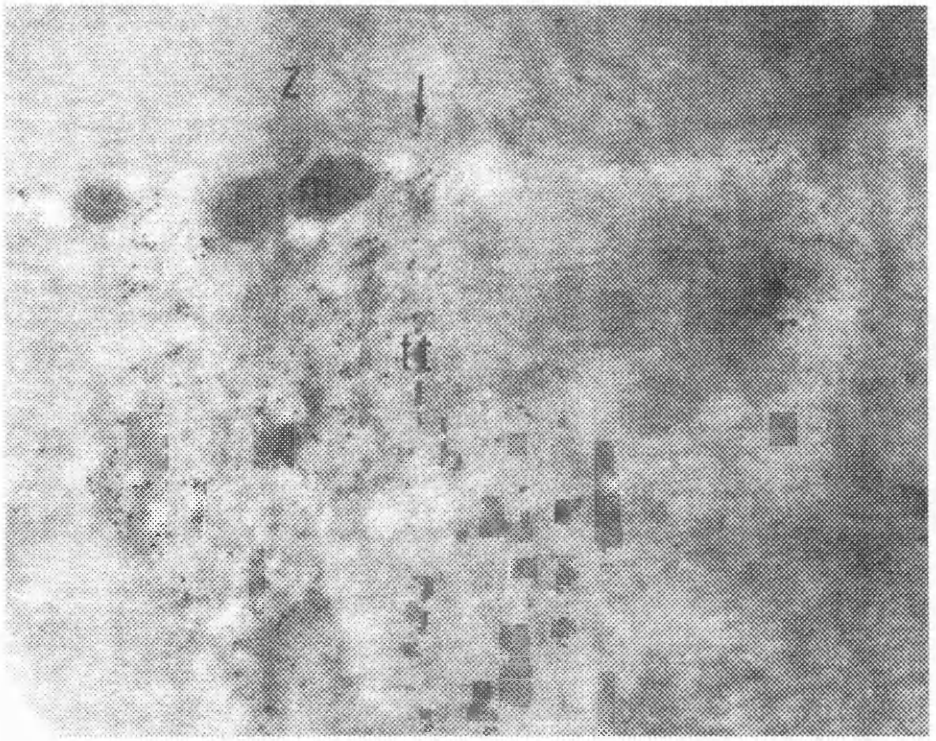


Figure 9.2
GLUT4 antisera 1:200, Protein A (10nm) 1:10 (magnification x 47,250)
Immunogold labelling observed in greater quantities with discrete labelling at the t-tubule (located at the A – I band junction) in cryo-ultrathin sections of rat skeletal muscle
Key:

