GLUCOSE UPTAKE
IN
SKELETAL MUSCLE

By

Cathryn Kolka, BSc (Hons)

A thesis submitted in requirement for the degree of

Doctor of Philosophy

Division of Biochemistry

University of Tasmania

2006

Table of Contents

STATEMENT

The work in this thesis has been undertaken exclusively for the use of a Ph.D. in the area of Biochemistry, and has not been used for any other higher degree or graduate diploma in any university. All written and experimental work is my own, except that which has been referenced accordingly.

Cathryn Kolka

AUTHORITY OF ACCESS

This thesis may be available for loan and limited copying in accordance with the Copyright Act 1968.

Cathryn Kolka

ABSTRACT

Glucose uptake occurs in skeletal muscle under basal conditions, and increases in response to stimuli such as insulin and exercise. Exercise is known to increase blood flow, and it appears that insulin has similar hemodynamic effects, including increased blood flow and capillary recruitment, which can modify the amount of glucose uptake occurring under each condition. Here we study factors affecting both basal and stimulated myocyte glucose uptake, with a particular focus on vasoactive agents.

Insulin stimulates the release of endothelin-1 (ET-1), a potent vasoconstrictor, from endothelial cells in culture. As yet it is unknown whether ET-1 is a type A (causing nutritive perfusion) or a type B (non-nutritive) vasoconstrictor, so here we use the pump-perfused rat hindlimb to characterize the distribution effects of ET-1. We show that ET-1 causes a type A vasoconstriction, stimulating basal metabolism at low doses, while at high doses the distribution of flow changes to become non-nutritive, inhibitory to metabolism. As a general vasodilator prevents both metabolic and hemodynamic effects, the effects on metabolism are due to the redistribution of flow. These redistribution effects are confirmed by the ability of high dose ET-1 to decrease aerobic tension development in the contracting hindlimb, and by the ability of low dose ET-1 to increase the interstitial glucose concentration.

Given this understanding of the effects of ET-1 alone, we can investigate the interactions between ET-1 and insulin. In the perfused rat hindlimb, insulin has not been observed to have any vasodilatory effect, whereas here for the first time insulin appears to have vasodilator-like actions against ET-1 mediated vasoconstriction. Also, the redistribution of flow by ET-1 does not appear to alter the metabolic effect of insulin to cause glucose uptake at either dose of ET-1 used.

Nitric Oxide (NO) is thought to be the mechanism by which insulin causes vasodilation in muscle. A previous study has shown that methacholine (MC), by increasing NO, was able to augment insulin-mediated glucose uptake and capillary recruitment, while other NO donors were unable to do so. Here we show that, at the dose used to increase glucose uptake in the previous study, MC has only a vasodilatory effect, and no direct effect on glucose uptake, in the perfused rat hindlimb. At higher doses, an effect on glucose uptake can be observed. This means that the increase in capillary recruitment by MC was responsible for the elevated insulin-mediated glucose uptake, and there was no direct effect of MC on glucose uptake.

A recent publication suggested that the Na^+-D-glucose cotransporter (SGLT1) was essential for insulin-mediated glucose uptake, although not required for basal glucose uptake. The implications of this detract from our proposed role of blood flow redistribution in insulin action. In attempting to reproduce these results in the perfused rat hindlimb we found that SGLT1 is not required for insulin-mediated glucose uptake, and confirmed this using a low sodium buffer, which would also inhibit the transporter. We conclude that SGLT1 is not required for insulin-mediated glucose uptake.

Our results therefore suggest that complex interactions are involved in insulin action, some of which involve hemodynamic actions that are capable of altering insulin-mediated glucose uptake, and others in which insulin itself can limit the action of other vasomodulators, such as ET-1. It is apparent, however that SGLT1 in the endothelium may not be necessary for the metabolic effects of insulin, and that blood flow distribution, or capillary recruitment is therefore of great importance in delivering glucose to myocytes.

ACKNOWLEDGMENTS

I wish to thank Prof Clark, for his guidance and supervision during the last four years, without him I am sure my work would not have gone as well, and the ‘Steve’s’ (Rattigan and Richards), for all their help and suggestions along the way.

I would also like to thank many past and present members of the muscle research group, including Cate, Hema, Lei, Georgie, Maree, Phil, Amanda, John, Carole, Merren and Geoffrey, for making my time here enjoyable. An extra special thanks to Renee and Eloise – for your friendship and help along the way.
Also thanks to those hard workers in the animal house, especially Marcus and Murray, and all the friends I have made in the rest of the building as well, particularly those in the MBU.

Thanks also go to my family, friends and to Justin for their love, support and friendship.

ABBREVIATIONS

A-V	arterio-venous
ANOVA	Analysis of variance
AII	Angiotensin II
e	extensor digitorum longus
EDRF	Endothelium-derived relaxing factor
ET	Endothelin
ET-1	Endothelin-1
ET_A	Endothelin receptor type A
ET_B	Endothelin receptor type B
GLUT	facilitative glucose transporters
Ins	Insulin
L-NAME	Nitro-L-Arginine Methyl Ester
L-NMMA	mono-methyl nitro-L-arginine
MC	Methacholine
NE	Norepinephrine
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
eNOS	endothelial NOS
iNOS	inducible NOS
nNOS	neuronal NOS
Nox	end-products of oxidized NO
p	plantarus
r	red gastrocnemius
R'g	Rate of glucose uptake
s	soleus

SGLT1	sodium-glucose co-transporter
SNP	sodium nitroprusside
t	tibialis
VO2	Oxygen consumption
w	white gastrocnemius
1-MX	1-methylxanthine
5HT	serotonin

PREFACE

Some of the data presented in this thesis has been published or presented at scientific meetings and has been listed below.

Publications arising directly from this thesis

Kolka CM, Rattigan S, Richards S, Clark MG. Metabolic and vascular actions of endothelin-1 are inhibited by insulin-mediated vasodilation in perfused rat hindlimb muscle. British Journal of Pharmacology. 2005 May 16

Kolka CM, Rattigan S, Richards SM, Barrett EJ, Clark MG. Endothelial Na+ -D-glucose cotransporter: no role in insulin-mediated glucose uptake. Hormone and Metabolic Research. 2005.

Mahajan H, **Kolka CM**, Newman JMB, Rattigan S, Richards SM, Clark MG. Vascular and metabolic effects of methacholine in muscle. Circulation Research.

Rattigan S, Zhang L, Mahajan H, **Kolka CM**, Richards SM, Clark MG. Factors influencing the hemodynamic and metabolic effects of insulin in muscle. Current Diabetes Reviews, in press (2006).

Kolka CM, Rattigan S, Richards S, Clark MG. Reduced exercise capacity in hypertension: a consequence of endothelin-mediated functional shunting. (Manuscript in preparation.)

Other publications

Wallis MG, Smith ME, **Kolka CM**, Zhang L, Richards SM, Rattigan S, Clark MG. Acute glucosamine-induced insulin resistance in muscle in vivo is associated with impaired capillary recruitment. Diabetologia. 2005

Posters at scientific meetings

American Diabetes Association 64th Scientific Sessions, June 4th-8th 2004, Orlando, Florida.

Endothelin-1 as a messenger for insulin has both stimulatory and inhibitory effects on perfused muscle metabolism via its vascular actions. **Kolka CM**, Rattigan S, Richards S, Clark MG.

Freycinet Conference on: Diabetes and Exercise: Impact of Muscle Blood Flow, Freycinet, Tasmania, 18th-20th August 2004.

Endothelin-1 via its vascular actions in muscle can be either supportive or antagonistic of insulin. **Kolka CM**, Rattigan S, Richards S, Clark MG.

European Association for the Study of Diabetes annual meeting, Sept 11th-14th 2005, Athens, Greece.

Endothelin-1 vascular and resultant metabolic actions in perfused rat hindlimb are opposed by insulin. **Kolka CM**, Rattigan S, Richards SM, Clark MG.

Glucosamine induces acute insulin resistance in muscle in vivo associated with impaired capillary recruitment. Clark MG, Wallis MG, Smith ME, **Kolka CM**, Zhang L, Richards SM, Rattigan S.

Heart Foundation Conference and Scientific Meeting, March 23rd-25th 2006, Sydney Australia

Reduced exercise capacity in hypertension: A consequence of endothelin-mediated functional shunting of blood flow in muscle. **Kolka CM**, Rattigan S, Richards SM, Clark MG.

CHAPTER 1

INTRODUCTION

GLUCOSE UPTAKE IN SKELETAL MUSCLE

Glucose and other fuels enter the body after a meal, and the body maintains a constant level of blood glucose by stimulating glucose disposal into muscle and adipose tissue and inhibiting hepatic glucose production. The main hormone responsible for these effects is insulin, which also has effects on cell growth and development, ion transport, and sympathetic nervous system activity (reviewed in (1)). Exercise is also capable of increasing glucose uptake into muscle, most likely through a combined effect including the increase in delivery of blood flow, and thereby glucose, to the exercising area, and a cellular effect involving translocation of GLUT4. Insulin is able to alter blood flow through muscle as well, which can affect the level of glucose uptake that can occur. The focus of this study will be the uptake of glucose into myocytes and factors that may alter both basal and insulin-mediated glucose uptake, particularly those involved in changing the blood flow distribution through muscle.

1.1 Metabolic effects of insulin in skeletal muscle

Skeletal muscle relies on glucose for much of its energy requirements, and as such is one of the major sites of glucose uptake in the body (2), although the brain requires large amounts of glucose under basal conditions. Insulin release after a meal allows circulating glucose to be taken into muscle, helping to maintain glucose homeostasis, and supplying energy to myocytes.

1.1.1 Insulin-mediated glucose uptake by muscle requires glucose transporters

Diffusion barriers exist for glucose at the cell membrane and there are two main groups of glucose transporters to overcome this barrier. They are the Na^+ dependent sodium-glucose cotransporters (SGLTs) and the Na^+ independent glucose transporters, or facilitative glucose transporters (GLUTs) (reviewed in (3)). Many of these glucose transporters are not specifically activated by insulin, and may only be involved in basal glucose uptake. Both forms of glucose transporters are relevant to the work involved in this thesis, and are therefore discussed further.

Sodium glucose transporters

At least three, and possibly up to six, SGLTs have been identified (4). Although originally these glucose cotransporters were thought to be primarily located in the intestine and kidney, recent studies have shown that SGLT1 may be located in the coronary artery (5) and in the endothelial cells of skeletal muscle capillaries (6).

SGLTs are primarily involved in absorption of glucose from the intestine, and reabsorption in the kidney. The Na^+-D-glucose cotransporter SGLT1 is found in epithelial cells of the intestine and proximal renal tubule, where it plays a central role in the absorption of glucose and galactose from food and the reabsorption of glucose from the glomerular filtrate (7). In both locations the process of uptake is coupled to a Na^+/K^+ ATPase positioned on the serosal side of the cell to pump out the co-transported Na^+ (4). The coupling to energy expenditure in the intestinal and renal epithelial cells provides a process whereby even small concentrations of glucose can be scavenged (discussed in (8)), transporting glucose against the concentration gradient (4). The SGLT proteins appear to be more active in low glucose situations, and glucose uptake may be inhibited by high cytosolic glucose (9). While the primary function appears to be absorbing and preserving dietary glucose, SGLT1 also seems to be located in areas where glucose is essential for body functions in situations of starvation, such as the brain (9), cardiomyocytes (10), and neurons (11). In these locations SGLT1 may be able to scavenge the little available glucose for tissues that are essential for survival, similar to another SGLT protein that has been isolated (12).

Early studies used phlorizin, an SGLT1 inhibitor, to block insulin mediated glucose uptake, as well as other sugars, in skeletal muscle and cardiomyocytes without affecting insulin-mediated amino acid transport (reviewed in (13)). Many studies suggested that these effects were specific to insulin action to increase glucose uptake (14, 15), although other studies discovered that phlorizin may also affect the basal level of glucose uptake as well (16) (reviewed in (13)). These early studies used doses of phlorizin that may have been high enough to also block GLUT1 and GLUT4 glucose transport (1-5mM) (14-16). There has been more recent evidence to indicate that SGLT1 in small intestine is regulated by insulin, as activity of the protein was increased in an experimental form of type 1 diabetes, and activity was returned to normal following treatment with insulin (17). In these diabetic rats, SGLT1 protein content was elevated, suggesting some translational control of SGLT1 by insulin.

Functional characterisation of these transporters is continuing, and a recent study has proposed a role of SGLT1 in insulin-mediated glucose transport from the blood vessel to the myocytes in skeletal muscle (6). This study used immuno-localization techniques to demonstrate the presence of SGLT1 on the endothelial cells of skeletal muscle capillaries, and then showed that phlorizin, at doses low enough to be a specific inhibitor of SGLT1 without affecting GLUT1 and GLUT4, was able to block insulin-mediated glucose uptake, with no apparent effect on basal glucose uptake. This suggests that SGLT1 is responsible for all glucose uptake into skeletal muscle that occurs with insulin. Such a finding presents a potential rate-limiting step for glucose uptake into muscle: a step that is regulated by insulin and thus relevant to this thesis.

Facilitative glucose transporters

The facilitative glucose transporters, or GLUTs, use the concentration gradient of glucose to allow diffusion of glucose across plasma membranes. The diffusion rate is greater than would be expected for a molecule of the size of glucose, so it is likely that transporters are required to lift this rate, possibly through a more complex mechanism than simple diffusion (reviewed in (3)). There are thirteen members in this family, which exhibit different substrate specificities and tissue distribution profiles. GLUT1 and GLUT4 co-exist in insulin-sensitive tissues including skeletal

muscle; GLUT1 is thought to play a role in basal glucose uptake, and is located in the myocyte plasma membrane (18). The insulin-responsive glucose transporter is GLUT4, and is found in brain, heart, skeletal muscle and adipose tissue. Insulin stimulates the translocation of GLUT4 from an intracellular store to the plasma membrane to increase glucose uptake in myocytes as well as in adipocytes (18-20). A greater level of GLUT4 translocation permits greater insulin sensitivity, as observed by glucose disposal (21), and different levels of GLUT4 protein content between muscle may account for insulin sensitivity differences between muscles (22). Exercise also may be able to induce the translocation of the GLUT4 protein to the plasma membrane (23), although appears to draw on a different intracellular pool of GLUT4 than insulin (reviewed by (24).

1.2 Insulin has hemodynamic effects in skeletal muscle

Insulin has been shown to have effects on blood vessels. Initially, insulin was found to cause an increase in blood flow to skeletal muscle (25), and since this first study, many other studies have helped to develop a more complete picture of insulin-mediated hemodynamic effects. The three main effects include an increase in blood flow, vasodilation and capillary recruitment.

Increased blood flow

Although it had been known for a least sixty years that high levels of insulin, often in the pharmacological range, increased cardiac output and skeletal muscle blood flow (26), Laakso et al were the first in more recent times to show, using a thermodilution technique, that insulin acts to increase leg, and therefore muscle, blood flow in a dose-dependent manner (25). Tack et al (27) showed that insulin was able to cause increases in total flow, but these increases were only occasionally noted at physiological insulin concentrations, and the greatest effect was noted after several hours. The fraction of blood flow through muscle increases as a function of total flow, and skeletal muscle accounts for most of the blood flow through the limb (28). Venous occlusion plethysmography has also been used in humans (27, 29, 30) and supports the evidence provided by other methods that shows an increase in flow. It

appears that insulin can cause hemodynamic effects that can increase blood flow and decrease vascular resistance: effects that are consistent with vasodilation (31).

Vasodilation

Evidence from studies of large vessel responses has shown that insulin can decrease the stiffness of the vessel wall, and cause vasodilation. Chaudhuri et al (32) observed an increase in diameter of the internal carotid artery that became significant at 15 minutes after treatment with insulin. Results obtained by Ueda et al (33) in humans showed insulin to be a weak local vasodilator, although this effect was amplified in the presence of glucose. This finding was supported by the research of Tack et al (27), who found that local physiological hyperinsulinemia induced a slow vasodilation that became maximal about three hours after the first infusion. In the presence of hyperglycemia, there was a quicker onset of vasodilation, but the same maximum was reached.

Capillary recruitment

Rattigan et al (31) suggested that insulin causes capillary recruitment, as measured in rats by a metabolic marker 1-methylxanthine (1-MX), which is targeted to endothelial xanthine oxidase. The products of this enzyme increased with insulin treatment, suggesting that more of the endothelium-bound enzyme had been exposed to the substrate 1-MX, which was constantly infused to maintain steady-state concentrations in the blood. This means that more of the capillary wall is exposed to blood flow, which implies that perfusion of previously unperfused capillaries has occurred, most likely in muscle. Another method that has been applied in rats is contrast enhanced ultrasound (34), an imaging technique, which showed that insulin increased the capillary blood volume, without increasing velocity of red blood cells. Laser Doppler flowmetry has also been used in the rat, where insulin was found to increase capillary recruitment (35). Vincent et al (36) discovered that skeletal muscle microvascular recruitment occurred with physiological hyperinsulinemia, as measured using 1-MX and contrast enhanced ultrasound in rats, and that this capillary recruitment occurred before any increase in blood flow was observed.

Serne et al (37) showed that capillary density is proportional to insulin sensitivity in human skin, as it decreases the diffusion distances from the capillary to the cells. If the capillary density decreased, then there was an observed increase in vascular resistance. The decrease in capillary density may be due to a decrease in the vasodilation at the precapillary level, preventing perfusion of the smaller capillaries.

Nutritive and non-nutritive flow routes in muscle

The concept of two distinct vascular routes in muscle was first proposed by Pappenheimer (38), who noted that various vasoconstrictors could have different effects of the oxygen uptake by muscle. It was found that in certain circumstances blood flow could continue, but the interior of the muscle would be less perfused (39). This suggests that the blood can pass through a shunt, lowering the perfusion of the muscle. Later, it was found that radiolabelled sodium disappeared at different rates from muscle depending on the area of injection, leading to the proposal of nutritive and non-nutritive pathways (40). The nutritive pathway is believed to be in contact with skeletal muscle cells, while the non-nutritive pathway is shunted through septa and tendons away from muscle (41), and is possibly associated with adipose tissue (42). This is shown by the inverse relationship between tendon blood flow and oxygen consumption, an indicator of metabolism (41). The regulation of blood flow through these pathways, by vasodilation and vasoconstriction, is proposed to control the metabolism of the muscle (43, 44).

1.2.1 The hemodynamic effects of insulin can increase insulin-mediated glucose uptake

It is possible that the hemodynamic effects of insulin may increase glucose and insulin delivery to tissues. Rattigan et al (45) showed in a simple experiment that the vascular system may be involved in insulin action on glucose uptake, as serotonin caused insulin resistance in the form of reduced glucose uptake in a perfused rat hindlimb preparation, but not in isolated incubated muscles. Baron (46) suggested two physiological roles for insulin-induced vasodilation: one to modulate vascular resistance and blood pressure, and the other to allow greater substrate and hormone delivery to insulin sensitive tissues for better control of fuel disposal. In this way, the

hormone that is responsible for increasing glucose uptake is also able to act on the vasculature to potentiate its own metabolic effect (47-49).

Total blood flow

In rats, in the absence of insulin, it was shown that a low flow of perfusate caused a lower level of glucose uptake when compared to high perfusate flow (50). This showed that glucose delivery to myocytes in the pump-perfused muscle preparation is the rate-limiting step for glucose uptake in skeletal muscle. Avogaro et al (51) suggested that the hemodynamic effects of insulin might amplify insulin-mediated glucose uptake. This was supported by the observation that insulin led to an increase in blood flow in the perfused rat hindlimb, which increased insulin sensitivity and glucose uptake *in vitro* (50). Also, *in vivo* an increase in flow achieved by methacholine vasodilation caused an increase in insulin-mediated glucose uptake (52).

In healthy human subjects Laakso et al (25) found that insulin could generate a two-fold increase in blood flow, which can account for up to 40% of insulin-mediated glucose uptake in the very high physiological range of insulin. Baron (1) suggested that, in theory, augmentation of either glucose extraction or flow could increase glucose uptake in the target muscle. However other researchers have shown that glucose uptake occurs before any observed changes in blood flow, and so therefore blood flow probably has no effect on the initial insulin-mediated glucose uptake in the acute stages of insulin exposure (3, 53, 54). Fugmann et al (29) suggest early insulin-mediated glucose uptake is due to the effect of insulin increasing glucose transport in the tissues. Eventually, insulin-mediated increases in blood flow can become a major determinant of the degree of glucose uptake.

Pendergrass et al (55) found that increasing forearm blood flow did not increase forearm glucose uptake in humans. Zierler (3) also found that increasing leg blood flow had no effect on glucose uptake. Nuutila et al (56) made a similar observation using bradykinin to increase the blood flow over that achieved by insulin, without observing an increase in glucose uptake. To explain this result, it was hypothesized that any increase in flow leading to a greater delivery of glucose and insulin to tissues will not increase glucose uptake unless previously unperfused areas are made available. For example, in the study by Nuutila et al (56), bradykinin was used to

increase blood flow. Bradykinin causes venodilation, which may not have any effect on capillary recruitment. Also, venodilation may change the nature of flow through the muscle, without increasing the muscle perfusion or recruiting more capillaries than insulin alone. Simply, insulin may have already caused capillary recruitment, and any further hemodynamic effects that will not augment microvascular perfusion will thus not affect glucose uptake.

As reviewed by Zierler (3), the effects of insulin on limb blood flow are controversial. Often the responses noted are due to pharmacological or super-physiological levels of insulin. Hernandez et al (57) showed that, after a meal, there were no significant changes in leg blood flow, and therefore that leg blood flow does not increase enough to be a major determinant in glucose uptake. However, the authors acknowledge that the nature of the meal may be involved in determining the extent of the increase in limb blood flow, and no measurements of insulin levels were taken, so an accurate correlation between limb blood flow and insulin levels, and the effects on glucose uptake, could not be performed. Also, Taddei and Salvetti (58) showed that hyperinsulinemia did not necessarily have to increase blood flow to potentiate vasodilation. Thus, vasodilation can occur without any changes to total flow. A study by Clark et al (35) focussed on the changes in flow upon the administration of insulin, and concurrently measured the amount of nutritive flow by Laser Doppler Flowmetry. It was found that an increase in nutritive flow was observed 30 minutes before a blood flow response was noted. These data suggest that any measurement of total flow as an indication of vasodilation does not take into account any local vasodilation or capillary recruitment. Therefore, capillary recruitment may be more effective than increasing limb blood flow at augmenting glucose uptake.

Capillary Recruitment

As reviewed in Baron (1) the vasomotion of capillaries allows flow through each capillary to be altered in response to different stimuli. If capillary recruitment occurs, a greater exposure of tissue to glucose can be expected, resulting in a larger volume of distribution of glucose. As mentioned previously, this may be a rate-limiting step of glucose disposal (50), and so with a larger area of diffusion for glucose, the rate-limiting step is reduced.

Baron et al (59) found that insulin sensitivity correlated well with leg glucose uptake and suggested that the perfusion of the leg was limiting to glucose uptake. It was hypothesized that observed reductions in insulin sensitivity may be due to alterations in regional blood flow, which causes an impaired delivery of substrate (glucose) or insulin to tissues, thereby dampening the insulin response (60). The ability of insulin to dilate the macrovasculature in skeletal muscle was directly proportional to the glucose metabolism that can be induced by insulin (61). In addition, the insulin-mediated vasodilation in skeletal muscle augments the effect of insulin to stimulate glucose uptake. Other studies support this finding, and the proposal that skeletal muscle blood flow can, by means of increased substrate delivery, account for up to 20-30% of insulin-mediated glucose uptake (52, 62).

Utriainen et al (63) using positron emission tomography showed that insulin was able to increase the absolute but not relative dispersion of flow, which is consistent with capillary recruitment, and was capable of redirecting blood flow to areas that perform glucose uptake. Under high levels of insulin, there was an increase in, as well as a redistribution of flow. This observation was not supported by Raitakari et al (28) or Bradley et al (64), who found that glucose uptake in response to insulin does not co-localize to the same area in muscle tissue as the observed increase in blood flow, suggesting that the hemodynamic and metabolic effects were separate. In rats, however, it was shown that flow was redirected preferentially to muscles with higher levels of oxidative type I fibres (63) which are more sensitive to insulin in terms of glucose uptake (65).

If elevated limb blood flow leads to increased muscle glucose uptake, the capillary surface area must have increased to allow the exposure of previously unperfused tissue to insulin (52). The kinetics of insulin-mediated glucose uptake suggest that if an increase in blood flow were to occur through a fixed volume of capillaries, the velocity of blood through the muscle would increase. However, if the increase in blood flow occurred through an increased capillary volume, the velocity of solutes in the blood does not increase. Results from Laakso et al (66) suggested that the volume of capillaries increased, implying that vasodilation, probably in the form of capillary recruitment, was observed. When represented as a model, if an increase in blood flow was to occur through a fixed volume of capillaries, it is likely that the increased

velocity through the vessels would lead to a decrease in glucose extraction from the blood, and therefore lower glucose uptake.

The degree of skeletal muscle perfusion has been suggested as an independent determinant of insulin-mediated glucose uptake, which tends to support the reputed role of dispersion of flow through the muscle. This is observed in studies that achieve augmentation of the leg glucose uptake response above the maximum effect achievable by insulin alone by increasing skeletal muscle perfusion (52). In studies of human skin microcirculation, capillary recruitment was found to correlate strongly with insulin sensitivity measured by insulin clamp technique, suggesting that microvascular function is an important aspect of insulin action (37). When compared to exercise, which increases both the capillary blood volume (recruitment of capillaries) and the velocity of the red blood cells, physiologic hyperinsulinemia only increases the capillary blood volume, with no effect on velocity, as assessed using contrast enhanced ultrasound (34). Higher doses of insulin may increase both capillary blood volume and red cell velocity.

1.2.2 Possible coupling between insulin-mediated glucose uptake and capillary recruitment

There is some controversy regarding the nature of the coupling of the metabolic and hemodynamic effects. Cleland et al (67) hypothesized that insulin-mediated glucose uptake in the endothelium leads to an increase in blood flow, and that the rate of glucose uptake determines vascular smooth muscle relaxation. This conclusion was based on evidence obtained in healthy men, where an intra-arterial infusion of insulin caused vasodilation, and this effect could be augmented by a concurrent infusion of glucose (67). However, it was determined that there could be no insulin-mediated glucose uptake in endothelial cells (68), and Petrie et al (69) found that the degree of insulin-induced vasodilation was determined by tissue glucose uptake, not the endothelium. Therefore glucose uptake in the muscle appears to increase vasodilation by insulin. This tends to suggest the involvement of a second messenger released after glucose uptake in muscle to cause vasodilation, although such a messenger has not yet been identified.

The findings by Petrie et al (69) have not as yet been corroborated by others, and in contrast, Utriainen et al (63) show that the rate of glucose uptake is not responsible for increases in flow. Their observation was that it is the presence of insulin, not the rate of glucose uptake, which determines the degree of vasodilation, as limb blood flow can increase in the early stages of non-insulin dependent diabetes mellitus (type 2 diabetes) in response to insulin, without a significant glucose uptake. A number of other groups also found data contrary to this concept. For example, Raitakari et al (28) showed that glucose uptake itself is not enough to cause vasodilation, as glucose oxidation under hyperglycemic conditions does not lead to vasodilation. Also, Tack et al (27) observed that maximal insulin-mediated glucose uptake occurred before the vasodilation induced by insulin, but agreed that insulin-mediated glucose uptake was not a determinant of the degree of vasodilation, as the degree of vasodilation was not dependent on glucose uptake, suggesting a direct effect of insulin to cause vasodilation. Vollenweider et al (70) and Meneilly et al (71) found that hyperinsulinemia is the main stimulus that causes vasodilation in skeletal muscle, and that glucose metabolism is an independent effect.

More recent results by Vincent and colleagues suggest that microvascular recruitment is an early event in hyperinsulinemia, as an increase in capillary blood volume measured by contrast enhance ultrasound was observed within 7 min (72). This suggested that the microvascular effects of insulin occurred prior to any significant insulin-mediated glucose uptake or increase in limb blood flow. It is possible that metabolism by myocytes initially receiving flow could lead to metabolic signals that permeate nearby tissue to enhance microvascular perfusion. The only argument against this is the observation that insulin-mediated capillary recruitment is more sensitive to insulin than insulin-mediated glucose uptake (73).

1.2.3 Insulin resistance in diabetes

Diabetes is characterized by insulin resistance – an effect that was initially thought to be a tissue (muscle) effect. Bak (74) reported that, under normal circumstances, insulin caused an increase in glucose uptake and glycogen synthesis in skeletal muscle, whereas in type 2 diabetes and cases of insulin resistance, the insulin-mediated glucose disposal is impaired. Endothelial dysfunction has since been noted

to be an important factor in diabetes, when the endothelium-dependent vasodilation is impaired, although insulin therapy can successfully improve both endothelium-dependent and –independent vasodilation (75).

1.2.3.1 Insulin resistance leads to endothelial dysfunction

A common but unsubstantiated view is that insulin resistance leads to endothelial dysfunction. For example, Pieper et al (76) have shown that hyperglycemia may lead to endothelial dysfunction, especially in those that suffer from type 1 diabetes, and in other cases can lead to hyperglycemia induced diabetes mellitus. In addition, Hsueh and Anderson (77) report that the vascular endothelium is a target organ of damage in diabetes, which can show altered function. Metabolic abnormalities resulting from hyperglycemia such as advanced glycosylation end products and reactive oxygen species can cause damage to the endothelial cells. They conclude that interventions that modulate insulin resistance can affect the endothelial response (77). There is evidence that endothelial damage in diabetes may be either the cause (78) or the consequence (51) of insulin resistance. Recent evidence that endothelial dysfunction occurs before insulin resistance has been shown in situations where offspring and relatives of insulin resistant people show impaired endothelial responses (79). As hypertensive people do exhibit both vascular impairment and insulin resistance, an important role of the vasculature in insulin sensitivity is suggested.

1.2.3.2 Flow deficit in diabetes

McVeigh et al (80) have used venous occlusion plethysmography to show that vasodilation is impaired in type 2 diabetes, involving both endothelium-dependent and –independent vasodilation. This finding is supported by Watts et al (81) who discovered that both endothelial cell and vascular smooth muscle cell function was impaired in type 2 diabetes. Nuutila et al (56) have also found a defect in the normal increase in limb blood flow observed with insulin stimulation in type 1 diabetes mellitus with hyperinsulinemia. As patients suffering from diabetes show no increase in limb blood flow, the insulin resistance observed as lower insulin-mediated glucose disposal may be due to the decrease in delivery of glucose and insulin to insulin sensitive tissues (82).

Cleland et al (83) reported that there were associations between endothelial function and both insulin sensitivity and insulin-induced vasodilation. It was hypothesized by Julius et al (82) that there was a link between insulin resistance and hypertension due to the decreased blood supply evident in both disorders. Any treatment aiming to improve the hemodynamic effects of insulin in diabetics may also be of benefit in hypertensive and obese patients, as Feldman and Bierbrier (47) propose that impaired insulin-mediated vasodilation contributes to increased peripheral vascular resistance. Mather et al (84) reported that, although insulin resistance can impair normal insulin-mediated increases in blood flow, metformin treatment can improve insulin resistance and endothelial function, although this was not supported by Natali et al (85). If the vascular system can be modified to increase skeletal muscle blood flow and perfusion, insulin resistance may be improved by correcting insulin sensitivity, glucose tolerance and hyperinsulinemia (86).

1.2.4 Mechanisms of the hemodynamic effects of insulin

Nitric oxide (NO) has long been thought to be involved in the hemodynamic effects of insulin, as the vasodilation observed with large doses of insulin can be prevented by infusing NO blockers, such as L-NAME and L-NMMA. Recent studies have indicated that the hemodynamic actions of insulin may in fact be a combination of two vasoactive substances. Misurski et al (87) showed that insulin induced biphasic responses in rat mesenteric vascular bed. An initial nitric oxide-mediated vasodilation was overcome by generation of endothelin-1 (ET-1) when the rat mesenteric vascular bed was exposed to high insulin concentrations, an effect that was blocked by an endothelin receptor antagonist. It is now thought that insulin causes the release of both ET-1 and NO, and it is a balance between these two vasoactive agents that causes the hemodynamic effects of insulin, as shown in isolated skeletal muscle arterioles (88), the rat aorta (89), and in the healthy human forearm (90). Ferri et al (91) suggested that endogenous insulin modulates the circulating concentration of ET-1, although this was determined through an oral glucose load, which was observed to cause an increase in both insulin and ET-1. Altering the balance of these two vasoactive agents (NO and ET-1) may modify normal insulin action in muscle.

1.3 Nitric Oxide is the vasodilator involved in insulin action

All hemodynamic processes that are elicited by insulin, including increased limb blood flow, vasodilation and capillary recruitment as discussed above, appear to be dependent on NO, a potent vasodilator, and are all impaired in insulin-resistant states. Steinberg (92) showed that insulin-mediated vasodilation is largely dependent on NO. As the metabolic effects of insulin appear to be dependent on limb blood flow, we can potentially alter the metabolic effects, measured by glucose uptake by modifying the available blood flow with a vasodilator such as methacholine, or by blocking the vasodilation elicited by NO using nitric oxide synthase (NOS) inhibitors.

1.3.1 Nitric Oxide

NO is a potent vasodilator that is produced by many different cells in the body, including macrophages, neurones, mast cells, hepatocytes and endothelial cells (93). NO was first found to be synthesized from L-arginine in porcine aortic endothelial cells in culture (94, 95). It has a very short half-life, and is degraded rapidly in the plasma to nitrite and nitrate, which suggests that any action elicited by NO is directed mainly at local areas, near the place of production. While NO is involved in platelet aggregation and adhesion, the main action in the circulation is vasodilation, primarily to maintain blood pressure and the control of resting vascular tone (96).

1.3.1.1 Nitric Oxide and Vasodilation

Initially, the substance that was involved in dilating the vasculature was described as endothelium-derived relaxing factor, or EDRF. This was later identified as NO, or as containing a highly labile nitroso compound capable of releasing NO, using chemical identification and comparative pharmacology, examining the effect of both EDRF and NO on vascular strips (97, 98). Endogenous NO was found to be a significant modulator of microvascular tone in skeletal muscle (99), where L-NMMA, an inhibitor of NO synthesis, was found to dose-dependently reduce microvascular diameters. NO activates guanylate cyclase by reversibly oxidising sulfhydryl groups on the enzyme for activation of guanylate cyclase (100), which elevates cGMP levels.

NO can also react with heme to form nitrosyl-heme complexes that can activate guanylate cyclase (reviewed in (101)).

In rabbit hindlimbs, blockade of NO (or EDRF) increased vascular resistance, and attenuated the normal production of cGMP in platelets. Also, the oxygen uptake of the hindlimb was reduced, which suggested that NO was involved in maintaining an adequate perfusion of the hindlimb and maintaining basal metabolism (102). Generally, NO appears to be responsible for a decrease in vascular tone through vasodilation, and is constantly released by endothelial cells to maintain a constant blood pressure and vessel tone.

L-NMMA and L-NAME are used regularly to block vasodilation, as they are inhibitors of nitric oxide synthase (NOS), the enzyme responsible for the synthesis of NO for L-arginine. There are three different NOS isoforms: eNOS (endothelial NOS), nNOS (neuronal) and iNOS (inducible).

1.3.1.2 NO in endothelial dysfunction and disease

A deficiency in the NO system appears to be involved in certain cardiovascular diseases (93, 103-105). Atherosclerosis and hypertension are associated with an impairment of the release or effect of NO, which may potentially lead to an increased blood pressure due to a lack of vasodilation. Animal models of sepsis demonstrate an increased circulating NO level, and raised levels of nitrate in human sepsis cases tends to suggest that this is true in humans as well (reviewed in (93)).

Endothelial dysfunction is characterized by a diminished blood flow in response to stimulation of eNOS (106). The response to injected methacholine or acetylcholine is often used as an indicator of endothelial dysfunction. As these cholinergic agonists activate nitric oxide synthase, a functional endothelium is required to cause vasodilation, and is used for endothelium-dependent vasodilation studies, as opposed to sodium nitroprusside, which does not require the endothelium to cause vasodilation, as the vascular smooth muscle responds directly to NO and NO donors. This process was used to examine the endothelial response to elevated free fatty acids, which were infused to mimic insulin resistant levels. In this case, methacholine-mediated vasodilation was impaired by 20%, while there was no effect on sodium

nitroprusside-mediated vasodilation (107). This suggests endothelial dysfunction, while normal NO-mediated endothelium-independent vasodilation was unaffected. Methacholine has also been used to examine endothelial dysfunction in many other studies (eg (108-112)). Endothelial dysfunction is particularly evident when insulin is used as an endothelium dependent stimulus (113). But it is not clear, even now, that insulin-dependent and methacholine-dependent vasodilation use the same mechanism, although it seems likely that NO is involved.

Even so, if eNOS is blocked with L-NMMA in rats, insulin resistance is observed (60). This insulin resistance occurred in liver and peripheral tissues of the eNOS knockout mouse, where the NOS protein is removed from the endothelial cells, compared to neuronal NOS (nNOS), where insulin resistance was only exhibited in the peripheral tissues. Zeng et al (114) found that insulin receptor tyrosine kinase (IRTK) was required for insulin-mediated NO production in human vascular endothelial cells.

1.3.2 Nitric Oxide and Insulin

It has been shown that insulin is able to activate the enzyme responsible for the release of NO (53, 115). Baron and Clark (61) reviewed data showing that insulin caused a doubling in the rate of production of venous Nox (the end products of oxidized NO), and suggested that insulin-mediated vasodilation is largely NO dependent. An association between insulin sensitivity and NO production was demonstrated (83), so insulin sensitivity is therefore proportional to insulin-mediated vasodilation.

1.3.2.1 Nitric oxide is involved in the hemodynamic effects of insulin

Blood Flow

Inhibition of NO release by L-NMMA was found to prevent any increase that would normally be observed in limb blood flow in response to insulin, and larger reductions in limb blood flow were observed in hyperinsulinemic studies compared to controls (53). Such results suggest that NO is responsible for the hemodynamic effect of

increased limb blood flow due to an insulin stimulus. As noted above, changes in limb blood flow are not the only hemodynamic effects involved in insulin action, and so a specific study of each of the effects is required.

Vasodilation

When insulin is introduced into first order arterioles from rat cremaster muscle, vasodilation was observed as an increase in the diameter of the arteriole. This increase was blocked by including L-NNA (nitro-L-arginine), a NO blocker, and by removing the endothelium (116). This suggests that the vasodilation observed in insulin action is dependent on both the endothelium and NO. While insulin-mediated vasodilation in a whole body study is difficult to assess, in a hyperinsulinemic euglycemic clamp in healthy humans L-NMMA prevented insulin-induced vasodilation, detected as an increase in limb blood flow (53, 92).

Capillary Recruitment

In rats *in vivo*, systemically infused L-NAME was shown to completely block insulin-mediated increases in blood flow and microvascular recruitment, as measured by both the 1-MX method, and contrast enhanced ultrasound (117), while having no significant effect on basal recruitment. However, this may be more complex than first imagined, as locally infused L-NAME in rats *in vivo* does not block insulin-mediated capillary recruitment (118). The site where systemic L-NAME inhibits insulin may be central (60).

1.3.2.2 Inhibition of Nitric Oxide on insulin-mediated glucose uptake

Roy et al (119) showed that inhibition of NOS reduced the ability of the body to dispose of glucose by approximately 16%, and reduced the rate of disappearance of the radiolabelled marker for glucose uptake by about 30%. It was also found that NOS inhibition had no effect on glucose transport by insulin, and caused a decrease in insulin-mediated glucose uptake with no relation to vascular, contractile or metabolic properties of the individual muscle fibres (119). The insulin-mediated vasodilation was independent of the extent of vascularization or the muscle fibre type. The reduced insulin-mediated glucose uptake that was observed with L-NAME treatment

was thought to be due to the decrease of NO release into the vasculature. But, as discussed above, there have been conflicting findings on the proposed effect of limb blood flow on glucose uptake.

In a separate study, inhibition of the hemodynamic effects by blocking NOS action was found to cause no change in whole body insulin-mediated glucose uptake, and so it was suggested that the effects of insulin on blood flow and perfusion had no effect on insulin-mediated glucose uptake (53). However, this was thought to be due to the infusion of the inhibitor only in the arm, which accounts for only 10% of the skeletal muscle mass in the body. As the NOS blockade may have only had local effects, whole body insulin-mediated glucose uptake may not have been an accurate indication of the effect of NO on insulin-mediated glucose uptake.

1.3.2.3 Increasing insulin-mediated glucose uptake with agents that stimulate the production of Nitric Oxide

Limb infusion of methacholine was able to increase insulin-mediated glucose uptake, observed concurrently with an increase in femoral blood flow in lean healthy men Baron, 2000 #874;Baron, 1994 #862}, and in hypertensive patients (120). In a further development, Mahajan et al (121) demonstrated that insulin-mediated glucose uptake could be increased by limb infusion of methacholine in a euglycemic hyperinsulinemic clamp in rats, while another vasodilator, bradykinin, had no effect. Similarly, methacholine, but not bradykinin, was able to augment the insulin-mediated capillary recruitment, as measured using the 1-MX method, though both vasodilators were able to augment the femoral blood flow response to a similar extent (121). It is interesting that two vasodilators, both acting by NO-dependent mechanisms, cause different effects on insulin sensitivity. In other studies that have attempted to increase insulin-mediated glucose uptake using both NO-dependent and –independent vasodilators, simply augmenting the flow did not change glucose uptake in either humans, using SNP (122), adenosine (123) and bradykinin (124), or in rats using epinephrine (31). It has been suggested that the differences in the metabolic action of these vasodilators may be due to the specific site of action of each vasodilator (52), and specifically their ability to alter the distribution of flow within the muscle. Methacholine may be the only one to selectively increase nutritive flow.

Increasing the skeletal muscle blood flow in elderly humans had no effect on glucose uptake. This study showed that the infusion of NO precursors did increase blood flow and corrects endothelial dysfunction, but it is possible that a defect in the tissue in elderly patients may have been responsible for the lack of an effect on glucose uptake (71).

In general, NO-dependent vasodilators have not shown any effect to increase insulin-mediated glucose uptake, however several studies using methacholine have demonstrated this ability (52, 92, 120, 121). As yet, whether this effect is due to the vasodilator abilities of methacholine, or due to a direct effect of methacholine on myocyte glucose uptake is uncertain.

1.3.3 NO action is impaired in diabetes

As discussed above, normal insulin-mediated vasodilation is dependent on NO. In patients with type 2 diabetes, an impaired NO mediated vasodilation by methacholine is observed (125) (endothelium-dependent), in conjunction with an impaired endothelium independent vasodilation (sodium nitroprusside). These defects could be due to a reduced NO release in diabetic patients and/or to an inhibition of the signalling mechanism NO normally activates to cause vasodilation (125). In patients with type 1 diabetes, synthesis of NO is decreased, and is thought to be due to decreased tetrahydrobiopterin (126), thereby reducing insulin-mediated vasodilation. In addition, treatment with tetrahydrobiopterin was found to be beneficial by improving NO-mediated vasodilation in type 2 diabetics. There is also data suggesting that the high plasma glucose observed in diabetic patients may actually reduce the amount of insulin-stimulated NO production (127). Glucose toxicity through free radical attack may cause inactivation or lowered expression of NO by a variety of mechanisms (128).

An impaired bioavailability of NO was observed in obese and diabetic patients, and it was thought to be due to elevated ET-1 levels, at least in obesity, where endothelial function could be restored using a specific ET-1 antagonist (129). However, in diabetes, the ET-1 antagonist was incapable of fully restoring NO bioavailability,

suggesting some other mechanism is involved at least at this late stage in diabetes that lowers NO availability (129).