- ↓ - A – I band junction
- Z - Z band
- tt - t-tubule
- m - mitochondria

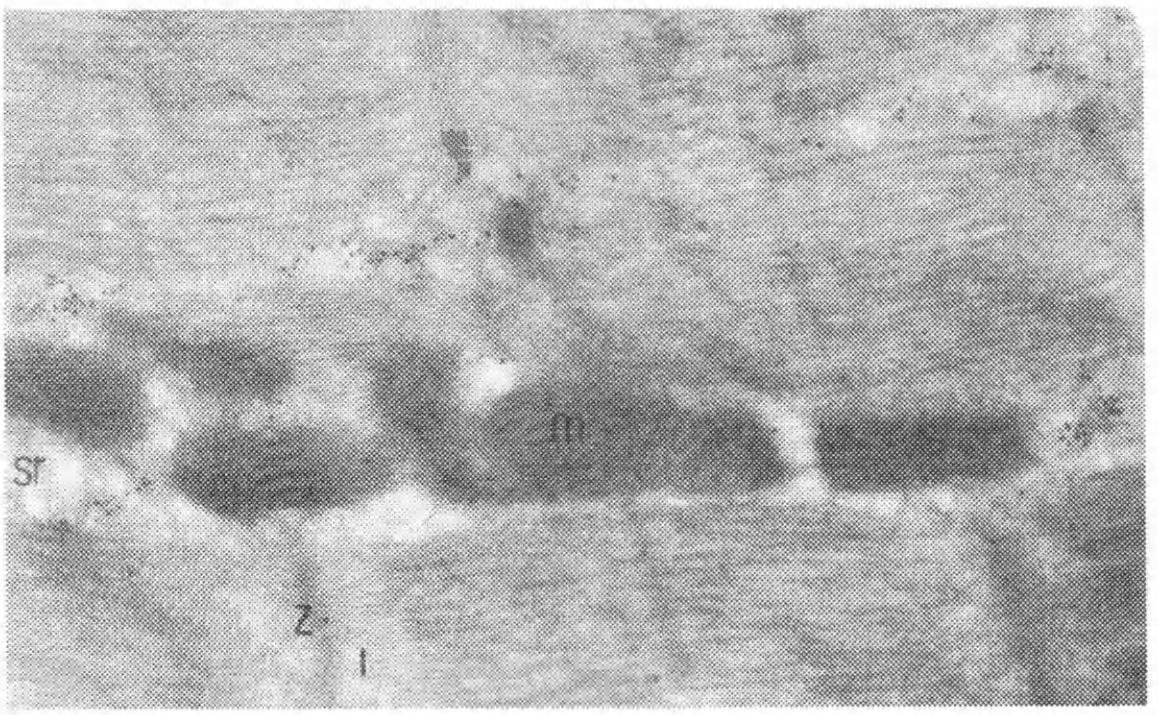


Figure 9.3
GLUT4 antisera 1:200, Protein A (10nm) 1:10 (magnification x 47,250).
Immunogold labelling is seen in close association with the sarcoplasmic reticulum (SR) in cryo-ultrathin section of rat skeletal muscle

Key:
Z - Z band
I - I band
SR - sarcoplasmic reticulum
T - triad
m - mitochondria

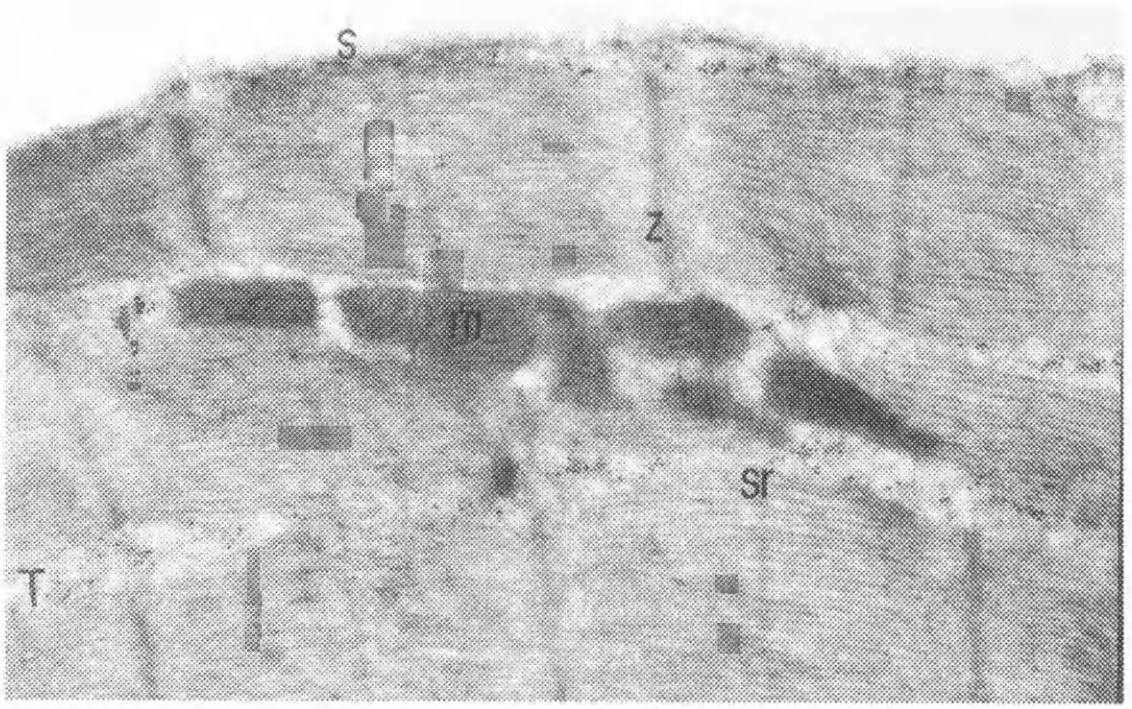


Figure 9.4
Immunogold labelling of the GLUT4 antigenic site is localised to the sarcolemmal surface of rat skeletal muscle in cryo-ultrathin section (magnification x 33,000).
GLUT4 antisera 1:200, Protein A (10nm) 1:10.