1.4 Vasoconstrictor effects on insulin action: ET-1

ET-1 has been identified as a potential vasoconstrictor involved in insulin action *in vivo*. In perfused muscle *in vitro* vasoconstrictors can redirect flow to either nutritive or non-nutritive routes in muscle with different effects on metabolism. These metabolic effects due to vasoconstrictor action can be observed when the vasoconstrictor is added alone, but may also modify the effects of other hormones including insulin. As the full metabolic effect of insulin on myocyte glucose uptake requires full nutritive perfusion of muscle, any modification of blood flow distribution with a vasoconstrictor such as ET-1 can potentially affect glucose uptake. Experiments such as these have previously been pursued in this lab using norepinephrine and serotonin (130). Based on the type of vasoconstrictor that is used, variable effects on metabolism can be observed. An increased nutritive distribution of flow throughout the muscle (for example, by low dose norepinephrine), termed type A vasoconstriction will cause an increase in metabolism, due to an increase in the delivery of nutrients to muscle, whereas a decreased nutritive flow through muscle (favouring non-nutritive flow, by an agent such as serotonin), or type B vasoconstriction, will lower the observed metabolism. As yet, no studies have determined whether ET-1 causes type A or type B vasoconstriction, and so the involvement of ET-1 in the hemodynamic effects normally observed with insulin is not clear.

1.4.1 ET-1

ET-1 is one of a family of 3 endothelins; all contain 21 amino acids, and differ in their sequence by up to 6 amino acids. Endothelins can be produced by various cells, particularly those involved in inflammation, including leukocytes, macrophages, smooth muscle cells, cardiomyocytes and mesangial cells (131). All isoforms can induce platelet aggregation and stimulate aldosterone production, as well as stimulate the production of cytokines and growth factors. Other effects include inducing the formation of the extracellular matrix proteins and fibronectin (131), and besides the

vasoconstrictor action, endothelins can be involved in wound healing and neurotransmission in the brain, as well as renal homeostasis. ET-1 is the only isoform produced by the endothelial cells – the vascular endothelium is the major source of ET-1 *in vivo*, and is found in greater concentrations in the plasma than the other endothelin isoforms. It was first isolated as a very potent vasoconstrictor, with long lasting effects.

ET-1 is relatively stable in plasma and blood, and is cleared mainly by the lungs, kidney and endothelial cells *in vivo*, with a relatively short half-life of approximately 1 minute due to this high clearance rate (132).

1.4.1.1 Vasoconstriction and blood flow

ET-1 is a potent arteriolar vasoconstrictor, more potent on smaller than larger vessels (133), which works in a dose-dependent manner in skeletal muscle (134). There are two ET-1 receptors; both are coupled to a G-protein receptor. Throughout the body, the receptors are expressed in a variety of cells, and are involved in different effects. The binding of ET-1 to either receptor on smooth muscle cells causes activation of phospholipase C, leading to an increase of inositol triphosphate, diacylglycerol and intracellular calcium ion, which causes long-lasting vasoconstriction (135). The two receptors elicit different responses: ET_A, which is located on the vascular smooth muscle cell, causes vasoconstriction by altering internal Ca^{2+} stores, and ET_B causes vasodilation if located on the endothelial cell, and vasoconstriction if located on the vascular smooth muscle cell (136). The activation of ET_B receptors limits the vasoconstrictor response of the ET_A receptors (137-139).

The mechanism by which ET_B receptors cause vasodilation is not yet certain, with some suggesting prostaglandins (132), others suggest NO (140, 141) or EDRF (endothelium-derived relaxing factor) (142), while in guinea pig trachealis muscle, arachidonic acid has been shown to be involved (143). It is generally accepted that NO is involved (144), while prostacyclin also appears to be involved in the vasodilator effects (145). Prostacyclin may also be responsible for a decreased permeability in the vascular wall with ET-1, as the main functions of prostacyclin involve the prevention of blood clotting by preventing platelet formation and aggregation (145). This finding of a reduced permeability coincides with increased

transcapillary absorption of fluid, probably due to an increased post-capillary constriction causing an elevated hydrostatic capillary pressure, leading to oedema formation. This effect can be blocked by ET_A receptor antagonist infusion, suggesting that the ET_A receptors located on the venules are responsible for this pressure-induced oedema development.

As the release of ET-1 is primarily directed abluminally (146), ET-1 is released towards the vascular smooth muscle cells, where it would cause vasoconstriction. If a high level of ET-1 is released, there will be a degree of overflow towards the endothelial cells and into the circulation, which would be limited by the action of ET-1 on the endothelial cell receptors (132, 141). The affinity of ET-1 for its receptors is very high (147). When given as a bolus in the healthy human forearm, ET-1 causes a transient vasodilation, which is followed by a slow-onset vasoconstriction (148). The role of ET-1 in the maintenance of basal vascular tone is debatable; some suggest it is involved (149-151), while others contest this (152). In cases of essential hypertension, increased ET-1 vascular activity appears to contribute to vascular tone (153).

ET-1 infusion *in vivo* appears to increase blood pressure, as expected, while decreasing splanchnic and renal blood flow, and decreasing splanchnic glucose release (154). The functional response to ET-1 varies due to the specific distribution and expression of the receptors in different tissues and vascular beds. It was noted that the vasoconstriction induced through ETA receptors caused a fall in oxygen uptake proportional to the decrease in blood flow during ET-1 infusion in canine small intestine (155). This effect was limited by ET_B receptors, as determined using BQ788, a specific ET_B inhibitor, and potentiated by ET_A receptors, discovered by using BQ123, a specific ET_A inhibitor.

1.4.1.2 Endothelial dysfunction, disease

ET-1 has been shown to be involved in disease and in cases of endothelial dysfunction. Ahlborg et al (154) have suggested that ET-1 may contribute to both vascular and metabolic responses in exercise and other pathophysiological conditions, as infusion of a low dose of ET-1 increases mean arterial pressure, decreases renal and splanchnic blood flow, and decreases hepatic glucose release. In another study,

continuous infusion of ET-1 caused arterial narrowing, which was reversible, but it was hypothesised that there could be other non-reversible morphological changes with prolonged exposure to maintain a narrowed lumen (156).

ET-1 plasma levels are elevated in various states including hypertension (157, 158), diabetes (159, 160), obesity (151) and chronic heart failure (161), as well as other examples of endothelial dysfunction.

The plasma levels of ET-1 are variable between studies, but are generally seen to be from 1 to 5 pM, although as discussed above the circulating concentration merely represents an overflow from the local environment (146). A previous discussion has focussed on why such a low concentration of circulating ET-1, which even when elevated is at most 25pM, is insufficient to cause contractions in isolated vessel incubations (162). Studies in animals have demonstrated that ET-1 levels can increase in congestive heart failure (163), renal failure (164) and in paradoxical sleep deprivation (165). In humans, ET-1 levels were shown to correlate with BMI, lipid parameters and systolic blood pressure in children and adolescents with hypertension, obesity or diabetes, all of which showed elevated ET-1 levels (166). In adults, ET-1 is elevated in patients with essential hypertension versus controls, and is thought to promote atherosclerosis in these patients (167).

There have been extensive studies on ET-1 involvement in vascular tone and blood pressure regulation in hypertension and obesity (151, 168-170), while not necessarily agreeing on the role in basal vascular tone, there is agreement that ET-1 is involved, probably through enhanced vascular activity. In several studies the normal ETB response to cause release of NO is inhibited in cases of hypertension (171, 172), possibly due to a down-regulation of ETB receptors.

1.4.2 ET-1 + insulin

Insulin is able to cause ET-1 release from endothelial cells in culture, although an *in vivo* effect was not observed in this study (173), probably due to the effect discussed above whereby 80% of the synthesized ET-1 is released abluminally (146), so an increase in plasma levels would be unlikely to be detected. In other studies, a

hyperinsulinemic euglycemic clamp led to increased ET-1 levels (91, 160), and in lean type 2 diabetic men there was a negative correlation between ET-1 plasma concentration and glucose uptake (160). ET-1 then appears responsible for a decrease in insulin sensitivity; this was hypothesized to be due to a decrease in blood supply to insulin-sensitive tissues, causing an increased insulin secretion to compensate for the slower insulin response. In healthy human subjects under a hyperinsulinemic euglycemic clamp, an additional ET-1 infusion causes an increase in mean arterial pressure, decreases splanchnic and renal blood flow, and has no effect on total blood flow in the leg (174), although the redistribution of flow was not measured. Based on the evidence of ET-1 blocking insulin-mediated vasodilation (88-90), and the fact that elevated levels of ET-1 have been observed in cases of diabetes (159, 160), it is possible that elevated levels of ET-1 may be causing, or increasing, insulin resistance.

1.4.2.1 In disease

Many studies have been performed on animal models of diabetes regarding ET-1 involvement. Diabetic rats were shown to have elevated ET-1 levels (175). Treatment with an ET_A blocker reduced hyperglycemia and restored plasma glucose clearance rates towards normal, and increased L-NAME sensitive relaxatory responses of jejunum. Miller et al (176) have observed that normal rats show insulin-mediated mesenteric vascular bed vasodilation, although this action in blocked in diabetic rats. Treatment with an ET_A inhibitor uncovered the insulin-mediated vasodilation in the diseased rats. Rats implanted with osmotic mini-pumps to deliver ET-1 for 5 days developed insulin resistance (177). There was a decrease in insulin-stimulated glucose disposal rates of about 30% into soleus muscle.

Mather et al (178) has shown in humans that ET-1 contributes more to the basal vascular tone in obese and type 2 diabetic patients than in lean healthy controls. The ET_A receptor appeared to be responsible for this, as treatment with an ET_A blocker lowered vascular tone in those patients with endothelial dysfunction. In these experiments, big ET-1 infusion decreased the arterio-venous difference of oxygen by 33% which suggests that big ET-1 increases blood flow in skeletal muscle (179). In healthy humans under a hyperinsulinemic euglycemic clamp, infusion of big ET-1 reduced insulin sensitivity and insulin clearance (179). ET_A receptor blockade can increase the clearance of insulin, but also increases the efficiency of insulin.

1.4.2.2 Interactions between insulin and ET-1

When insulin signalling proteins were investigated in rats implanted with osmotic mini-pumps that delivered ET-1 constantly for five days, a decrease in expression of IRS-1, IRS-1 associated p110a, and AKT activation (phosphorylation) was observed, accompanied by an increase in insulin resistance as measured by glucose uptake (177). Therefore, it would appear that elevated ET-1 levels can lead to insulin resistance by impaired insulin signalling. However another study by Idris et al (180) has demonstrated that 24 h exposure to ET-1 had no sustained effect on insulin-mediated glucose uptake in a study on L6 myoblasts, and only a transient effect on insulin-mediated glucose uptake by 3T3-L1 adipocytes. Based on this evidence, the researchers hypothesized that the insulin resistance observed with high levels of ET-1 would be likely due to an indirect effect of vasoconstriction, leading to reduced substrate delivery and insulin delivery to insulin-sensitive skeletal muscle (180).

Further studies into a mechanism of ET-1-mediated insulin resistance have shown that insulin signalling was reduced in chronic ET-1 treated 3T3-L1 adipocytes, inhibiting the MAPK pathway (181). In this way, chronic ET-1 exposure caused IRS degradation, inhibiting insulin-mediated glucose uptake and GLUT4 translocation, effects which could be blocked by incubation with an ETA antagonist. The study by Idris et al (180) used only a 2 hour time frame to observe an inhibition of ET-1 on insulin action, whereas Ishibashi et al (181) observed effects only after exposing adipocytes to ET-1 for 24 hours. It is possible that a significant effect of ET-1 to block glucose uptake in cells may have developed by this time in the study by Idris et al (180); however, it is likely that, due to the constriction elicited by ET-1, a delivery effect may still be involved in the insulin resistance *in vivo*.

Therefore, the role of ET-1 on insulin sensitivity is not well defined. It is possible that ET-1 restricts blood flow to skeletal muscle, which may lead to secondary effects causing insulin resistance due to reduced delivery of insulin and glucose. However the results by Ishibashi et al (181) suggest that ET-1 is able to have direct, chronic cellular effects on the insulin pathway leading to glucose uptake, at least in adipocytes. It is possible that differences between tissues may account for why direct

effects of ET-1 on adipocytes to block insulin action are not also observed in myocytes.

1.5 Summary of Aims

The primary focus of this laboratory is the hemodynamic changes that can control blood and nutrient distribution within the muscle bed, primarily capillary recruitment. This study will focus more specifically on the delivery of glucose to the myocytes by insulin's own vascular actions – primarily by using vasoactive substances to cause the redistribution of flow within muscle to observe any effects on glucose distribution and delivery within the muscle.

A recent study from this laboratory has shown that methacholine, probably by a NO-dependent mechanism, is able to augment both insulin-mediated capillary recruitment and glucose uptake, while bradykinin was not. The possibility arises that methacholine may be able to increase glucose uptake independent of its nitro-vasodilator effects, by a direct effect on the myocyte. Thus a detailed study of methacholine needs to be undertaken to assess its effects on microvascular perfusion and metabolism. The aim is to separate the direct metabolic effect of methacholine from its vasodilatory or redistribution effects on skeletal muscle glucose uptake.

A recent paper suggested that a Na^+-glucose cotransporter present in muscle endothelium may be an important control in insulin-mediated glucose uptake, although not involved in basal glucose uptake. As this represents a potential control site for glucose access to muscle, this particular study aims to determine or confirmthe involvement of this transporter in insulin-mediated glucose uptake using appropriate controls.

As discussed above, ET-1 is elevated in insulin resistance and diabetes. The perfused rat hindlimb has previously been used to investigate the effects of various vasoconstrictors on insulin-mediated glucose uptake, and provides a system in which the hemodynamic and metabolic effects of ET-1 can be studied without interference from other tissues. The aim is to more fully understand the role of ET-1 in the vasculature, specifically its effects on metabolism, as well as the interactions that may occur between ET-1 and insulin.

There are three main control points involved in glucose uptake by myocytes: these include the delivery of glucose by blood to the area, the transendothelial transport of glucose, and crossing the myocyte membrane to enter the cell. This study will focus on two of these: the transendothelial transport of glucose, specifically the involvement of SGLT1, and vascular changes that affect blood delivery, including both vasoconstriction by ET-1 and vasodilation by NO. The overall purpose of this study is to examine in more detail the steps involved in insulin-mediated glucose uptake and glucose delivery to the myocyte, with the hypothesis that blood flow redistribution is a major event in determining the amount of glucose uptake.

CHAPTER 2

METHODS

2.1 Introduction

To assess the effect of various vasomodulators and insulin on both metabolic and hemodynamic effects *in vitro*, the constant flow pump-perfused rat hindlimb was used. The surgery and general perfusion protocol is described below, with any additional details or changes from this protocol noted in the relevant chapter.

2.2 Perfused rat hindlimb

2.2.1 Animals

Male hooded Wistar rats were used throughout the study, and were housed at 22°C in conditions consistent with the Australian code of practice for the care and maintenance of laboratory animals. Rats were fed a commercial diet (Gibsons, Hobart) containing 21.4% protein, 4.6% lipid, 68% carbohydrate and 6% crude fibre, with added vitamins and minerals. Water was freely available.

The University of Tasmania Ethics Committee approved all the experiments conducted in this study.

2.2.2 Perfusion Buffer

Regular Krebs Henseleit buffer

Krebs Henseleit buffer consisted of

118 mM NaCl

4.74 mM KCl

1.19 mM KH_2PO_4

1.18 mM $MgSO_4$

25 mM $NaHCO_3$

8.3 mM glucose

Modified low sodium Krebs buffer

A modified Krebs buffer was used in Chapter 7, which consisted of

118 mM choline chloride

4.74 mM KCl

1.19 mM KH_2PO_4

1.18 mM $MgSO_4$

25 mM $NaHCO_3$

8.3 mM glucose

The perfusion buffer contained the respective Krebs buffers as indicated above and 40 g/L BSA (4%). This buffer was gassed for >30 min with carbogen (95% O_2: 5% CO_2) before $CaCl_2$ was added to a final concentration of 2.54mM. All buffers were filtered through a 0.45µm filter before use.

Modified low Calcium Krebs buffer

A low Calcium buffer was used in Chapter 5. In this buffer the $CaCl_2$ from the regular Krebs Henseleit buffer was not added and instead EGTA was added at the same time point to a final concentration of 100mM.

2.2.3 Surgery

Animals (180 – 200 g) were anaesthetized with an intra-peritoneal injection of sodium pentobarbital i.p. (Vibrac Australia) (minimum 6g/100g body weight) prior to all surgery, and animals remained alive but anaesthetized throughout any surgical procedures.

Surgery was performed to isolate blood flow to the skeletal muscle of a single hindlimb. The surgical procedures were essentially the same as those in Ruderman et al (182) with modifications by Colquhuon et al (183). After anaesthetizing the animal, ties were placed around the tarsus of the right hindlimb and at the base of the tail. A ventral incision was made along the midline, through the skin and body wall. Ligatures were placed around the superior and inferior epigastric vessels, and the muscle layer trimmed to fully expose the intestines. The right common iliac vessels were tied to prevent blood flow to the skin of the hindlimb. Ligatures were tied around the duodenum immediately below the stomach and the large bowel level with the bladder, allowing the intestines to be completely removed. A small, plastic pipe was then positioned under the rat, raising the dorsal body wall. A single ligature was placed around the seminal vesicles and bladder and the seminal vesicles were dissected out. The testes were pushed into the body cavity, tied off and removed. The epigastric vessel of the left leg was tied off, and a single ligature was placed around the internal spermatic vessels, ureter and ilio-lumber artery and vein on either side of the rat. This required that connective tissue holding the vessels to the dorsal body wall to be gently teased away. In a similar fashion, connective tissue surrounding the vena cava and dorsal aorta was removed, allowing them to be separated. Double strands of silk thread were then placed beneath the right femoral artery and vein, vena cava and dorsal aorta. The vein and artery leading to the right leg were ligated to allow perfusion of only a single leg. Two hundred microlitres of heparin (1000U/ml) was injected into the vena cava in the region below the stomach before ligatures were tied around the left femoral artery and vein. The upper ligature was tied around the vena cava immediately below the stomach, preventing blood flow back to the upper body and allowing cannulation of the vein with an 18G, 1 ¼ inch cannula (Terumo). A second tie was then placed around the vein and cannula, holding it in place. The dorsal aorta was cannulated similarly, with a 20G, 1 ¼ inch cannula (Terumo). As the

aorta didn't inflate after the upper ligature was tied, it was cut halfway through with fine scissors and held open with a needle threader whilst the 20G cannula filled with 0.9% saline was inserted. Figure 2.1 is a diagram showing ligature and cannula placement.

In order to reduce the amount of time the hindlimb was unperfused, cannulation was performed as quickly as possible and connection of the rat to the perfusion apparatus occurred as soon as the arterial cannula was secure; this was performed within two minutes. The arterial cannula was connected to the line supplying oxygenated perfusion buffer, allowing the exit of buffer through the vena cava cannula. Once connected, the rat was euthanased with intracardiac sodium pentobarbital and a body ligature was tied around the rat at the level of the L3 vertebra to prevent blood flow to the lower back muscles during a rise in perfusion pressure. The entire surgical procedure was completed within 20 minutes.

2.2.4 Perfusion apparatus

A non-recirculating perfusion was performed using a Cole-Parmer Masterflex pump to maintain a constant flow rate of either 8 or 15 ml/min as indicated in the relevant chapter. The femoral flow rate in a healthy rat *in vivo* is approximately 1ml/min; as such the flow rates used here are much higher. This allows an adequate amount of oxygen to be delivered to the hindlimb, and yet shear stress should be minimal, as the pressure across the hindlimb is negligible when compared to arterial pressure in rats *in vivo*. The chamber and perfusate temperatures were maintained at 32°C. The perfusate was oxygenated by passage through silastic tubing in a lung constantly equilibrated with carbogen (95% O_2: 5% CO_2). A small infusion port for the infusion of test substances was located prior to the pressure gauge attached to a bubble trap to measure arterial perfusion pressure. A 500µL chamber containing a Clark-type oxygen electrode measured the oxygen content of venous perfusate and both perfusion pressure and venous oxygen content were constantly recorded on WINDAQ computer program (Dataq Instruments, USA). The oxygen electrode was calibrated with air and 100% O_2. Figure 2.2 is a diagram of the perfusion apparatus.

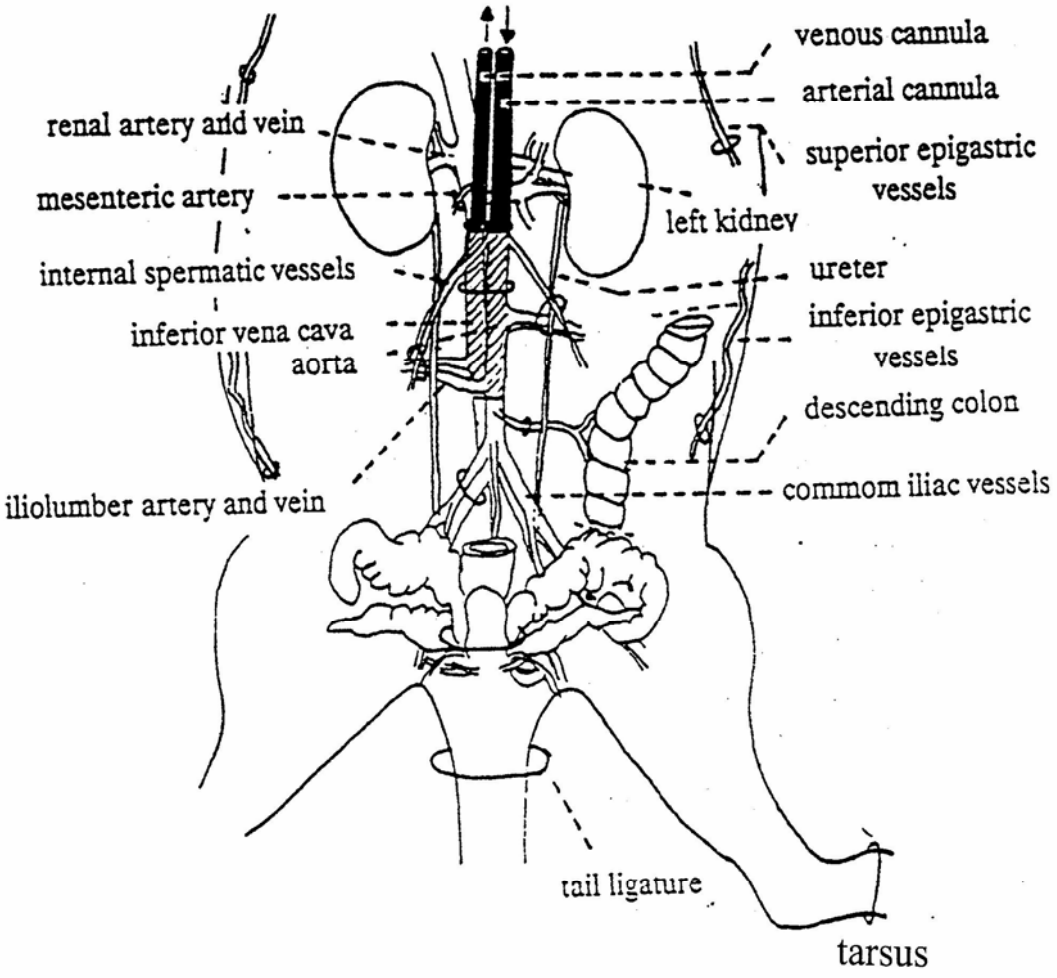

Figure 2.1 Ventral view of the rat, showing vessel ligation and cannulation performed to isolate blood flow to a single hindlimb. (adapted from Ross, 1972 (184)and Greene, 1968 (185)).