Key:
M - mitochondria
Z - Z band
SR - sarcoplasmic reticulum
S - sarcolemma
T - triad

9.4 Discussion

In collaboration with colleagues in membrane biochemistry and electron microscopy, the technique of immunogold-EM has been successfully established with a view to characterising the response of GLUT4 to insulin stimulation in skeletal muscle cells. The technique has been refined so that GLUT4 protein can be consistently and clearly demonstrated in relation to intracellular membrane structures. Background staining has been minimised and contrast has been optimised resulting in high quality images on a par with previously published work from other groups.

The next stage of this ongoing work is to visualise GLUT4 in myocytes following insulin stimulation. Translocation of GLUT4 to t-tubules and sarcolemma is predicted and, hopefully, it will be possible to quantify the degree of translocation to these sites compared with basal conditions. Once this has been established, a number of potential studies can be initiated. Using biochemical techniques it may be possible to characterise the key steps of the insulin signalling pathway which stimulate GLUT4 translocation. Studies in muscle from insulin resistant animals may reveal differences in GLUT4 translocation in response to insulin. Finally, it is hoped to extend these studies to human muscle, with the ultimate aim of studying muscle from subjects in whom detailed characterisation of *in vivo* insulin action has been obtained.

Chapter 10

General discussion

In recent years there has been intense interest in physiological mechanisms linking aspects of metabolism with vascular control. This has been fuelled by observations that insulin resistance and vascular endothelial dysfunction are common features in both metabolic disorders, such as obesity and type 2 diabetes, and cardiovascular disorders, such as essential hypertension and heart failure. The demonstration of insulin as a vascular hormone, causing vasodilation by endothelium-dependent mechanisms, has led to speculation that a generalised defect in insulin action might be important as a determinant of both metabolic and vascular dysfunction.

Prior to this thesis commencing, the predominant hypothesis concerning insulin action and blood flow (Baron's group, USA) was that primary endothelial dysfunction results in blunting of insulin-mediated vasodilation with subsequent reduction of nutrient blood flow to skeletal muscle vascular beds; this, in turn, causes reduced insulin-mediated glucose uptake in muscle cells. This hypothesis depended on muscle blood flow and substrate delivery being rate-limiting to the process of limb glucose uptake. Baron's results from the early 1990s (discussed in detail in section *1.5.3*) appeared to support this hypothesis

A number of European investigators (most influential being Yki-Jarvinen, Finland) went on to refute Baron's conclusions, criticising the unphysiological nature of his

studies and questioning the interpretation of his results. A number of studies involving manipulation of limb blood flow showed no effect on limb glucose uptake suggesting that substrate delivery was unlikely to be rate-limiting for glucose uptake. The current feeling is that Baron's hypothesis is no longer tenable, although recent work by Clark in Australia has introduced the concept of 'nutritive' and 'non-nutritive' vascular beds within skeletal muscle which might be differentially stimulated by insulin, and the notion that insulin redirects blood flow in order to optimise nutrient uptake remains teleologically attractive.

In addition to providing evidence against Baron's hypothesis, Yki-Jarvinen (1998) and others proceeded to argue that there was unlikely to be any functional coupling between insulin's vascular and metabolic actions, although these same investigators are tending to concede in recently published studies that their results are, indeed, compatible with a common physiological mechanism linking glucose uptake and vasodilation.

The alternative hypothesis that reduced insulin-mediated glucose uptake is the primary event leading to blunting of insulin's vascular action (and associated vascular endothelial dysfunction) has been previously discounted, mainly on the basis of one published study by Vollenweider et al (1993) which showed that a fructose-mediated increase in intracellular metabolism did not cause vasodilation. However, this result is still compatible with the notion extended in this thesis that insulin's vascular action and promotion of glucose uptake may have a common intracellular signalling pathway.

Indeed, there is increasing evidence from *ex vivo* studies (section *1.4.1*) that cellular glucose uptake plays a key role in insulin's vascular effect, both directly in VSMCs and indirectly, via endothelial mechanisms. In addition, we demonstrated in the human forearm that co-infusion of glucose with insulin caused significant augmentation of vasodilation compared with co-infusion with an isomeric control (Ueda et al, 1998). Taking account of previous results demonstrating a positive association between insulin sensitivity and basal endothelial NO production (Petrie et al, 1996), a working hypothesis has been generated (figure 7.15). The studies described in this thesis relate to this hypothesis and a general discussion of the major findings follows.