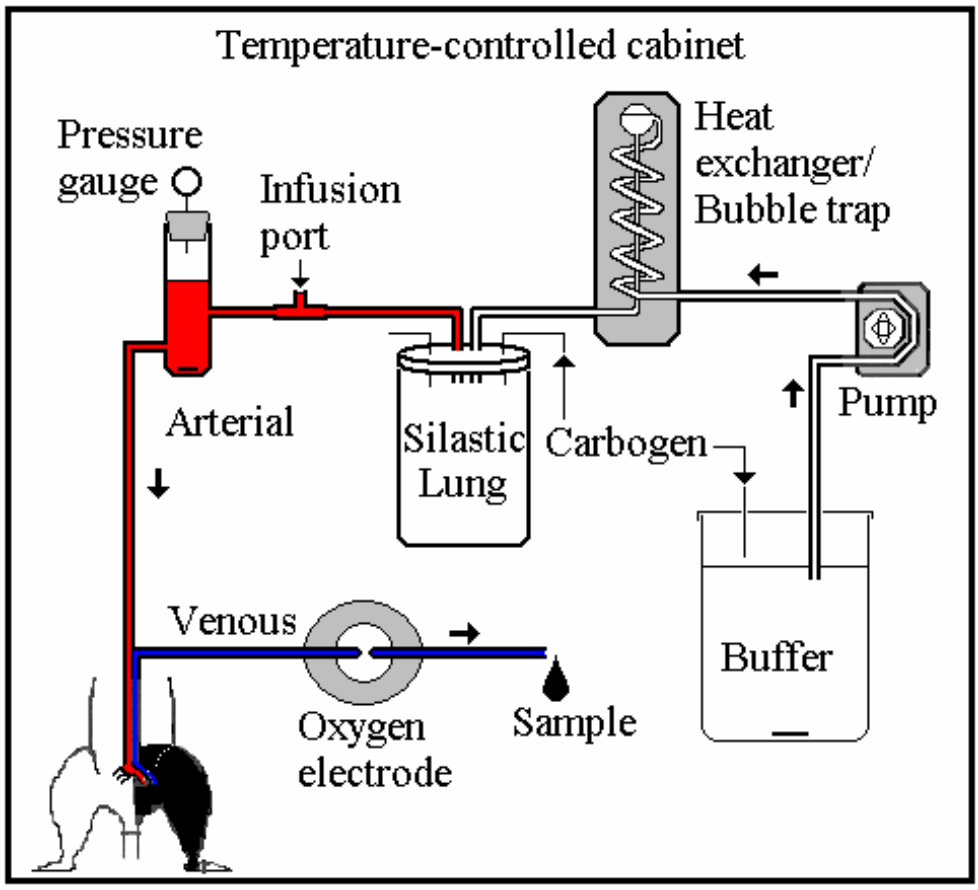

Figure 2.2 Apparatus for the perfusion of a single rat hindlimb.

2.2.5 Perfusion protocols

All protocols allowed a 30 – 40 min equilibration time after hookup to establish stable baseline oxygen, pressure and glucose and lactate readings. Venous samples were taken as indicated in protocols, and arterial samples from the buffer reservoir for glucose and lactate determination using the YSI 2300 STAT Plus Glucose Analyser (Yellow Springs). All protocols involved the infusion of test substances into the infusion port proximal to the hindlimb by a syringe pump (Sp101i, WPI.). The perfusion protocols and the concentrations of the infusions will be outlined in the specific chapters.

2.2.6 Calculation of oxygen consumption (VO2)

Muscle oxygen consumption was calculated using the Fick Principle:

$$VO_2 = \frac{ß \times (P_aO_2 - P_vO_2) \times (flow/1000) \times 60}{\text{Muscle weight (g)}}$$

Where ß = calculated from the Bunsen coefficient

ß = α / (22.4 x 760)

= 0.0230 ml/L / (22.4 mM x 760mmHg)

= 1.351 µmol/L/mmHg

(α = the volume (ml) of oxygen dissolved per ml of plasma at 0^oC and 760 mmHg. α is 0.0230 in plasma at 32^oC.)

Where P_aO_2 = arterial PO_2 using the calibrations from the oxygen electrode for the arterial, air and oxygen and using their known PO_2.

$$P_aO_2 = \frac{\text{cal Art} - \text{cal Air}}{\text{cal 100\% } O_2 \text{ cal Air}} \times (PO_2 \text{ at 100\%} - PO_2 \text{ in Air}) + PO_2 \text{ in Air}$$

where: cal Art = electrode arterial calibration

cal Air = electrode air calibration

cal 100%O_2 = electrode oxygen calibration

PO_2 at 100% = 760mmHg – 36mmHg (H_2O vapour pressure at 32^oC, due to the use of a wet oxygen electrode).

=724mmHg

PO_2 in Air = 154mmHg

Where P_vO_2 = venous PO_2 calculated the same as the P_aO_2 however the value for cal Art was replace by the value for the venous effluent.

Where flow = perfusion flow rate in ml/min, defined in each chapter.

Where muscle weight = total perfused muscle mass, which has previously been determined to be $1/12^{th}$ of the body weight for a single perfused hindlimb (186).

2.3 Radiolabelled glucose uptake

2.3.1 Infusion solutions

30 min 2-deoxyglucose solution

[^{3}H] 2-deoxyglucose was infused at a constant rate for 30 min prior to the end of the perfusion.

160µl [^{3}H] 2-deoxyglucose made up to 16ml with saline.

10 min 2-deoxyglucose solution

[^{3}H] 2-deoxyglucose was infused at a constant rate for 10 min immediately prior to the end of the perfusion.

540µl [^{3}H] 2-deoxyglucose made up to 16ml with saline.

2.3.2 Protocol

Solutions were infused at a rate of 1/200th of the flow (at 8ml/min, this is 40µl/min). Venous samples taken during 2-deoxyglucose infusion and a final arterial sample were analysed to give total plasma dpm, which was averaged over the time of infusion of the radiolabelled glucose. At the end of the perfusion, muscles were immediately dissected out and freeze-clamped under liquid N_2. Soleus, plantaris, gastrocnemius red and white, extensor digitorum longus and the tibialis were excised and stored at –20^{o}C. Frozen muscles were powdered under liquid nitrogen and homogenized in 1.5ml distilled H_2O using an Ultra Turrax™ or Silentcrusher ^{TM}. The homogenates were centrifuged at 13,000g for 10 minutes at 4^{o}C, and free and phosphorylated [^{3}H] 2-deoxyglucose were separated by ion exchange chromatography using an anion exchange resin (AG1X8) (187, 188). Columns were washed with distilled water to elute the unbound glucose, which was counted in 16ml scintillation fluid (BCS). Columns were then washed with 1M HCl to elute the phosphorylated 2-deoxyglucose that was bound to the column, which were also counted as above. A 100µl sample of the supernatant was also counted to determine the total counts that should be eluted

from the column. Biodegradable counting scintillant was added to each radioactive sample and radioactivity determined using a scintillation counter.

The R'g ($\mu g.g^{-1}.min^{-1}$) was calculated from the following expression:

$$\frac{\text{phosphorylated } [^3H] \text{ 2-deoxyglucose dpm in muscle}}{\text{wet wt muscle (g) x 30 min}} \times \frac{\mu g \text{ per ml glucose in perfusate}}{^3H \text{ dpm per ml in perfusate}}$$

2.4 Contraction

If exercising muscle was used, the right leg was isolated instead of the left for perfusion. Skin was removed from the knee area. After hook-up to the perfusion apparatus, the leg was stabilized in the jig, with the pin secured on the bone around the knee. Sharp forceps were inserted between the Achilles tendon and the bone for the insertion of the hook of the isometric transducer previously calibrated to 0 and 500 g. The isometric transducer measured the tension constantly, and data was recorded on a WINDAQ program. Tension was adjusted to 40g during the equilibration period, and this was maintained as much as possible. Electrodes were placed through the skin of the rat above the knee and through the Achilles (without touching the tension wire).

Flow was increased to 15 ml/min before stimulation was commenced. Using a Nerv-Muskel-Reizgerat stimulator (Hugo Sachs Elektroniks), twitch stimulation was performed initially to reach full fibre recruitment. 0.1 ms pulses were applied at 0.5 - 0.3 Hz. Voltage initially began at 1V, and was increased in 1V increments until maximum tension was reached, indicating full fibre recruitment.

An anaerobic phase of contraction was observed at first, with an increase in tension development, but this dropped away to a stable baseline tension development indicating aerobic exercise. Once this stable phase was reached (regularly required 5 – 10 minutes to reach the steady state tension development), the test substances were infused.

2.5 Statistics

All tests were performed using the SigmaStat^{TM} statistical program (Jandel Software Corp.). The statistical significance of differences between groups of data was assessed by one or two way analysis of variance (ANOVA) for sets of experiments containing multiple groups. Significant differences were recognized at $P<0.05$. Symbols to designate significance are defined in each chapter.

CHAPTER 3

METABOLIC AND HEMODYNAMIC EFFECTS OF ENDOTHELIN-1 IN THE PERFUSED RAT HINDLIMB

3.1 Introduction

As discussed in chapter 1, it is likely that vasoactive agents may alter flow distribution in the perfused hindlimb to increase or decrease metabolism, as demonstrated by Newman et al (130) using type A (nutritive) and type B (non-nutritive) vasoconstrictors respectively. One method of measuring this change in flow distribution is the microdialysis technique, which has previously been used in this laboratory to observe the effects of both type A and type B vasoconstrictors on the blood flow around the probe, and to calculate the interstitial glucose concentration (189). Type A vasoconstrictors such as NE and AII tend to increase, while type B vasoconstrictors including 5HT decrease interstitial glucose concentration by modulating the blood flow through muscle – greater flow will deliver glucose more efficiently to the interstitial space, and increased flow past the probe will remove more radiolabel from the microdialysate solution. As yet, ET-1 has not been fully characterised, although it is one of the most potent vasoconstrictors known, so it is unknown whether ET-1 can cause nutritive or non-nutritive flow. Insulin stimulates ET-1 release (173) and so it is likely that ET-1 may alter blood flow during insulin action.

This chapter will focus on the metabolic and hemodynamic effects of ET-1 alone in the perfused rat hindlimb. In this study the aim was to determine whether ET-1 can manipulate blood flow within the muscle in either a nutritive or non-nutritive manner by observing effects on metabolism. The constant-flow pump-perfused rat hindlimb was used as it allows for a more invasive study involving the measurement of both hemodynamics and metabolism, without interference from homeostatic mechanisms that may mask the direct effect of ET-1 alone *in vivo*.

3.2 Methods

3.2.1 Solutions

Solutions were made up to be 200 times the required final concentration to allow for infusion at 1/200th of the flow into the infusion port. Therefore, at a constant flow rate of 8ml/min, substances were infused at 40µl/min unless otherwise indicated.

Endothelin-1

ET-1 was dissolved in a minimum amount of acetic acid (1mg in 125µl), and then made up to volume with saline. Infusion solutions of 200nM and 2µM were infused at 1/200th of the flow rate into the infusion port to give final concentrations of 1nM and 10nM respectively. Other final concentrations used in this chapter were achieved by altering the infusion rate.

Vehicle

Vehicle infusions contained an equal volume of acetic acid as used in the 10nM final ET-1 experiments.

Nitroprusside

Sodium nitroprusside (SNP) was dissolved and diluted to the required volume with saline, and infused at 1/200th of the flow rate to give a final concentration of 50µM. Higher concentrations of SNP were attempted, however a direct inhibitory effect on the muscle oxygen uptake was observed, possibly due to liberated cyanide ions.

3.2.2 Perfusion conditions

Hindlimb perfusions were performed at 32^{o}C through only the right leg, with 2.54mM $CaCl_2$ and 8.3mM glucose. The flow rate was maintained at 8ml/min using a pump, and the regular Krebs buffer was used (section 2.2.2).

3.2.3 Perfusion protocol

After equilibrating the rat hindlimb for 40 minutes, ET-1 was infused at an appropriate rate to give a known concentration of ET-1 in the perfusate. This infusion was maintained for 40 minutes as shown in Figure 3.1. SNP when used was infused for the same period of time as ET-1 to give a final concentration of 50μM.

During the experiments, perfusate samples were taken at 5-10 minute intervals for analysis for glucose uptake and lactate release. Perfusate samples were analysed for glucose and lactate using a YSI 2300 STAT Plus Glucose Analyser.

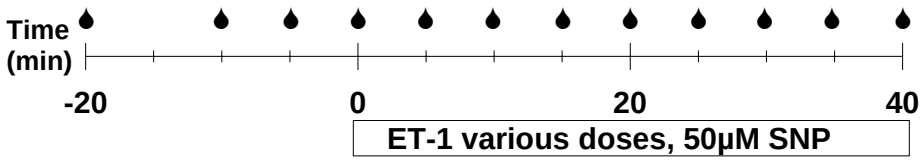

Figure 3.1. Protocols used for experiments performed in this chapter. Venous samples were collected for analysis of glucose and lactate as indicated by the drops (⧫). Infusions of various solutions are shown by the open bar as labelled.

3.2.4 Statistical Analysis

Two-way repeated-measures ANOVA was performed using SigmaStat (SPSS Science; Chicago, IL) for the time-course experiments, and one-way ANOVA for the end-point histograms and 2-deoxyglucose results, with comparisons made between conditions using the Student-Newman-Keuls post hoc test. Significance was assumed at the level of $P < 0.05$. Data are presented as means ± SE.

3.3 Results

3.3.1 ET-1 dose curve

ET-1 was infused for 40 min, during which time changes in pressure, oxygen consumption, as well as glucose and lactate uptake were measured. Figure 3.2A shows that ET-1 causes a slow-onset, dose-dependent increase in perfusion pressure. This vasoconstriction appeared to continue to increase over time, and only plateaued at lower doses of ET-1. No effect of ET-1 to vasodilate in this preparation was observed, possibly due to the fact that the perfused rat hindlimb is already fully vasodilated. When ET-1 was infused against a background of vasoconstriction by either 5HT or AII, there was no significant effect of any concentration of ET-1 to dilate against this prior vasoconstriction (Figure 3.2B).

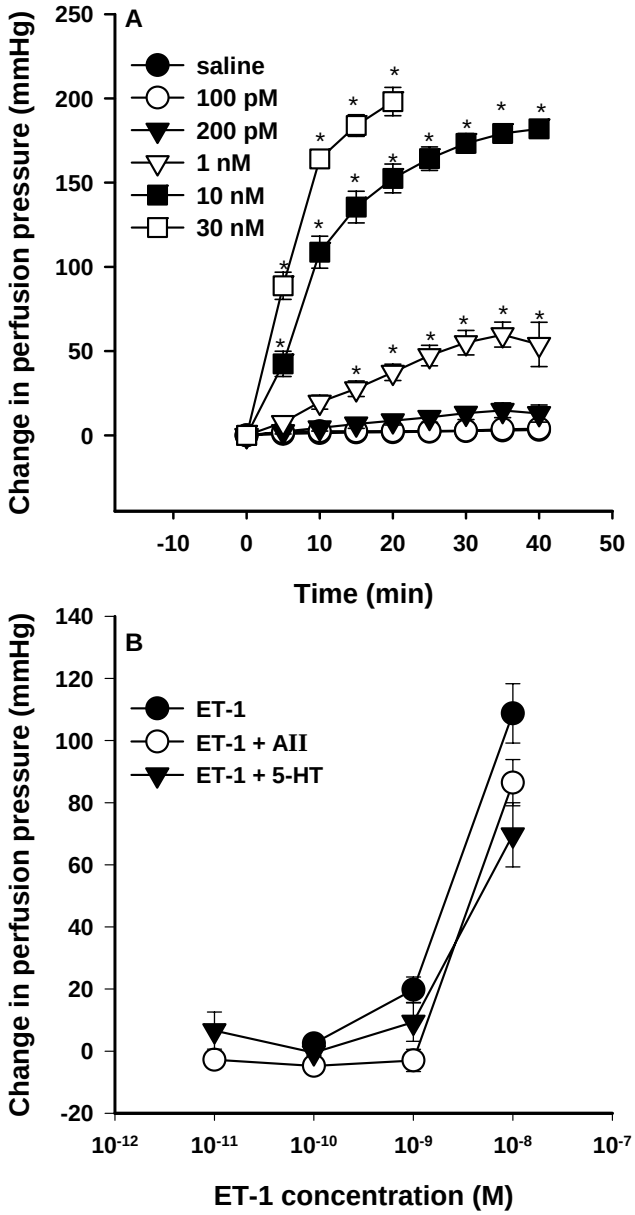

Figure 3.2. Effect of ET-1 on perfusion pressure, with and without prior constriction by angiotensin II or serotonin. Saline or ET-1 were added at t = 0 min according to the protocol shown in Figure 3.1. A) The concentrations of ET-1 were 100pM, 200pM, 1nM, 10nM and 30nM (n= 6-12). B) Change in perfusion pressure following infusion of ET-1 alone, or following constriction due to either 7.5nM AII, or 0.45μM 5-HT (n= 5-12) as in the protocol in Figure 3.1. Values were at 10 min after ET-1 addition. *, significantly different ($P < 0.05$) from saline using a two-way repeated measures ANOVA.

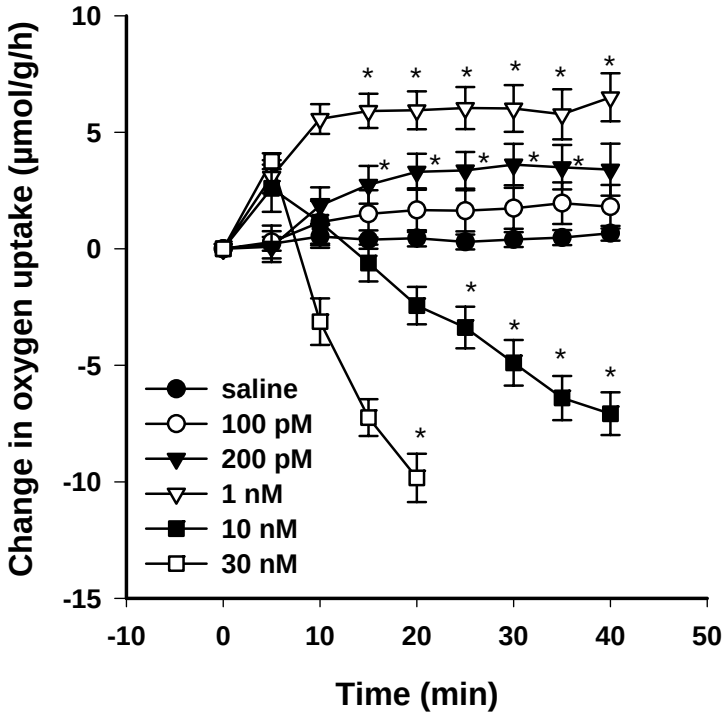

Figure 3.3. Effect of ET-1 on oxygen uptake. Saline or ET-1 were added at t = 0 min according to the protocol in Figure 3.1. The concentrations of ET-1 were 100 pM, 200 pM, 1nM, 10nM and 30nM, (n= 6-12). *, significantly different ($P < 0.05$) from saline using a two-way repeated measures ANOVA.

Figure 3.3 shows the biphasic effects of ET-1 on oxygen uptake through the hindlimb. Lower doses caused a sustained increase in oxygen uptake, which was significant at 1nM and 200pM from 15min. Higher doses of ET-1, including 10nM and 30nM, first induced a transient increase in oxygen consumption, which was followed by a sustained inhibition of oxygen uptake. These inhibitory effects became significant at 20min for 30nM and 25min for 10nM ET-1.

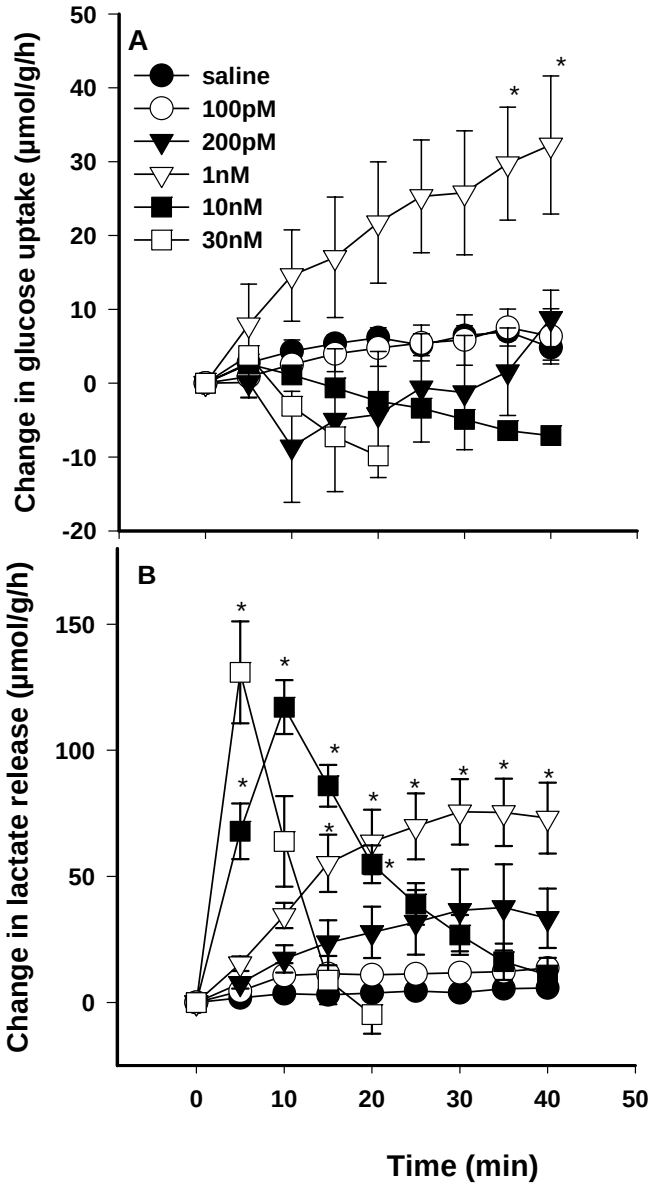

Figure 3.4. Effect of ET-1 on the changes in glucose uptake (A) and lactate release (B). Saline or ET-1 were added at t = 0 min according to the protocol in Figure 3.1. The concentrations of ET-1 were 100 pM, 200 pM, 1nM, 10nM and 30nM (n= 6-12). *, significantly different ($P < 0.05$) from saline using a two-way repeated measures ANOVA.

ET-1 had similar dose-dependent effects on both glucose uptake and lactate release, as shown in Figure 3.4. 1nM ET-1 caused a significant stimulation in both glucose uptake and lactate release, which were significant from 35min and 15min respectively. Higher concentrations of ET-1 (10nM) caused a non-significant inhibition of glucose uptake that continued to increase the inhibition with time. These doses caused an initial stimulation of lactate release (Figure 3.4B) that often coincided with a washout of red blood cells. This significant stimulation effect then subsided, tending towards an inhibition (30nM) or no net effect (10nM) by the end of the protocol.

3.3.2 ET-1 and SNP

The experiments above were repeated in the presence of sodium nitroprusside. A dose of SNP was chosen that had no cyanotic effects alone, but which was able to vasodilate to block at least some of the vasoconstrictor effects of ET-1.

Figure 3.5 below shows the time-course of the inhibition of SNP on ET-1-mediated pressure and oxygen effects. SNP is able to almost completely block 1nM ET-1 mediated increases in both pressure and oxygen uptake. While SNP is unable to completely block high dose (10nM) ET-1 effects, it is able to significantly block perfusion pressure, and ameliorated the inhibitory effects of ET-1 on oxygen uptake (Figure 3.5).

In terms of glucose uptake and lactate release, Figure 3.6A demonstrates that the effect of ET-1 on glucose uptake at both low and high doses is difficult to interpret. The stimulation by 1nM ET-1 normally observed is less obvious, and the trend of ET-1 10nM to initially stimulate, then tend to inhibit glucose uptake is indiscernible from control data. For lactate release, the stimulation caused by both 1nM and 10nM ET-1 was reduced (Figure 3.6B).

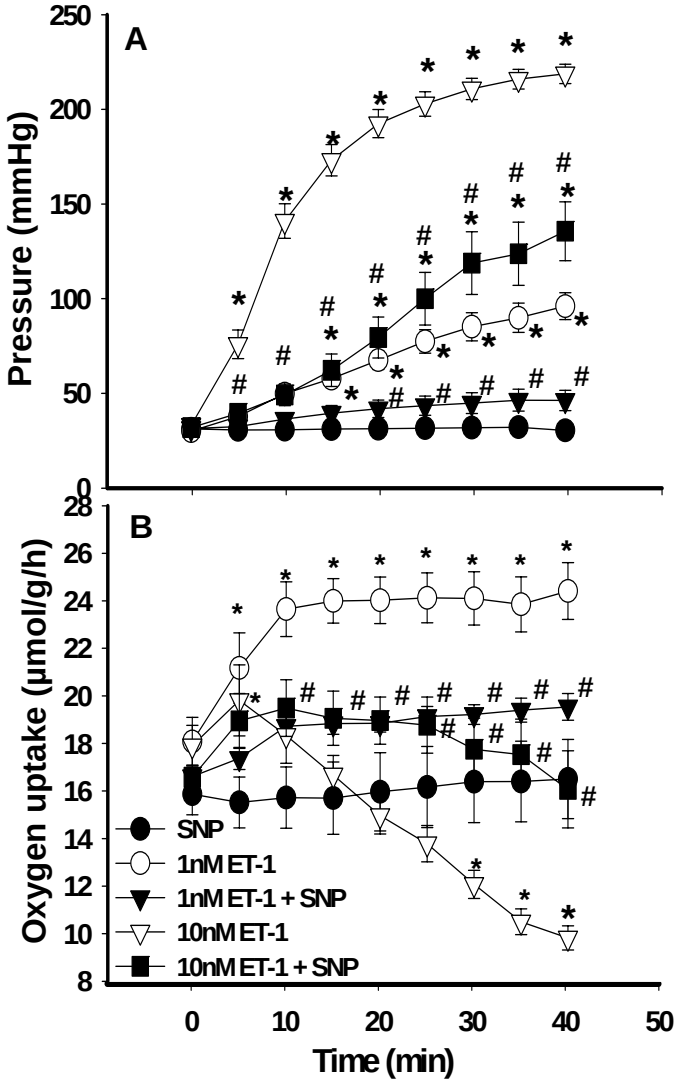

Figure 3.5. Effect of SNP 50µM to block ET-1 mediated increases in perfusion pressure (A) and oxygen uptake (B). SNP and ET-1 were infused from t = 0 as shown in the protocol in Figure 3.1. *, significantly different ($P < 0.05$) from SNP and $^{\#}$, significantly different from corresponding dose of ET-1 alone (n=6-12) using a two-way repeated measures ANOVA.

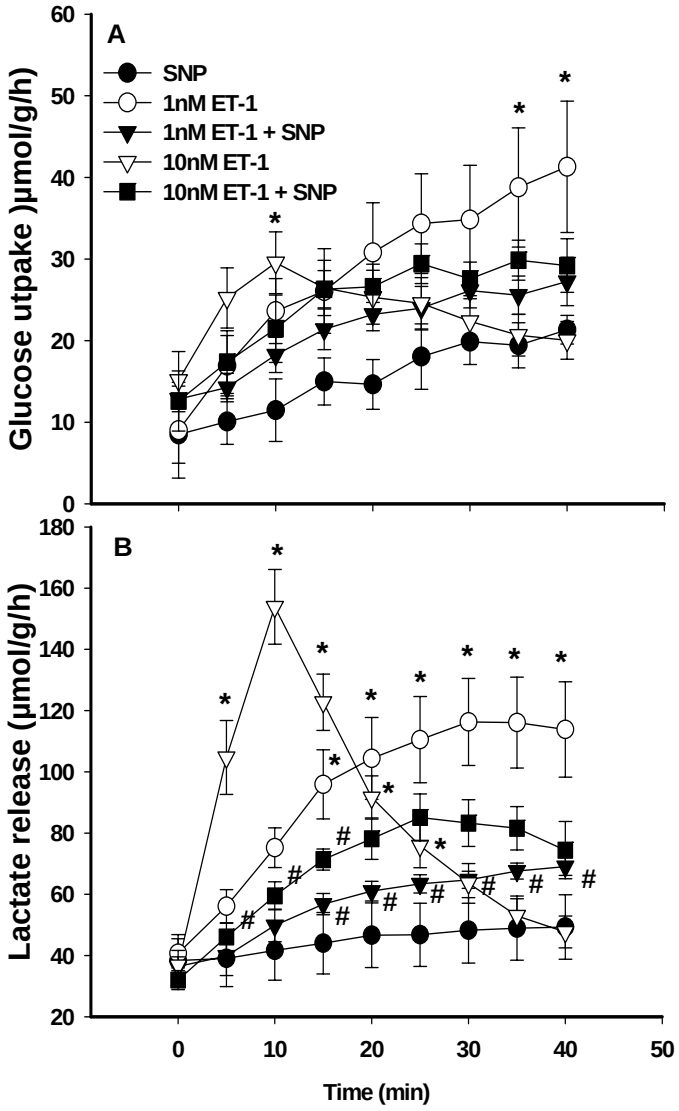

Figure 3.6. Effect of SNP 50µM to block ET-1 mediated increases in glucose uptake (A) and lactate release (B). SNP and ET-1 were infused from t = 0. SNP and ET-1 were infused from t = 0 as shown in the protocol in Figure 3.1. *, significantly different ($P < 0.05$) from SNP and #, significantly different from corresponding dose of ET-1 alone (n=6-12) using a two-way repeated measures ANOVA.

It is difficult to get a clear indication from the graphs above regarding the effect of SNP on the ET-1 response for each parameter measured. For ease of comparison between groups, the end point of each of the SNP experiments is plotted along side its control as a histogram. It is evident then that SNP is able to prevent the increase in pressure and oxygen uptake induced by 1nM ET-1 (Figures 3.5 and 3.7). While SNP was not able to completely block the effect of 10nM ET-1 to increase the perfusion pressure, it appeared to be able to prevent the inhibition of oxygen uptake (Figures 3.5 and 3.7). However, when observing the time course of SNP on 10nM ET-1 oxygen uptake, it is apparent that SNP slowed the time course of ET-1, as there is still a transient stimulation of oxygen uptake, which was subsiding into a general inhibition, although at the final time point it appears as if there is no net effect (Figure 3.5B).

When looking at the other metabolic parameters measured, SNP again appears able to completely block the 1nM ET-1 mediated stimulation of both glucose uptake and lactate release (Figures 3.6 and 3.8). The 10nM ET-1 dose has no net effect on either glucose or lactate alone at the time point chosen, although sodium nitroprusside appeared to push each into an apparent stimulation (Figures 3.6 and 3.8). As with the oxygen data above, the time course seems to imply that including SNP slows the progression of the type A and type B effect. The transient stimulation of oxygen uptake seen at 10 minutes after infusion of 10nM ET-1 is lost when SNP is infused. Also, the lactate release caused by ET-1 is never as substantial when SNP is infused, although at the end of the time course of measurement, the lactate release with 10nM ET-1 and SNP appears to be decreasing, possibly heading towards a net inhibition (Figure 3.6B).

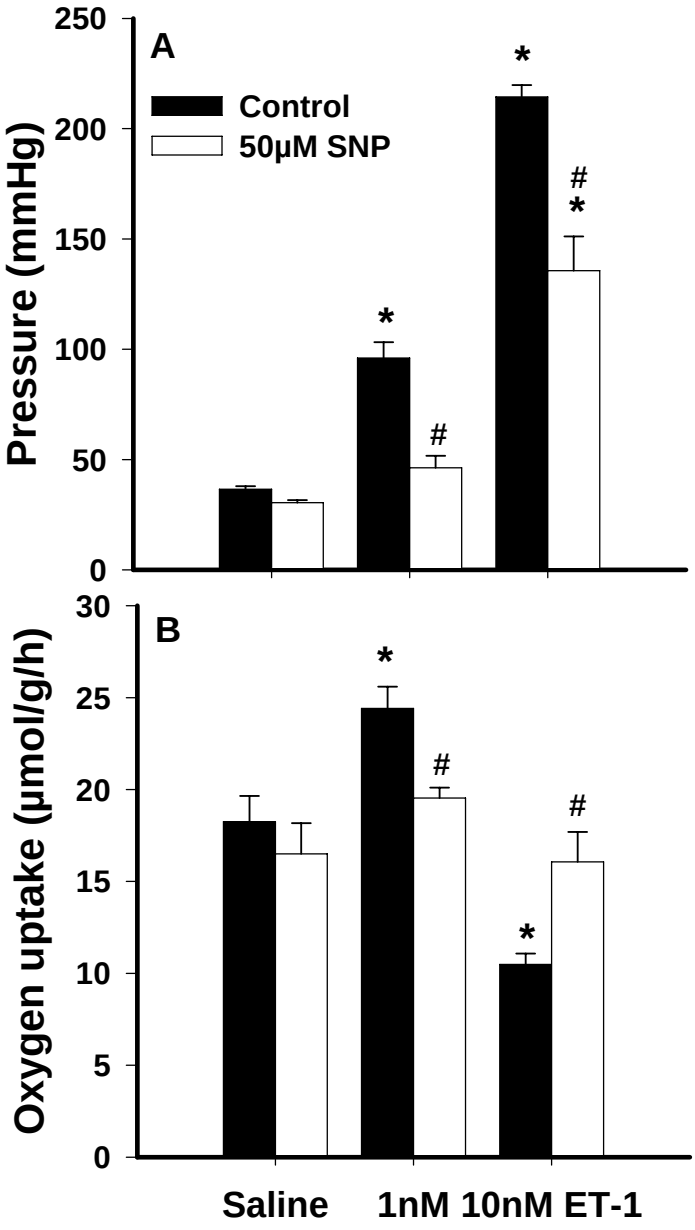

Figure 3.7. Effect of ET-1 on perfusion pressure (A) and oxygen uptake (B) with and without sodium nitroprusside. Filled bars indicate ET-1 or vehicle infusion without SNP, white bars are for results with SNP infusion. Saline, SNP or ET-1 ± SNP were added at t = 0 min, according to the protocol in Figure 3.1. Values were at 40 min. *, significantly different ($P < 0.05$) from saline, and $^{\#}$, significantly different from corresponding dose of ET-1 alone (n=6-12) using a one-way ANOVA.

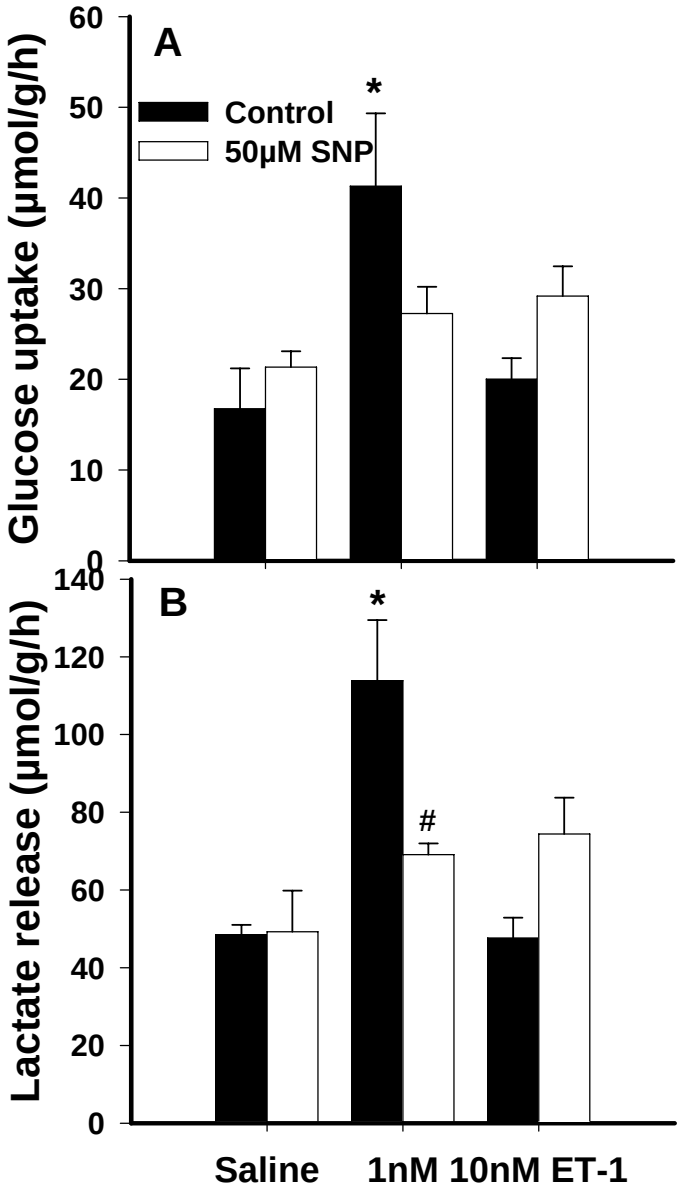

Figure 3.8. Effect of ET-1 on glucose uptake (A) and lactate release (B). Filled bars indicate ET-1 or vehicle infusion without SNP, white bars are for results with SNP infusion. Saline, SNP or ET-1 ± SNP were added at t = 0 min, according to the protocol in Figure 3.1. Values were at 40 min. *, significantly different ($P < 0.05$) from saline and $^{\#}$, significantly different from corresponding dose of ET-1 alone using a one-way ANOVA.

3.4 Discussion

The present study shows that ET-1 has marked metabolic effects in the constant-flow pump-perfused rat hindlimb. These include changes in oxygen and glucose uptake as well as lactate release. At low doses of 1nM or less, ET-1 is stimulatory with significant increases in oxygen and glucose uptake and lactate release. These metabolic increases were always associated with an increase in perfusion pressure and both the pressure and metabolic changes due to the low dose of ET-1 were blocked by the NO-vasodilator SNP. On this basis it appears likely that the metabolic effects of ET-1 are attributable to flow redistribution effects associated with the vasoconstrictor activity. As such, the stimulatory effects of low dose ET-1 are similar to a number of other vasoconstrictors that have been characterized in the constant-flow perfused rat hindlimb under a variety of perfusion conditions (i.e. at different temperatures, various albumin concentrations, and with or without red blood cells). These other vasoconstrictors include angiotensin, vasopressin, and low dose norepinephrine, and have been termed type A vasoconstrictors because of their general stimulatory effects on metabolism and their effects to recruit nutritive flow in this constant-flow preparation (190). Thus like ET-1, the increases in metabolism due to the type A vasoconstrictors were blocked by vasodilators, regardless of the mechanism of action of the vasodilator (183).

At higher doses, for example 10nM, ET-1 either inhibits metabolism or neutralizes the stimulatory effect of the lower dose by an inhibitory effect to render a situation where there is no net change. This is particularly evident when the time courses of the higher doses are examined. Initially, there is a marked transient stimulation of metabolism which then subsides to a gradual inhibitory influence that becomes dominant with time. For VO_2 there was a significant net inhibition for both 10 and 30nM ET-1. Much of the pressure increase and the inhibitory effect on VO_2 due to high doses of ET-1 were fully blocked by SNP, suggesting again that the metabolic effects were attributable to a redistribution of flow within the muscle, and thus similar to the type B vasoconstrictors that have been characterized in earlier publications (45, 130, 191). Clearly the dose of 50μM SNP was insufficient to fully block the pressure due to the high dose of 10nM ET-1 and higher doses of SNP used produced cyanotic effects. As a consequence, only a partial blockade of the inhibitory effect on VO_2 was evident. For glucose uptake and lactate release where the high dose of 10nM ET-1 had no net

effect, SNP tended to give rise to a net stimulation, although not significant. This may be due to a residual low dose effect of ET-1, as the time course suggests that the stimulation is slower to develop, and a gradual inhibitory effect, or attenuation of the stimulation, only just begins to occur at the end of the time course used.

Most noticeable in this study was the relative slowness of the response to ET-1. This vasoconstrictor, unlike either AII or NE (which took less than 5 min to plateau following commencement of infusion (190)), required 10min or more for VO_2 to plateau. Glucose uptake had not reached a plateau by 40min. Thus when high doses of ET-1 were infused initial low dose stimulatory effects occurred and as time progressed these declined, and eventually led to high dose inhibitory effects. Comparison of the time courses for high dose 10nM ET-1 effects on VO_2, glucose uptake, and lactate release indicate that the latter two were even slower to develop than VO_2 and this might account for why a net inhibition at 40min had not been attained. Other researchers have also noted that the time of onset of the effects of ET-1 were delayed (138). It is unknown whether prolonged exposure to a low dose of ET-1 would lead to either further stimulation of metabolism over time, or begin causing a net inhibition similar to high dose ET-1.

ET-1 is known to have vasodilatory effects largely mediated by ET_B receptors on endothelial cells (192). Pre-constriction of the hindlimb was essential before the vasodilatory activity of ET-1 could be assessed. To achieve this the hindlimb was constricted by constant infusion of either AII, a type A vasoconstrictor, that increases nutritive flow by decreasing non-nutritive flow (190), or 5-HT, a type B vasoconstrictor, that decreases nutritive flow and increases non-nutritive flow (190). Since the flow distribution effects of these two vasoconstrictors are opposite (130), it is quite likely that the sites in the vascular tree where vasoconstriction takes place are also different. Vasodilators may also show site-specific responses, dilating at one site but not another. Accordingly, dose curves were constructed for ET-1 against AII- or 5-HT-pre-constricted hindlimbs. There was a stronger trend for ET-1 to oppose the pressure due to AII than due to 5-HT, however the difference was not significant.

As no significant vasodilation by ET-1 was noted in this preparation, it is unlikely that the ET_B receptors located on the endothelial cells were activated. It has been suggested that ET_B receptors are not located on the endothelial cells of skeletal

muscle, which would explain the lack of an effect of ET-1 to vasodilate. It is difficult to determine, based on these results, whether ET_A or ET_B receptors on vascular smooth muscle cells are responsible for the vasoconstriction observed, although in human blood vessels ET_B receptors are present at lower levels than ET_A receptors, and so do not contribute substantially to vasoconstriction (193). Unpublished results from this laboratory have shown that ET-1 antagonists have no significant effect on ET-1-mediated vasoconstriction, and as such further conclusions regarding the involvement of either receptor subtype can not be made. Previous studies have shown that ET-1 receptor antagonists are not necessarily effective against ET-1 due to the high affinity of ET-1 for its receptor (147). As SNP was able to block both the vasoconstriction and metabolic effects of low dose ET-1 (1nM), it would appear that all metabolic effects of the lower dose of ET-1 are mediated by the vascular effects. This would very likely rule out direct effects of low dose ET-1 on muscle. The effects of higher concentrations of ET-1 are less clear, even though SNP blocked some of the vasoconstriction with a simultaneous amelioration of the inhibitory metabolic response, consistent with a net response of an intermediate dose of ET-1. This could mean that direct effects of ET-1 even at the higher doses are unlikely, and receptors are largely absent. Previous studies in cultured cells have indicated that skeletal muscle may not express ET_B receptors, although ET_A receptor expression was detected using PCR (180). These same researchers found that incubation of cultured L6 myoblasts with both 1 and 10nM ET-1 had no effect on insulin-mediated glucose uptake, indicating that there was no effect of ET-1 on insulin-stimulated myocyte metabolism (180). Such findings would weaken the notion of functional ET-1 receptors on myocytes.