(i) Physiological mechanisms of insulin-mediated vasodilation in man

Having developed experience with the forearm model, the first studies of this thesis were designed to examine potential mechanisms for insulin-mediated vasodilation. These studies were complicated by the long time-course of insulin's vascular effects necessitating restrictions in study design and also the somewhat unexpected results. The hypothesis was that local inhibition of NO synthase would abolish insulin-mediated vasodilation in the forearm. To control for the fact that basal NO production contributes to vascular relaxation and therefore inhibition by L-NMMA would cause vasoconstriction, a 'control' endothelium-independent vasoconstrictor (angiotensin II) was used. Co-infusion with L-NMMA abolished vasodilation to insulin/glucose but a similar pattern emerged with angiotensin II as well as in a sub-group who had local inhibition of Na^+K^+-ATPase by ouabain infusion. These results

can be interpreted in a number of ways, as discussed in chapter 3. Unfortunately, it is difficult to draw any definite conclusions concerning mechanisms of insulin's vascular action from this study. However, it allows some insight into the pitfalls of interpreting results from similarly-designed studies, many of which have been widely cited as providing evidence for insulin as an endothelium-dependent vasodilator. The correlation analyses in chapter 3 involve small numbers of subjects but are of some interest, especially with regard to results obtained by similar methods from older subjects, described in chapter 7.

In order to examine insulin's vasoactive mechanisms in more detail it is proposed to study small arteries obtained by gluteal biopsy using myographic techniques; this work is in the preliminary phase and will not be discussed in detail as part of this thesis.

(ii) Insulin-mediated vasodilation and glucose uptake are functionally linked in man

According to the working hypothesis (figure 7.15) insulin resistance should be associated with blunting of insulin-mediated vasodilation. By virtue of the three- to five-fold variation in these parameters in health, it was possible to study the relationship between insulin's metabolic and vascular actions in young healthy male volunteers. The conclusion of this study, described in chapter 4, is that an association does indeed exist between insulin-mediated glucose uptake and insulin vasodilation, which is independent of potential confounding factors such as age, BMI and blood pressure.

While this supports the notion of functional coupling of insulin effects, there is no evidence of causality, although in the context of the 'D and L-glucose infusion' study it seems plausible that insulin-mediated glucose uptake is a primary determinant of insulin vasodilation.

(iii) Systemic inhibition of endothelial nitric oxide synthesis does not impair insulin sensitivity in man.

If Baron's hypothesis is valid, then inducing a temporary and artificial state of relative endothelial dysfunction should result in reduced insulin-mediated glucose uptake secondary to reduced glucose delivery to skeletal muscle vascular beds. This was tested in the study described in chapter 5, with the conclusion that systemic inhibition of NO synthase had no effect on whole-body insulin sensitivity. This supports the notion that substrate delivery is not rate-limiting for glucose uptake and that primary endothelial dysfunction is not the main factor leading to relative insulin resistance.

However, this study is also open to criticism. Ideally, a local infusion of L-NMMA should have been given on a background of systemic hyperinsulinaemia and any changes in forearm glucose uptake. Unfortunately, due to restrictions imposed by the Ethics Committee concerning the lumen size of brachial artery needles, it was impossible to obtain the arterial samples necessary for measurement of forearm glucose uptake. Delivering L-NMMA systemically may not have the same effect as local infusion due to differential sensitivity to this substance in renal and splanchnic

vascular beds. Thus, it cannot necessarily be assumed that limb vasoconstriction occurs in response to systemic L-NMMA during clamp studies. In a sub-study, forearm vasoconstriction was achieved in fasting conditions, but this could not be assessed in the context of systemic hyperinsulinaemia, since previous studies in the department have indicated that the procedure of hand-warming can affect blood flow measurements. Furthermore, while this dose of systemic L-NMMA is sufficient to exert haemodynamic effects, it is unclear to what degree endothelial function is blunted. To test this further, local endothelium-dependent vasodilators could be infused on a background of L-NMMA to give an indication of the degree of induced endothelial dysfunction.

However, despite retrospective criticism of study design, the result supports the working hypothesis which emphasises that metabolic derangement is the primary abnormality resulting in vascular dysfunction. For further study, it would be of interest to administer an agent which is known to induce temporary insulin resistance (for example, corticosteroid) and examine whether endothelial function deteriorated as a consequence. Of course, it could be argued that any such agent might be exerting direct effects on the endothelium as well as influencing insulin action.

(iv) Insulin action and endothelial function in type 2 diabetes and essential hypertension

Having studied aspects of insulin action and endothelial function in young healthy volunteers, the next step was to study similar variables in older subjects and, specifically, in patients with essential hypertension and type 2 diabetes, conditions

characterised by resistance to insulin-mediated glucose uptake. The results are presented in chapter 7 and, in general terms, similar conclusions apply. The numbers in each sub-group are small (n=9) due partly to the time-consuming nature of the study days and difficulties in recruiting subjects according to strict inclusion and exclusion criteria. To facilitate a 'clean' study, participating patients tended to have very mild disease, certainly not typical of the average clinic patient with multiple problems and on multiple drugs. While this ensured that sub-groups were well-matched for possible confounders such as lipids, renal impairment and co-existing macrovascular disease, it led to significant overlap with 'healthy controls' in the major variables studied, rendering most inter-group comparisons meaningless.

Nevertheless, a number of conclusions can be made from the data:

- Insulin's vascular action persists in older men although vasodilation is not as marked as in younger leaner subjects.
- The time-course of insulin vasodilation is similar in young and old, but the predominant mechanisms may be different. Comparing data from chapters 3 and 7, there is a good correlation between insulin-mediated vasodilation and basal endothelial NO production in the older subjects but not in the younger group, where basal Na^+K^+ATPase activity appeared to be most closely associated with insulin vasodilation.
- The delayed vasoconstrictor action of L-NMMA on a background of systemic hyperinsulinaemia supports the notion that insulin stimulates endothelial NO production

- In support of previous data in young volunteers, insulin sensitivity and basal endothelial function are significantly related in older subjects. However, in multiple regression analysis, insulin sensitivity does not persist as an independent predictor of NO production when insulin vasodilation is included in the model. Although not direct evidence of causality, this supports the concept of insulin's stimulation of endothelial NO as a key intermediate mechanism linking insulin-mediated glucose uptake and basal endothelial function. It must be emphasised that insulin-mediated glucose uptake is predominantly into skeletal muscle cells rather than endothelial cells, and there is no evidence to date for the existence of GLUT4 transporters in vascular endothelial cells. However, this does not rule out a functional link since either the post-receptor insulin-signalling pathway for glucose metabolism may be preserved in endothelial cells or a product of intracellular glucose metabolism in skeletal muscle cells may diffuse back to stimulate endothelial NO production.

(v) *Ex vivo* studies on insulin action in skeletal muscle and platelets

To complement the clinical physiology studies, a number of small *ex vivo* studies were undertaken, the results of which are presented in chapters 6, 8 and 9.

There is published evidence to suggest that insulin inhibits platelet aggregation and that this function is defective in insulin-resistant subjects. Using a washed platelet technique it was possible to quantify aggregation to thrombin (section *2.10*) in a number of subjects who were attending for clinical studies. The main conclusion from this preliminary study was that neither whole-body insulin sensitivity or local

insulin/glucose-mediated vasodilation was associated with platelet sensitivity to thrombin-induced aggregation (chapter 8). Based on the experience gained in this study, further studies are planned involving *in vitro* exposure of platelets to varying doses of insulin and glucose. It is also intended to explore possible mechanisms of aggregation inhibition which may be related to platelet NO production.

It has been suggested that inherent structure/function characteristics of skeletal muscle may partly explain the observed association between insulin action and vascular endothelial function since predominance of fibre type, which has been linked with insulin sensitivity, tends to differ in parallel with capillary density, which may contribute to measurements of endothelial function. In chapter 6 the hypothesis was tested that variations in local insulin/glucose-mediated vasodilation were related to capillary density, measured by immunohistochemical techniques on samples of skeletal muscle biopsied from vastus lateralis. This was essentially a negative study, suggesting that denser capillary beds do not appear to influence the vasodilator response to insulin.

Finally, a collaboration was initiated with experts in membrane biochemistry and electron microscopy with a view to explore cellular mechanisms of insulin resistance (section *1.10*) using immunogold electron microscopy techniques to directly visualise GLUT4 in skeletal muscle cells (section *2.11*). This difficult technique has been developed to a stage where a number of interesting studies can be proposed. The primary aim of this work is to characterise possible defects in GLUT4

translocation which may be responsible for cellular insulin resistance. The progress of this project is discussed in detail in chapter 9.