It appears that although probably fully vasodilated, the muscles of the hindlimb are not fully perfused. It is interesting that a vasoconstrictor is able to increase the perfusion of the muscle (as observed by increased metabolism) when, conceptually, full vasodilation would in theory cause the most homogenous flow and greatest perfusion of the entire muscle. Considering this, it appears that low dose ET-1 is able to constrict blood vessels to more efficiently perfuse muscle, probably by constricting the non-nutritive pathway so that a greater portion of the flow is directed towards the nutritive network.

High doses of ET-1 (10nM and 30nM) led to the development of oedema, which developed more rapidly with the higher concentrations. This oedema was coincident with a decrease in flow which varied from 10 – 50% between experiments (data not shown) and an increase in pressure. The pressure effects of ET-1 could therefore not be separated from the oedema effects at these doses. Once oedema sets in, it appears to be self-perpetuating, causing a further increase in pressure and accumulation of fluid. Therefore, it is difficult to determine whether perhaps a plateau of pressure development due to ET-1 infusion was reached at each dose, while the progression of oedema may be responsible for the continuing increase in pressure. Other studies have also demonstrated that ET-1 leads to oedema (145, 194, 195). ET-1 was shown to increase capillary pressure and transcapillary fluid loss, leading to an increase in the interstitial fluid volume (194). Results presented by Bentzer et al (145) showed that ET-1 was capable of reducing the capillary filtration coefficient in a dose-dependent manner, which appears to be ET_B dependent, with an increase in total vascular resistance. The capillary filtration coefficient is dependent on the fluid permeability of the capillary wall and the surface area available for filtration. That study, performed on denervated calf muscles of the cat, concluded that the reduction in capillary filtration coefficient was not due to a change in the number of perfused capillaries, vascular tone or blood flow (although this may occur), but to an alteration in microvascular fluid permeability (145). As the change could be blocked by tranylcypromine, a prostacyclin synthesis inhibitor, it is likely that ET-1 binding to the ET_B receptor causes prostacyclin release, altering the permeability of the capillary walls. A change in vascular permeability was not observed in a study in humans by Dahlof et al (195), although oedema was still evident, probably due to an increase in postcapillary resistance and hydrostatic pressure causing transcapillary fluid transfer to the tissue. Therefore, while different results in terms of vascular permeability have been recorded, oedema appears to be induced by ET-1, which elicits increased hydrostatic capillary pressure; this may coincide with an increase in vascular permeability elicited by ET_B-mediated prostacyclin release.

Insulin causes ET-1 release (88-90), and plasma ET-1 is known to be elevated in states of hyperinsulinemia, particularly type 2 diabetes (160). Insulin relaxation is impaired by high concentrations of ET-1, which induces an ET_A-mediated vasoconstriction (176). A negative correlation between glucose uptake and ET-1 concentration (160) suggests that ET-1 inhibits the insulin sensitivity of the target

tissue. This could be explained by the redirection of blood flow away from the nutritive areas. There was no correlation performed between glucose uptake and ET-1 concentration in non-diabetic healthy subjects with low plasma ET-1. Given the current results, it is possible that 'healthy' levels of ET-1 may actually increase glucose uptake.

In conclusion, ET-1 has a marked stimulation of metabolism at low doses in the constant flow perfused rat hindlimb that is the result of a direct vasoconstriction activity to alter flow distribution as has been reported earlier for a number of other vasoconstrictors. At higher doses the vasoconstriction intensifies and the metabolic stimulatory effect subsides and becomes inhibitory. The mechanism behind these dose-dependent effects is not yet certain and will be discussed in more detail in later chapters.

CHAPTER 4

HIGH DOSES OF ET-1 INHIBIT TENSION DEVELOPMENT AND ARE RESISTANT TO EXERCISE-MEDIATED VASODILATION

4.1 Introduction

Exercise in healthy subjects leads to a decrease in peripheral vascular resistance, although this response is impaired in patients with hypertension, and can be responsible for reduced exercise capacity (196). Treatment with an ET_A receptor blocker, BQ123 can restore the reduced vasodilator response to exercise in these patients to near normal levels (197) as ET-1 levels are elevated in hypertensive patients (153, 168) and in patients with other cardiovascular disorders (160, 198). Although so far there is inconclusive evidence regarding the effect of ET-1 on basal vascular tone (153), ET-1 is thought to aid in the exercise-induced redistribution of blood flow (199-201). In humans performing single-leg exercise, the ET-1 concentration in the non-working leg was found to be higher after exercise (201). This was thought to cause local vasoconstriction, thereby decreasing blood flow in non-working muscle and directing it towards the working muscle (200). Also, a study in rats revealed that ET_A receptors were responsible, at least in part, for decreased blood flow to internal organs measured by microspheres during treadmill exercise (199). This is consistent with the fact that ET-1 is released locally, not systemically, into the blood stream, and is mainly released abluminally (146), and so there may be regional heterogeneity of ET-1 release. For this reason, any measures of systemic ET-1 are likely to underestimate the ET-1 concentration in the local environment, and such figures may possibly represent an overflow effect.

The previous chapter has shown that ET-1 has both stimulatory and inhibitory effects on metabolism in a dose-dependent manner due to the ability of ET-1 to redistribute flow by vasoconstriction. As low dose ET-1 redirects flow to areas that stimulate metabolism, it is possible that ET-1 levels in normal subjects also exert a slight

stimulation of metabolism, by directing flow to nutritive areas, where a greater level of basal metabolism can occur (180). However, hypertensive patients may be subject to the inhibitory metabolic effects due to their higher circulating ET-1 levels (153, 168), which potentially lead to a reduction of flow through muscle, or non-nutritive perfusion. As an ET_A receptor blocker can restore the exercise-mediated dilation in hypertensive patients (197), we suggest that higher concentrations of ET-1 found in these patients may be resistant to exercise hyperemia. We hypothesize that at higher concentrations ET-1 may exert inhibitory effects on aerobic tension development, while at low concentrations there may be no inhibition of aerobic tension. Thus, in this study we aim to determine whether ET-1 has a similarly biphasic effect on the aerobic tension development in the perfused rat hindlimb, with comparisons made to other known vasoconstrictors, including serotonin (5HT), Angiotensin II (AII) and norepinephrine (NE). Also, we observe the effect of exercise-mediated hyperemia on ET-1-mediated vasoconstriction. We have used this system as it allows for more invasive study involving measurement of hemodynamics and metabolism, and prevents any sympathetic effects from masking the direct effect of ET-1 in muscle.

4.2 Methods

4.2.1 Solutions

Solutions were made up to be 200 times the required final concentration to allow for infusion at $1/200^{th}$ of the flow into the infusion port. Substances were infused into a small magnetically-stirred bubble trap located in the arterial perfusion line at 75 µl/min, equivalent to 1/200 of the pump flow rate.

Endothelin-1

ET-1 (1mg, Calbiochem) was dissolved in 125µl acetic acid and made up to volume with saline. Vehicle infusions were conducted using acetic acid concentrations equivalent to the 10nM ET-1 dose. Final concentrations used were 1nM and 10nM ET-1, so infusion solutions were made up to 200nM and 2µM respectively.

Vehicle

Vehicle infusions contained an equal volume of acetic acid as used in the 10nM final ET-1 experiments.

Serotonin, Norepinephrine and Angiotensin II

5HT, NE and AII were made up to the required volume with saline, and infused at $1/200^{th}$ of the flow to give final concentrations as shown in the relevant graphs.

4.2.2 Perfusion conditions

Hindlimb perfusions were performed at $32^{o}C$, with 2.54mM $CaCl_2$ and 8.3mM glucose. Flow rate remained at 8ml/min, and the regular Krebs buffer was used (section 2.2.2). The surgery was performed as in section 2.2.3, but the right hindlimb was ligated, and flow was restricted to the left hindlimb, as the jig used to secure the knee was located on the left.

4.2.3 Contraction

The skin was removed from the thigh of the left hindlimb, and the knee secured. Electrodes were placed through the skin above the knee and the Achilles tendon for field stimulation, and the Achilles tendon was attached to a Harvard Apparatus isometric transducer, allowing transmission of tension development from the gastrocnemius-plantarus-soleus muscle group. The hindlimb was allowed to equilibrate for 30 minutes at 8 ml/min; the flow rate was then increased to 15 ml/min as indicated in the protocols (Figure 4.1) and the resting length of the muscle was adjusted to obtain maximal active tension on stimulation. A Nerv-Muskel-Reizgerat stimulator (Hugo Sachs Elektroniks) administered 0.1 ms pulses applied at 0.5Hz with appropriate voltage for maximal stimulation (191, 202) as indicated in the protocols.

4.2.4 Perfusion protocol

Two different protocols have been used in this chapter. In each, the hindlimb was allowed to equilibrate for 30 minutes at a constant flow of 8 ml/min before the flow was increased to 15 ml/min. Infusions of test solutions and contraction were initiated as shown in Figure 4.1. The perfusion pressure and venous effluent oxygen content were continuously monitored, and venous effluent was sampled as indicated for measurement of glucose and lactate using a glucose analyzer (Yellow Springs Instruments, model 2300 Stat Plus), and the remainder discarded.

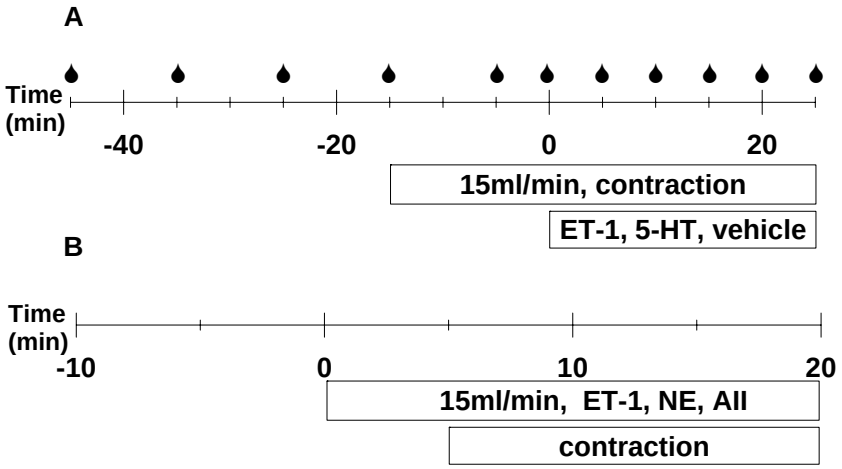

Figure 4.1. Experimental protocols for the surgically isolated pump-perfused rat hindlimb. A) The hindlimb was allowed to equilibrate for 30 minutes at a constant flow of 8 ml/min before the flow was increased to 15 ml/min and twitch stimulation commenced as described in the methods. A further equilibration period of 15 minutes was allowed before infusion of 1 μmol/L serotonin (5-HT), 1 nmol/L ET-1 or 10 nmol/L ET-1 (final concentration) or a suitable vehicle. The perfusion pressure and venous effluent oxygen content were continuously monitored, and venous effluent was sampled as indicated by the drops (♦) for glucose and lactate determination. B) In other experiments, flow was increased to 15ml/min after at least 30 minutes of equilibration at 8ml/min and vasoconstrictors were infused as indicated. After 5 minutes, contraction was initiated, and effects on pressure were observed.

4.2.5 Statistical Analysis

Two-way repeated-measures ANOVA was performed using SigmaStat (SPSS Science; Chicago, IL), with comparisons made between conditions using the Student-Newman-Keuls post hoc test. Significance was assumed at the level of $P < 0.05$. Data are presented as means ± SE.

4.3 Results

4.3.1 Vascular and metabolic effects of ET-1

The administration of 1μM 5-HT and 10nM ET-1 raised the perfusion pressure to a similar extent, which was significant by five minutes (Figure 4.2A), although the effects on metabolism, as measured by oxygen consumption, varied considerably. 5-HT caused a significant reduction in oxygen consumption from 5 minutes after infusion was commenced, shown in Figure 4.2B. 10nM ET-1 induced a transient stimulation of oxygen consumption (P=0.015 at 5 minutes vs vehicle), before a significant inhibition of this response was observed from 15 minutes until the end of the protocol. 1nM ET-1 also caused a significant stimulation of oxygen consumption above that of the vehicle infusion, although in this preparation the increase in pressure was not significant.

A significant effect of 1nM ET-1 to increase glucose uptake was noted at 5 minutes, and there was an increase in lactate washout that was significant for 1nM ET-1 at 10 minutes until the end of the protocol (Figure 4.3). 10nM ET-1 had no significant effect on glucose uptake, but significantly increased lactate release over the course of the experiment, with a tendency to drop back towards basal levels over time (Figure 4.3). 5-HT caused a significant decrease in lactate release at all time points measured.

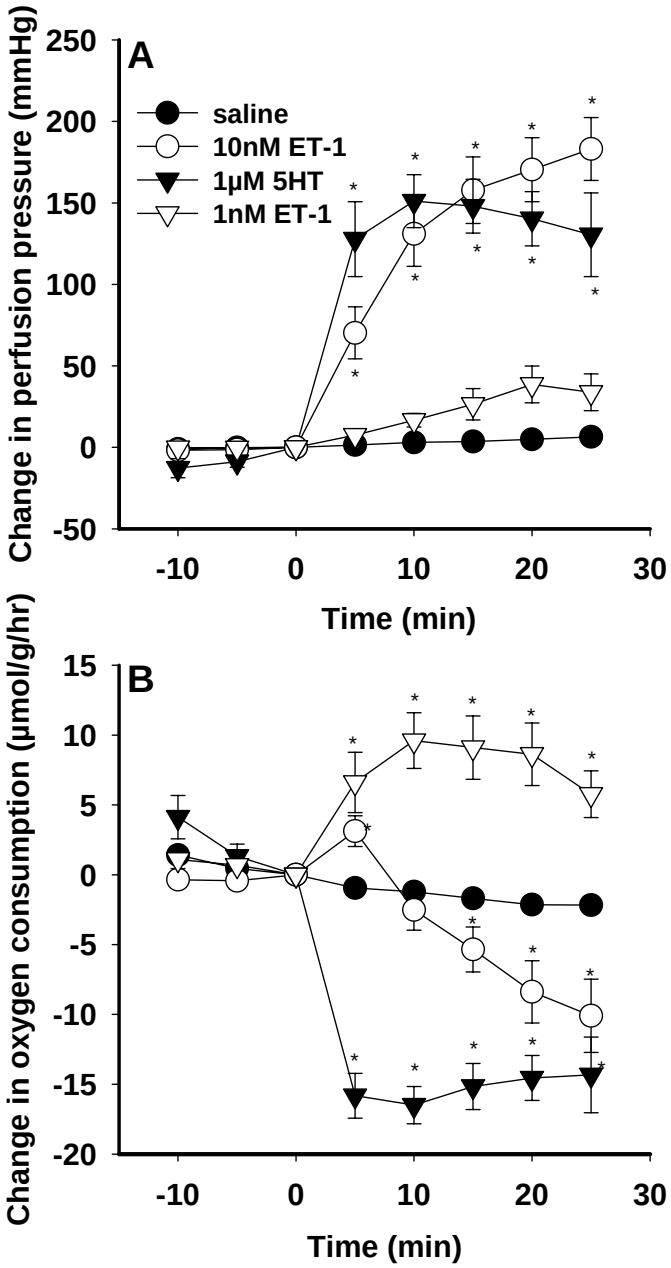

Figure 4.2. Effects of vehicle, 1nM, 10nM ET-1 and 1μM 5HT on the change in perfusion pressure (A) and oxygen consumption (B). n=6 for each point, using Protocol A from Figure 4.1. *, significantly different to vehicle ($P<0.05$) using two-way repeated measures ANOVA.

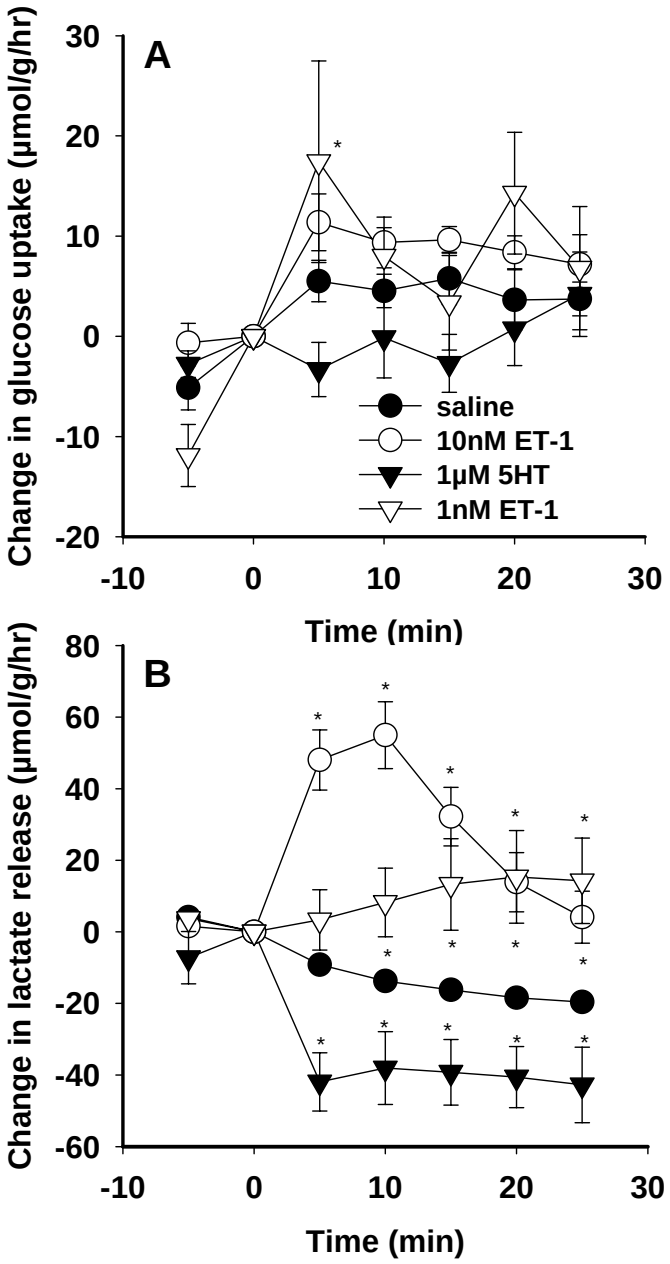

Figure 4.3. Effects of vehicle (●), 1nM ( ), 10nM ET-1 (○) and 1µM 5HT (▼) on the change in glucose uptake (A) and lactate release (B). n=6 for each point, using Protocol A from Figure 4.1. *, significantly different to vehicle ($P<0.05$).

4.3.2 Effects on aerobic tension development

Electrical field stimulation resulted in an initial anaerobic phase of maximum tension development, which eventually plateaued after ten to fifteen minutes representing a relatively constant level of aerobic tension development. Infusion of test substances was initiated at this point of plateau. Vehicle infusions showed a slight decrease in tension development over the time of the experiment, so all results were compared to the corresponding vehicle value at the same time point. Infusion of 1μM 5-HT showed an immediate decrease in the change in developed tension to a value significantly below vehicle by 5 minutes (Figure 4.4), which remained inhibitory over the course of the protocol. 10nM ET-1, however, caused an initial stimulation of aerobic contraction, which was significantly different to vehicle at 5 minutes ($P=0.049$), but which then dropped away to a significant inhibitory effect from 15 minutes until the end of the perfusion. 1nM ET-1 caused a milder, non-significant stimulation of the aerobic contraction.

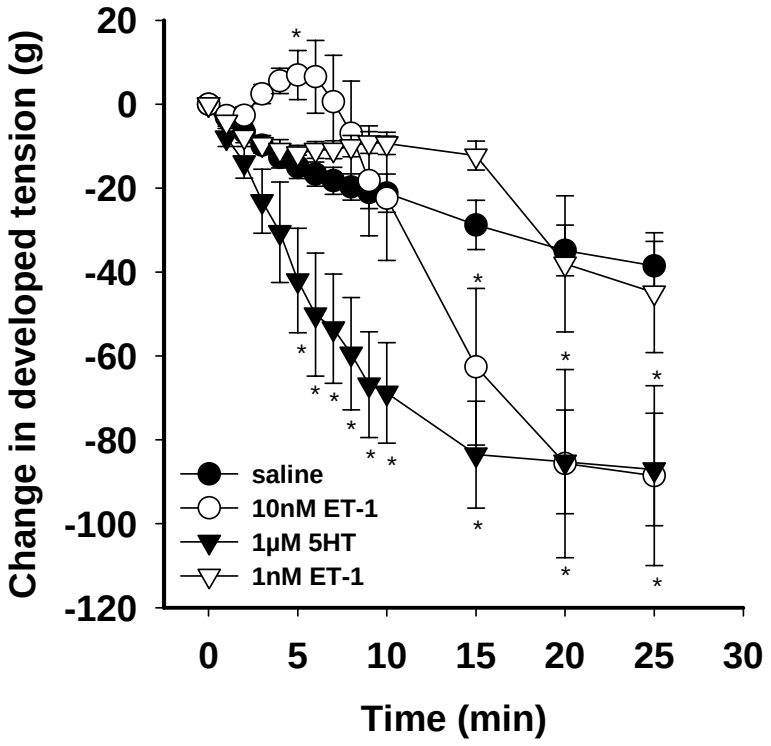

Figure 4.4. Effects of vehicle (●), 1nM ( ), 10nM ET-1 (○) and 1µM 5HT (▼) on the change in developed tension. n=6 for each point, using Protocol A from Figure 4.1. *, significantly different to vehicle ($P<0.05$) using two-way repeated measures ANOVA.