As a side-arm to the EM project the intention was to measure key steps of the insulin signalling pathway (*1.10.4*) such as IRS-1, IRS-2, PI3-K, PKB and GLUT4 in skeletal muscle tissue obtained by percutaneous biopsy. Unfortunately, while histochemical analysis and EM processing only requires a small amount of tissue, significantly more is required for biochemical assays which limited the feasibility. In view of this problem, the technique of myoblast cell culture has been learned and this is currently being developed with a view to studying the insulin signalling pathways in myoblasts cultured from subjects who have been well characterised for insulin action *in vivo*.

Conclusions

- Insulin is a vasoactive hormone.
- Mechanisms of insulin-mediated vasodilation include stimulation of endothelial NO production and membrane hyperpolarisation via stimulation of Na^+K^+ATPase.
- Insulin-mediated vasodilation occurs gradually over 40-50 minutes suggesting involvement of intermediate mechanisms.
- Cellular glucose uptake augments insulin-mediated vasodilation.
- Whole-body insulin-stimulated glucose uptake and local insulin/glucose vasodilation are functionally linked.

- Systemic inhibition of NO production does not impair whole-body insulin-mediated glucose uptake.
- Skeletal muscle capillary density is not associated with insulin's vascular action.
- Insulin resistance is not associated with increased sensitivity of washed platelets to thrombin-induced aggregation.
- Insulin-mediated vasodilation is preserved in older men but is blunted in parallel with reductions in insulin sensitivity.
- Vasoconstriction in response to local inhibition of NO synthase is delayed during hyperinsulinaemia compared with fasting conditions suggesting that insulin stimulates eNOS activity.
- Insulin sensitivity correlates with basal endothelial NO production in both young and older men.
- Insulin-mediated vasodilation correlates with basal endothelial NO production in older, but not in young men.
- Insulin's stimulation of endothelial NO production may be a key intermediate mechanism linking insulin sensitivity with basal endothelial function.

Publications containing work undertaken for this thesis:

i) Reviews

Cleland SJ, Petrie JR, Ueda S, Elliott HL, Connell JMC. (1998) Insulin as a vascular hormone - implications for the pathophysiology of cardiovascular disease. *Clin Exp Physiol Pharm* 25: 175-184.

Cleland SJ, Petrie JR, Ueda S, Elliott HL, Connell JMC. (1998) Mechanisms of insulin-mediated vasodilation in man. *Current Opinion Endocrin Diab* 5: 217-222.

Cleland SJ, Petrie JR. (1998) Insulin resistance in hypertension. *Curr Med Lit - Cardiol* 17: 95-100.

Petrie JR, **Cleland SJ**. (1997) Insulin sensitivity and endothelial function: a physiological relationship with pathophysiological significance? *Nutr Metab Cardiovasc Dis* 7: 113-116.

ii) Papers

Cleland SJ, Petrie JR, Ueda S, Elliott HL, Connell JMC. Insulin-mediated vasodilation and glucose uptake are functionally linked in man. *Hypertension* (in press - Jan '99).

Ueda S, Petrie JR, **Cleland SJ**, Elliott HL, Connell JMC. (1998) NO more arginine?: insulin vasodilatation and the 'arginine paradox'. *Lancet* **351:** 959-960.

Ueda S, Petrie JR, **Cleland SJ**, Elliott HL, Connell JMC. (1998) The vasodilating effect of insulin is dependent on local glucose uptake - a double-blind, placebo-controlled study. *J Clin Endocrin Metab* 83: 2126-2131.

Cleland SJ, Petrie JR, Morris AD, Ueda S, Dorrian CA, Connell JMC. (1996) FIRI: a fair insulin resistance index? (letter) *Lancet* 347: 770.

Cleland SJ, Petrie JR, Ueda S, Elliott HL, Connell JMC. Systemic inhibition of endothelial nitric oxide synthesis does not cause insulin resistance in man. (submitted for pub^{n} to *J Hypertension*)

Cleland SJ, Petrie JR, Ueda S, Elliott HL, Connell JMC. Physiological mechanisms of insulin-mediated vasodilation in man. (Submitted for pub^{n} to *Q J Med*)

Oral Presentations to Learned Societies containing work undertaken for this thesis:

Caledonian Society of Endocrinology, Peebles: 1995
"The vasodilating effect of insulin is dependent on local glucose uptake".

Scottish Society of Experimental Medicine, Edinburgh: 1996
"Increased local concentrations of glucose and nitric oxide enhance insulin-mediated vasodilation in man".

British Hypertension Society, Cambridge: 1996
"Systemic inhibition of endothelial nitric oxide synthesis enhances insulin sensitivity in man".

British Diabetic Association, Exeter: 1996
"Systemic inhibition of endothelial nitric oxide synthesis enhances insulin sensitivity in man".

British Hypertension Research Group, Hexham: 1997
"Direct vascular effects of insulin - an exploration of underlying mechanisms in man"

British Hypertension Society, Bristol: 1997
"Insulin vasodilation is abolished by L-NMMA, angiotensin II and ouabain"

Caledonian Society of Clinical Pharmacology, Dundee: 1997 **(Prize presentation)**
"Direct vascular effects of insulin - an exploration of underlying mechanisms in man"

Scottish Society of Experimental Medicine, Dundee: 1997 **(Prize presentation)**
"Insulin vasodilation is abolished by L-NMMA, angiotensin II and ouabain"

Young Investigators Initiative Group of the European Society of Hypertension, Amsterdam: 1997
"Direct vascular effects of insulin: exploring underlying mechanisms in man using venous occlusion plethysmography"

Scottish Society of Experimental Medicine, Glasgow: 1997
"Insulin-mediated vasodilation and glucose uptake are functionally linked in man"

Hypertension in Diabetes Study Group (EASD), Budapest: 1998
"Insulin-mediated vasodilation and glucose uptake are functionally linked in man"

Estonian Society of Hypertension, Tartu: 1998 **(Invited lecture)**
"Insulin resistance, endothelial dysfunction and essential hypertension"

American Council for High Blood pressure research, Philadelphia: 1998
"Insulin-mediated vasodilation and glucose uptake are functionally linked in man"

Caledonian Society of Endocrinology, Peebles: 1998 **(Caledonian Prize lecture)**
"Insulin action and endothelial function"

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Abbreviations:

ACE	angiotensin converting enzyme
AGEs	advanced glycosylation end-products
ANGII	angiotensin II
ANOVA	analysis of variance
ATP	adenosine triphosphate
BMI	body mass index
BP	blood pressure
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
CIRU	Clinical Investigation & Research Unit
ED50	estimated dose required for 50% maximal effect
EGP	endogenous glucose production
ELISA	enzyme-linked immunosorbent assay
EM	electron microscopy
eNOS	endothelial nitric oxide synthase
FBF	forearm blood flow
FBFR	forearm blood flow ratio
FFA	free fatty acid
FVIIc	factor VIIc
GLUT	glucose transporter
GSA	glucose specific activity

HDL	high density lipoprotein
HOT	Hypertension Optimal Treatment
IDDM	insulin-dependent diabetes mellitus
IGF-1	insulin-related growth factor type 1
IGT	impaired glucose tolerance
IQ	interquartile
IRS	insulin receptor substrate
LDL	low density lipoprotein
L-NMMA	N_G-monomethyl-L-arginine
LPL	lipoprotein lipase
M	metabolised glucose (insulin sensitivity)
MAP	mean arterial pressure
MCR	metabolic clearance rate of glucose
NO	nitric oxide
NOS	nitric oxide synthase
PAI-1	plasminogen activator inhibitor type 1
PBS	phosphate buffer saline
PET	positron emission tomography
PI3K	phosphatidyl inositol 3-kinase
PKB	protein kinase B
PRC	plasma renin concentration
PRP	platelet rich plasma
R_a	rate of appearance
R_d	rate of disappearance

RAMAP	rabbit anti-mouse alkaline phosphatase
RAMPo	rabbit anti-mouse peroxidase
RIA	radioimmunoassay
SC	space correction
SD	standard deviation
SE	standard error
SHEP	Systolic Hypertension in the Elderly Program
SPG	Sorenson phosphate buffer
SSPG	steady-state plasma glucose concentration
tPA	tissue plasminogen activator
UC	urinary correction for glucose loss
UKPDS	UK Prospective Diabetes Study
VLDL	very low density lipoprotein
VSMC	vascular smooth muscle cell
vWF	von Willebrand factor