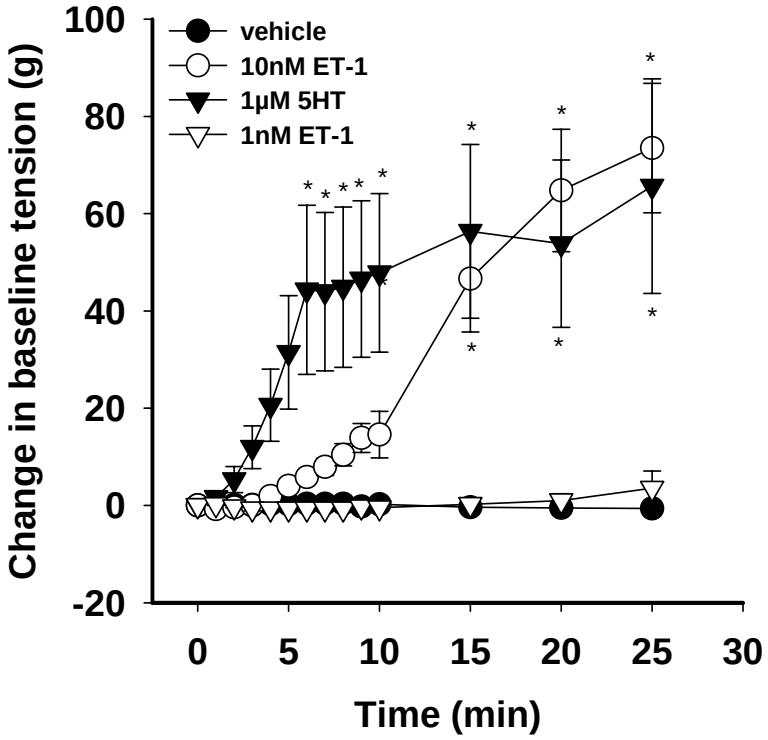

Figure 4.5. Effects of vehicle (●), 1nM ( ), 10nM ET-1 (○) and 1µM 5HT (▼) on the change in baseline tension. n=6 for each point, using Protocol A from Figure 4.1. *, significantly different to vehicle ($P<0.05$).

As seen in Figure 4.5, the baseline of the tension development curve tended to rise with both 5-HT and high concentrations of ET-1, an effect that seemed to coincide with the development of edema. The rising baseline effect was mirrored by increasing the height of the venous outflow, which simulates venoconstriction by increasing the venous pressure. This technique led to an increase in perfusion pressure, as well as the increase in baseline tension (data not shown).

4.3.3 Exercise-mediated hyperaemia

Exercise can cause vasodilation in the constricted hindlimb, as shown below in Figure 4.6. Exercise is able to dilate against both NE and AII, this effect is significantly different to the vasoconstrictors alone by 2 minutes after the initiation of contraction.

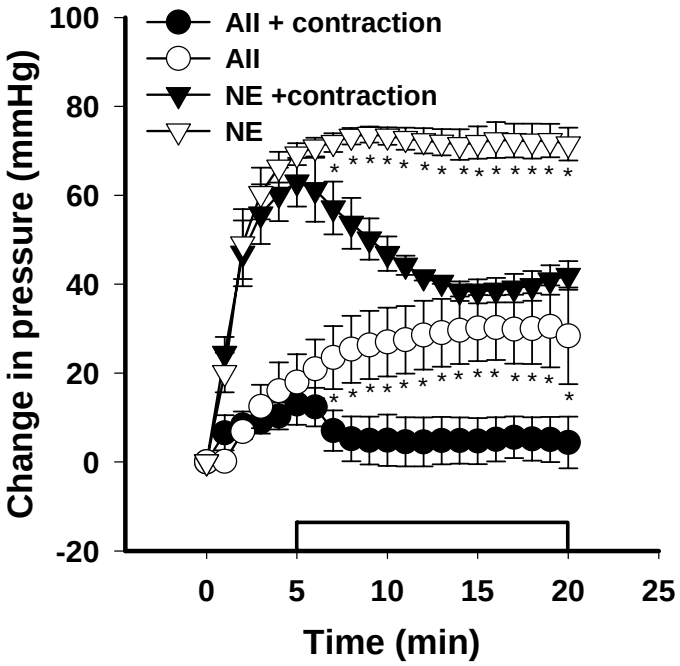

Figure 4.6. Effects of exercise-mediated vasodilation on the pressure developed by different vasoconstrictors. Vasoconstrictors were infused from 0 to 20 minutes, and contraction was initiated at 5 minutes (as shown by the bar) and continued until the end of the perfusion according to protocol B in Figure 4.1. The acute effects of exercise hyperaemia against AII (2nM) and NE (70nM) are shown. At least three experiments were performed for each group.*, significantly different to the corresponding time point for the same vasoconstrictor with contraction

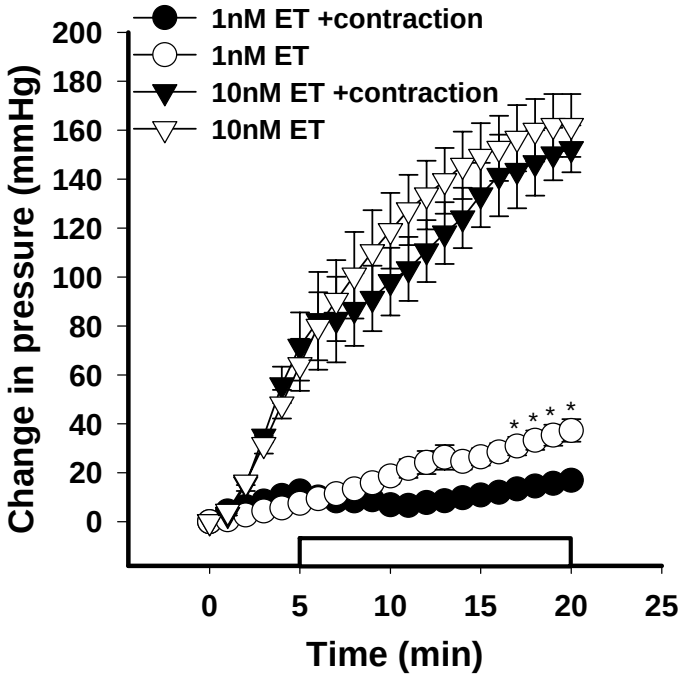

Figure 4.7. Effects of exercise-mediated vasodilation against ET-1-mediated vasoconstriction at both 1 nmol/L and 10 nmol/L. Vasoconstrictors were infused from 0 to 20 minutes, and contraction was initiated at 5 minutes and continued until the end of the perfusion (indicated by the bar), as shown in protocol B in Figure 4.1. At least three experiments were performed for each group. *, significantly different to corresponding dose of ET-1 alone at that time point ($P < 0.05$) using a two-way repeated measures ANOVA.

Figure 4.7 demonstrates that exercise is able to vasodilate efficiently against 1nM ET-1; this dose normally causes a significant increase in pressure over the 20 minutes studies, but the effect was lost during muscle contraction, and at 20 minutes there was a significant difference between the groups for 1nM ET-1. The higher dose of 10nM ET-1 appeared to be resistant to exercise-mediated vasodilation, as there was no significant difference between either group with or without muscle contraction. Both groups were significantly elevated above the pressure observed at the 0 minute time point.

4.4 Discussion

The main finding of the present study is that ET-1 can both stimulate and inhibit aerobic muscle tension development dependent on dose and time. As we have previously shown that the metabolic effects of ET-1 in muscle are due to the vascular effects (chapter 3) by blocking vasoconstriction with sodium nitroprusside, we suggest that the effects of ET-1 on aerobic contraction of muscle are also due to vascular effects, specifically redistribution of flow. Also, while lower concentrations of ET-1 may be sensitive to exercise-mediated hyperaemia, doses of 10nM appear to be resistant to any vasodilation that exercise can cause.

The results shown here confirm and extend the conclusions of the previous chapter: that ET-1 may be able to regulate the delivery of oxygen to contracting muscle, an effect that is consistent with the ability of ET-1 to redistribute flow to prevent delivery of nutrients and hormones (180), at least at high concentrations. High doses of ET-1 (10nM) transiently stimulated the aerobic tension development, before an inhibition became the more dominant force, while 1nM ET-1 had no significant effect on tension development. As the previous chapter was able to demonstrate that SNP could block the stimulation of oxygen consumption by ET-1, it is concluded that the effects of ET-1 on aerobic tension development were due to the vascular effects to redistribute flow.

This study also evaluated the ability of exercise to vasodilate against different vasoconstrictors, which could be observed in each case as a decrease in perfusion pressure. Exercise was able to successfully dilate against NE, AII and low dose ET-1 (1nM) in every experiment. However, ET-1 was able to continue constricting, and the pressure in the 1nM ET-1 group began to increase (non-significantly) over the time course, while NE and AII did not develop further pressure after exercise. Exercise did not appear to be able to acutely vasodilate against the vasoconstriction induced by 10nM ET-1, and the total pressure developed did not vary at all from that expected in the absence of contraction. There was however a significant difference between the two groups using a lower concentration of ET-1, whereby muscle contraction was able to vasodilate to significantly lower the pressure induced by 1nM ET-1. These results are similar to those of the previous chapter, where the dose of SNP used was not sufficient to completely dilate against the higher doses of ET-1 used. It is possible

that a higher concentration of SNP, or in this case, a more intense exercise, may have a greater effect to dilate against the ET-1-mediated vasoconstriction.

Hypertensive patients have an impaired vasodilator response to exercise that can be corrected to near normal levels by treatment with an ET_A receptor blockade (197); increased vascular activity of ET-1 in these patients might contribute to the reduced exercise tolerance. The contribution of each receptor type to the vasoconstrictor response observed has never been clarified, although in human blood vessels, ET_B receptors are present at much lower levels that ET_A receptors, and so are not thought to contribute substantially to vasoconstriction (193). Experiments using specific receptor blockers in the perfused rat hindlimb have so far been unable to demonstrate conclusively whether ET_A or ET_B receptors are involved in the rat ET-1-mediated vasoconstrictor response (our unpublished observations). Our results suggest that, irrespective of which receptor type is responsible, an increase in the concentration of ET-1 could change the perfusion of the muscle from predominantly nutritive (type A), or at least with no net effect on metabolism, to a predominantly non-nutritive (shunt) perfusion (type B), which reduces oxygen delivery to working muscle. This would result in reduced exercise tolerance due to inadequate perfusion of the working muscle. As hypertensive patients appear not to show the normal vasodilatory effects of exercise (196), it is possible that the elevated levels of ET-1 observed in cases of hypertension (153, 168) may be resistant to this vasodilation, according to the results from this chapter. Healthy humans with low concentrations of ET-1 would likely still be sensitive to exercise hyperaemia.

A limitation of this study is that the doses of ET-1 used are much higher than plasma levels of both normal and hypertensive patients. As yet, no study has been able to examine the concentrations of ET-1 in the local environment of where it is believed to be released, and it is thought that the plasma levels are a result of overflow from the local environment. Wagner et al (146) have shown that 80% of the ET-1 released by endothelial cells is directed abluminally, and have suggested that the 20% found on the luminal side may be due to diffusion. The local concentration can not be calculated from the systemic concentration, due to the possibility of heterogeneity of ET-1 release throughout the body, and the lack of confirmation that this 80:20 ratio in secretion occurs *in vivo*. Therefore we have used higher concentrations of ET-1 than are commonly found in the plasma to mimic the concentrations that may be found in

the local environment. The perfused hindlimb prevents any systemic or sympathetic response from the body in response to this high level, and in theory permits examination of the effects of ET-1 directly on the muscle and its vasculature.

Others have suggested that treatment with ET_A or mixed receptor blockade may aid in improving exercise capacity(197), lowering blood pressure and protecting against cardiovascular injury (151, 168). The results indicated here suggest that antagonism of ET-1 receptors would be beneficial in the above cases if the plasma levels of ET-1 were found to be in excess. However, it must be noted that low dose, positive effects of ET-1 on metabolism and oxygen delivery are possible. Further studies are required to investigate the effects of low dose ET-1 *in vivo* and in humans to determine if this effect is real. We suggest that ET-1 may have concentration-dependent biphasic effects on metabolism and exercise capacity, which will become inhibitory in disease states due to lower perfusion of the muscle.

CHAPTER 5

INTERACTIONS BETWEEN ET-1 AND INSULIN

5.1 Introduction

Previous studies have demonstrated that is possible to alter insulin-mediated glucose uptake effects by changing the properties of the blood flow distribution within muscle. For example, by infusing methacholine into human subjects, an augmentation of the normal glucose uptake response seen with insulin occurred, suggesting that increasing the blood flow distribution also increases the insulin sensitivity (59). Conversely, insulin resistance can be induced in rat muscle by the infusion of a vasoconstrictor such as serotonin (45) due to the restriction of flow to insulin-sensitive areas.

There is now growing evidence that the vascular actions of insulin may be the net result of vasodilation by NO and the vasoconstrictor activity of ET-1 (88-90). Eringa et al (88) have recently shown, with isolated blood vessels from muscle, that in the presence of a nitric oxide synthase inhibitor there is an insulin-mediated vasoconstriction that is blocked by an ET receptor antagonist. This observation combined with the previous chapter showing a nutritive effect of ET-1 alone at low doses might suggest that insulin-mediated release of ET-1 is intended to aid in the recruitment of capillary blood flow in muscle. Conversely, high levels of ET-1 reported in type 2 diabetics (198), obese patients (160) as well as hyperinsulinemic states in hypertensive individuals (198) may be antagonistic of insulin's metabolic and vascular effects and a significant contributor to the elevated blood pressure, a conclusion supported by the high dose ET-1 alone data presented in chapter 3.

It would appear, then, that the interaction between insulin and ET-1 is a complex one, quite likely dose-dependent. This chapter will further examine the dose-dependent effects of ET-1 on insulin action, assessed by glucose uptake, and also the effects of insulin on ET-1 action, particularly the vasoconstrictor effects on perfusion pressure.

5.2 Methods

5.2.1 Solutions

Solutions were made up to be 200 times the required final concentration to allow for infusion at 1/200th of the flow into the infusion port. Therefore, at a perfusion of constant flow rate 8ml/min, substances were infused at 40µl/min.

Endothelin-1

ET-1 was dissolved in a minimum amount of acetic acid (1mg in 125µl), and then made up to volume with saline. Infusion solutions of 200nM and 2µM were infused at 1/200th of the flow into the infusion port to give final concentrations of 1nM and 10nM respectively.

Vehicle

Vehicle infusions contained an equal volume of acetic acid as used in the 10nM final ET-1 experiments.

5.2.2 Perfusion conditions

Hindlimb perfusions were performed on the right hindlimb of the rat at 32^{o}C, with buffer containing 2.54mM $CaCl_2$ and 8.3mM glucose. Flow rate was maintained at 8ml/min, and the regular Krebs buffer was used (section 2.2.2).

In some experiments, Ca^{2+} free Krebs was used. In these experiments, $CaCl_2$ was not added to the perfusion medium, and instead EGTA was added to achieve a final concentration of 100mM.

5.2.3 Perfusion protocol

After equilibrating the rat hindlimb for 40 minutes, ET-1 was infused at an appropriate rate to give a known concentration in the perfusion medium. This infusion was maintained for 40 minutes, during which time, perfusate samples were taken at 5 minutes intervals for analysis for glucose uptake and lactate release. Perfusate samples were analysed for glucose and lactate using the YSI 2300 STAT Plus Glucose Analyser.

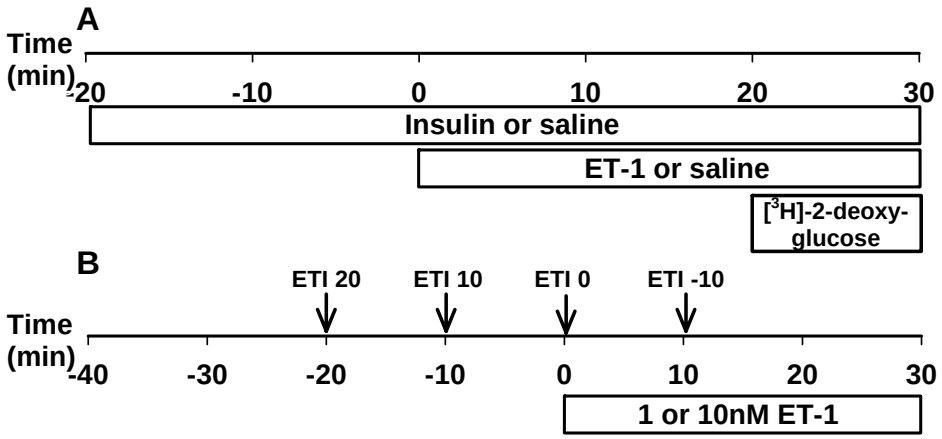

Figure 5.1. Experimental protocol for the surgically isolated pump-perfused rat hindlimb. A perfusate flow of 8ml/min was used, and the hindlimb was allowed to equilibrate for 40 minutes after surgery prior to the infusion of any test substances. Venous perfusate samples were taken every 10 minutes for analysis for glucose and lactate in both protocols. A) Insulin infusion began at -20 minutes, ET-1 infusion was initiated at 0 minutes, and 2-deoxyglucose when used was infused for the final 10 minutes, as shown by the lower bar; all infusions continued until the end of the experimental protocol at 30 minutes after ET-1 infusion. B) ET-1 infusion began at 0 minutes and continued for 30 minutes in all experiments. Insulin infusion began as indicated by the arrows, and then continued at a constant rate until the end of the experiment; labels indicate that a combination of ET-1 and insulin was used, and correspond to labels in the relevant graphs.

5.2.4 Radiolabelled glucose uptake

The ^{3}H-2-deoxyglucose assay has been described in chapter 2.3. 2-Deoxyglucose was infused for 10 min prior to the end of perfusion as shown in Figure 5.1A, at which time muscles were dissected out and freeze-clamped. The muscles collected were soleus, plantaris, gastrocnemius (red and white muscle analysed separately), extensor digitorum longus, and tibialis. These were then powdered under liquid nitrogen and analysed as described previously (section 2.3)

5.2.5 Statistical Analysis

Two-way repeated-measures ANOVA was performed using SigmaStat (SPSS Science; Chicago, IL) for the time-course experiments, and one-way ANOVA for the end-point histograms, with comparisons made between conditions using the Student-Newman-Keuls post hoc test. Significance was assumed at the level of $P < 0.05$. Data are presented as means ± SE.

5.3 Results

The first set of experiments involved the infusion of insulin for 20 minutes prior to the infusion of ET-1 as shown in protocol A in Figure 5.1. In these experiments, insulin appeared to completely block the vasoconstrictor activity of low dose ET-1 (1nM) measured by pressure (Figure 5.2A). Consistent with this, the normal oxygen response for ET-1 at this dose was completely blocked (Figure 5.2B). While the stronger pressure response observed with higher concentrations of ET-1 (10nM) was not completely blocked, there was still a significant attenuation by insulin (Figure 5.2A), and there was still an effect of insulin to significantly block the oxygen response (Figure 5.2B). The inhibition of oxygen uptake normally observed with ET-1 did not occur with insulin, resulting in no net effect of either dose of ET-1 on oxygen uptake in the presence of insulin. There was no significant effect of insulin alone on either pressure or oxygen uptake.

As shown in previous chapters, 1nM ET-1 can stimulate glucose uptake, while higher doses (10nM) tend to have no net effect by the end of the time course used, but appear to be heading into a general inhibition. Insulin itself can stimulate glucose uptake (Figure 5.3A) and this effect did not appear to be significantly modified by either dose of ET-1 used when the end-point is compared, yet there may be a slight trend for either dose of ET-1 to stimulate insulin-mediated glucose uptake when the entire time-course is considered. Effects on lactate release were slightly more complicated, and the sharp peak in lactate release observed with 10nM ET-1 appeared to be extended with insulin, and stimulation was still apparent at the end of the protocol used (Figure 5.3B). The effect of 1nM ET-1 to increase lactate release was blocked by insulin, such that this dose of ET-1 had no effect above the lactate release induced by insulin alone.

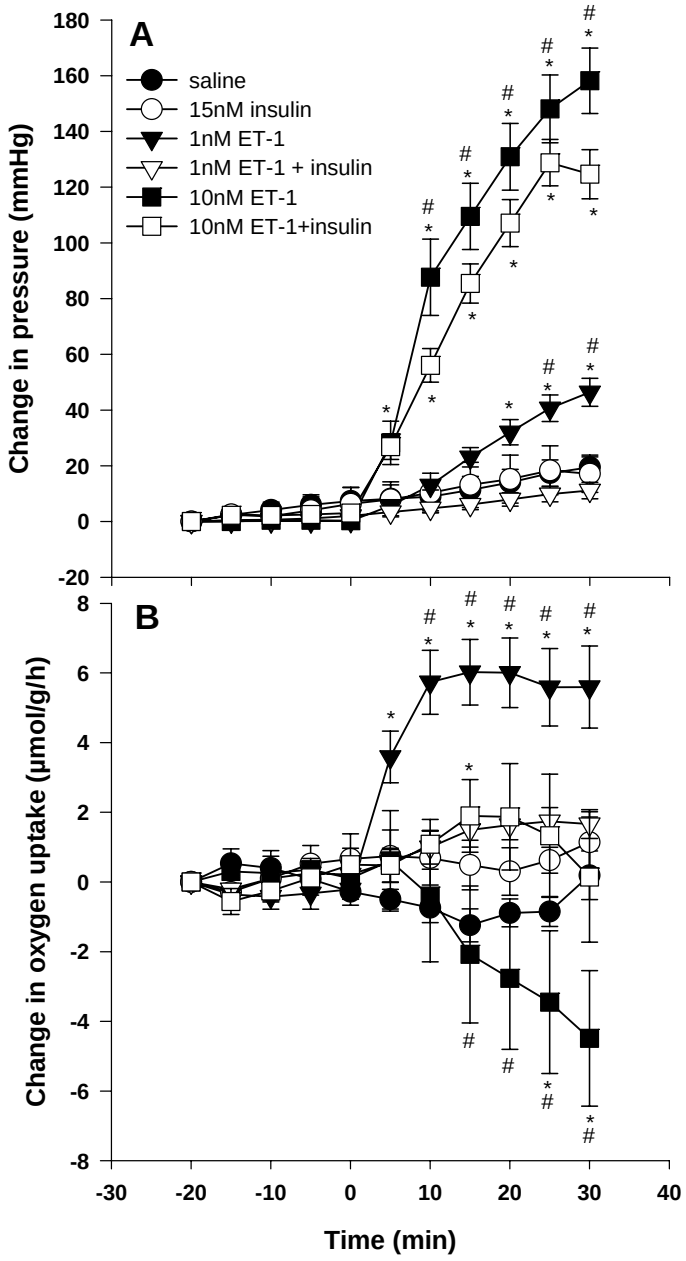

Figure 5.2. Effects of ET-1 and insulin on perfusion pressure (A) and oxygen uptake (B). Insulin infusion began at -20 min and ET-1 infusion commenced at 0 min as shown in Protocol A in Figure 5.1. n = at least 6 for each point. *, significantly different to vehicle, and #, significantly different to corresponding dose of ET-1 + insulin at that time point ($P<0.05$) using two-way repeated measures ANOVA.

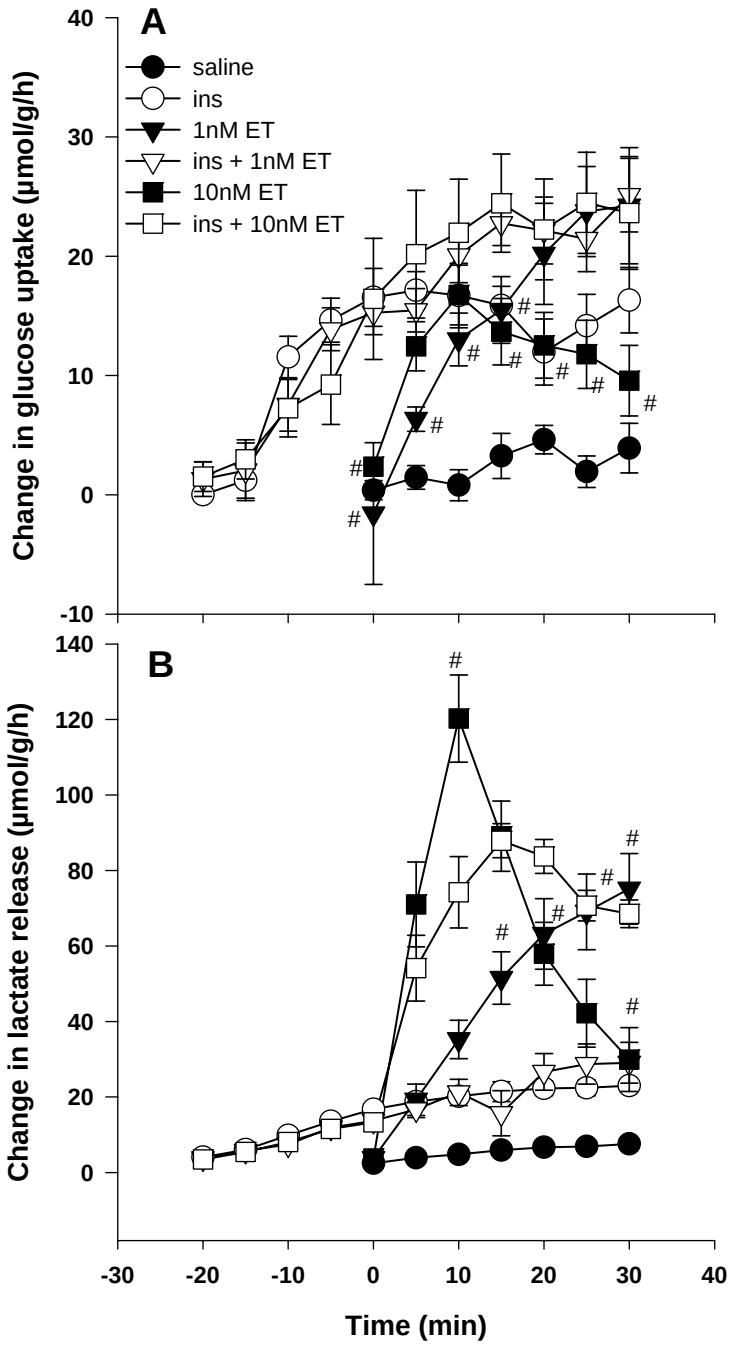

Figure 5.3. Effect of insulin on ET-1 mediated effects on glucose uptake (A) and lactate release (B). Insulin infusion began at -20 min and ET-1 infusion commenced at 0 min as shown in Protocol A in Figure 5.1. n = at least 6 for each point. # indicates that the black symbol is significantly different to the corresponding dose of ET-1 and insulin ($P<0.05$) using two-way repeated measures ANOVA.

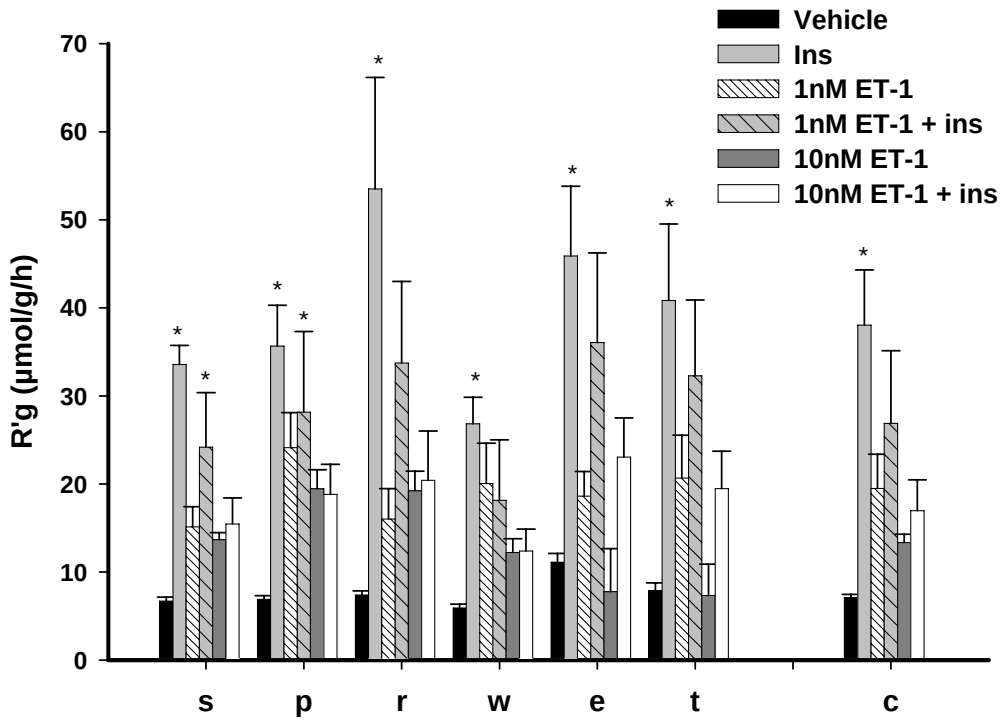

Figure 5.4. Effect of insulin and ET-1 on 2-deoxyglucose uptake (R'g). Protocol A in Figure 5.1 was used for each of these experiments. n = at least 6 for each point. X-axis headings refer to muscles measured: s, soleus; p, plantaris; r, gastrocnemius red; w, gastrocnemius white; e, extensor digitorum longus; t, tibialis; c, combined results from all muscles measured. *, significantly different to vehicle in that muscle ($P<0.05$) using two-way repeated measures ANOVA.

Figure 5.4 demonstrates that insulin itself is able to stimulate radiolabelled 2-deoxyglucose uptake in the muscle of the calf, and in this measure ET-1 is able to dose dependently inhibit this insulin-stimulated glucose uptake. The significant effect of insulin to cause glucose uptake is lost in most muscles by infusion of either 1nM or 10nM ET-1. These effects differ from the glucose uptake data shown above calculated from the A-V glucose difference.

The second aspect of this study was to examine the effect that varying the time of exposure to insulin had on the ability of insulin to block ET-1 effects. This was performed using protocol B in Figure 5.1. Insulin (15nM) was observed to have more profound effects against 1nM ET-1 as opposed to 10nM ET-1, confirming the results above. Insulin was more effective at blocking ET-1-mediated vasoconstriction when it had been infused for a greater length of time (Figure 5.5A). The oxygen consumption normally induced by ET-1 was blocked by infusing insulin 20 minutes prior to and throughout ET-1 infusion, but a shorter exposure to insulin had less effect to block the oxygen uptake (Figure 5.5B).

When insulin is infused prior to ET-1 there is an additive effect on glucose uptake as measure by A-V glucose difference (Table 5.1). When insulin infusion begins with or after ET-1 infusion, there appears to be no additive effect of insulin and ET-1. Any time of exposure to 15nM insulin blocked the lactate release (Table 5.1).

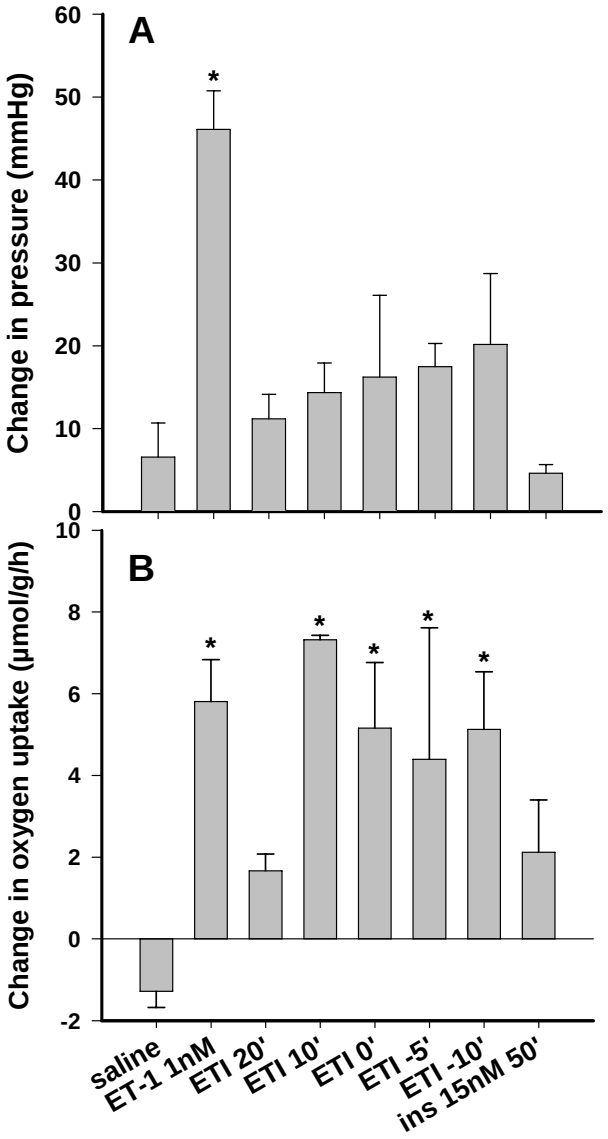

Figure 5.5. Effect of different times of exposure to insulin on the normal 1nM ET-1 response on perfusion pressure (A) and oxygen uptake (B). Insulin infusion began at various time points and ET-1 infusion commenced at 0 min as shown in Protocol B in Figure 5.1 using the relevant labels. ET-1 is infused alone, ETI 20, insulin is infused 20 minutes prior to ET-1; ETI 10, insulin is infused 10 minutes before ET-1; ETI 0, insulin and ET-1 are infused at the same time; ETI -5, insulin infused 5 minutes after ET-1; ETI -10, insulin infused 10 minutes after ET-1. The value given for insulin 15nM was after 50 minutes of insulin infusion alone. n = at least 3 for each point. *, significantly different to saline ($P<0.05$) using one way ANOVA.

	Change in glucose uptake (µmol/g/h)					Change in lactate release (µmol/g/h)			
	n	**Mean**		**SE**		**Mean**		**SE**	
Saline	6	2.78	±	1.71		4.62	±	2.05	
ET-1 1nM	6	15.19	±	1.67		75.17	±	9.35	*
ETI 20	6	25.12	±	3.09	*	29.07	±	5.37	
ETI 10	3	26.78	±	8.72	*	27.87	±	7.00	
ETI 0	3	11.49	±	1.98		19.49	±	4.64	
ETI -5	3	16.07	±	6.34		15.59	±	5.45	
ETI -10	5	19.17	±	1.77	*	34.40	±	8.79	*
Ins 15nM 50 min	3	16.35	±	4.66		23.00	±	1.57	

Table 5.1. Effect of different times of exposure to insulin on the normal 1nM ET-1 response on glucose uptake and lactate release. Insulin infusion began at various time points and ET-1 infusion commenced at 0 min as shown in Protocol B in Figure 5.1 and as described in Figure 5.5. The value given for insulin 15nM was after 50 minutes of insulin infusion alone. *, significantly different to saline ($P<0.05$) using one way ANOVA.

Infusion of 10nM ET-1 causes a strong vasoconstriction, and 15nM insulin was unable to block this significantly at any time of exposure (Figure 5.6A). Even though there was no significant effect to block the pressure development, exposure to insulin changed the oxygen response from a typically inhibitory response to a stimulatory effect (Figure 5.6B). Exposure to 10nM ET-1 and 15nM insulin over any time appeared to have an additive effect on glucose uptake (Table 5.2), although the table only indicates the glucose uptake observed after 50 minutes of insulin infusion. From Figure 5.3A, it can be seen that the glucose uptake induced by insulin reaches a plateau after 20-30 minutes, which implies that the value for glucose uptake after 50 minutes of insulin infusion can be applied to experiments where the exposure time to insulin is over 30 minutes. The lactate release results are complex, with an apparent additive effect occurring when insulin was infused 20 minutes prior to ET-1, although the other exposure times to insulin do not seem to follow any clear pattern (Table 5.2).

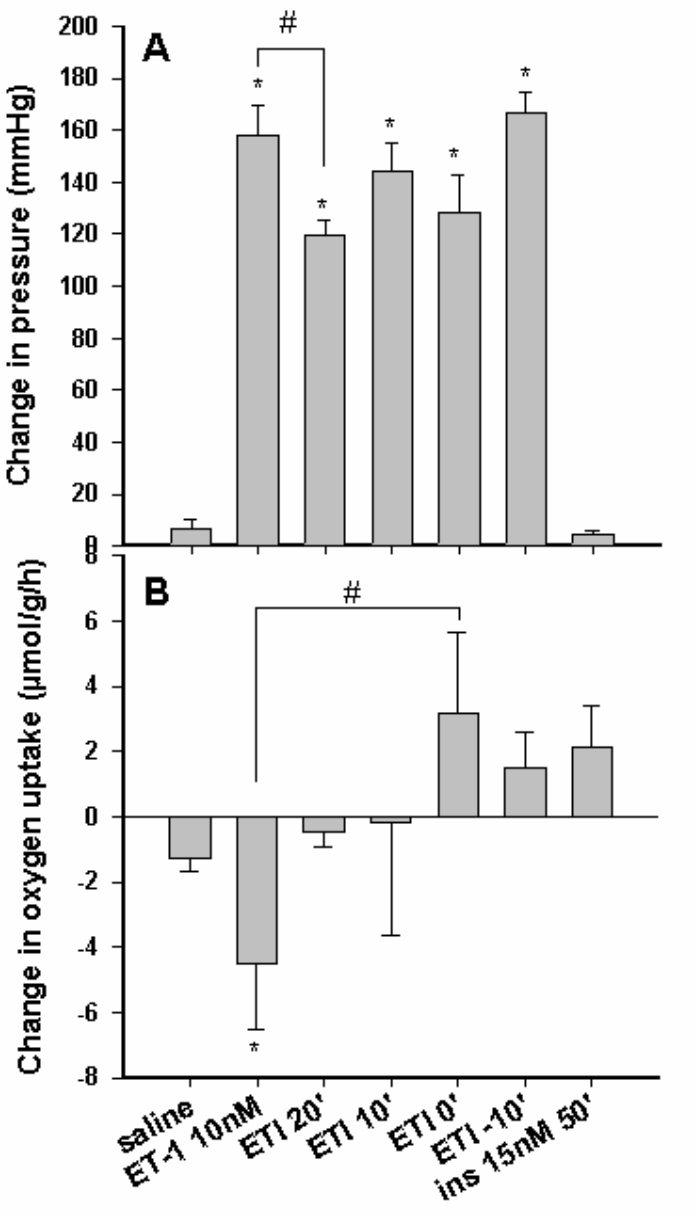

Figure 5.6. Effect of different times of exposure to insulin on the normal 10nM ET-1 response on perfusion pressure (A) and oxygen uptake (B). Insulin infusion began at various time points and ET-1 infusion commenced at 0 min as shown in Protocol B in Figure 5.1 and as described in Figure 5.5. The value given for insulin 15nM was after 50 minutes of insulin infusion alone. n = at least 3 for each point. *, significantly different to saline ($P<0.05$), #, significantly different as indicated ($P<0.05$) using one way ANOVA.

	Change in glucose uptake (µmol/g/h)					Change in lactate release (µmol/g/h)			
	n	**Mean**		**SE**		**Mean**		**SE**	
Saline	6	2.78	±	1.71		4.62	±	2.05	
ET-1 10nM	6	8.62	±	4.79		30.54	±	19.59	
ETI 20	6	23.62	±	4.72	*	68.42	±	3.65	
ETI 10	3	25.71	±	4.79	*	30.21	±	17.71	
ETI 0	3	25.3	±	3.71	*	40.64	±	13.12	
ETI -10	5	26.73	±	4.84	*	50.41	±	10.58	
Ins 15nM 50 min	3	16.35	±	4.66		23.00	±	1.57	*

Table 5.2. Effect of different times of exposure to insulin on the normal 10nM ET-1 response on glucose uptake and lactate release. Insulin infusion began at various time points and ET-1 infusion commenced at 0 min as shown in Protocol B in Figure 5.1 and as described in Figure 5.5. The value given for insulin 15nM was after 50 minutes of insulin infusion alone. *, significantly different to saline ($P<0.05$) using one way ANOVA.

In a further experiment a higher concentration of insulin (45nM) was able to more significantly inhibit the vasoconstriction induced by ET-1 (Figure 5.7). When 45nM insulin was infused for 20 minutes prior to ET-1, the pressure developed at the end of 20 minutes 10nM ET-1 infusion was only 100mmHg, compared to the normal value at this time point of 200mmHg, or around 150mmHg with 15nM insulin. Therefore, a higher dose of insulin is able to more effectively block the ET-1 vasoconstrictor response.

Another experiment Figure 5.8 demonstrated that insulin may be able to vasodilate against lower doses of ET-1. When 400pM ET-1 was included in the buffer, the pressure increase due to ET-1 was stopped and began reversing once an insulin infusion of 30nM was initiated. Only one experiment of this nature was performed. In other experiments the pressure due to ET-1 was higher, and was not modified by insulin infusion.

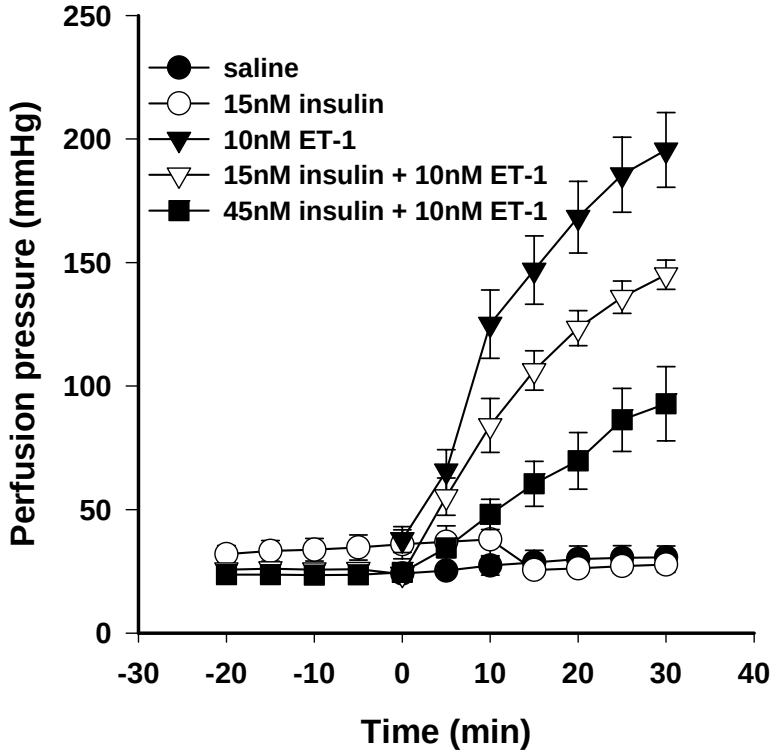

Figure 5.7. 45nM insulin on ET-1 mediated vasoconstriction. n=4 for 45nM insulin +10nM ET-1, n=6 or more for all other groups. Protocol A from Figure 5.1 was used.

Ca^{2+} free buffer

Experiments were carried out using buffer with no added calcium to prevent vasoconstriction by ET-1. As shown in Figure 5.8, removing calcium from the perfusion medium resulted in a much smaller increase in pressure due to ET-1 (Figure 5.8A), while decreasing the stimulatory effects of 1nM ET-1, and diminishing the inhibition of 10nM ET-1 on oxygen uptake (Figure 5.8B).

An effect of calcium omission on glucose uptake was difficult to discern (Figure 5.8C), although the normal effects of both low and high doses of ET-1 on lactate release were greatly reduced (Figure 5.8D). Thus it appears that Ca^{2+} is essential for the vasoconstriction by ET-1, and the corresponding metabolic effects associated with the pressure increase.

The effect of removing Ca^{2+} after the ET-1 response has already been initiated is shown in Figure 5.9A. Removal of Ca^{2+} causes a decrease in pressure that appears to be delayed; however the bars in the graph indicate only when the buffer reservoir was changed. At a flow rate of 8ml/min it would take approximately 5 minutes for the Ca^{2+} free buffer to pass through the approximately 50ml of perfusion apparatus and reach the rat hindlimb. Taking this into account, it is likely that the Ca free buffer has an almost immediate effect to begin to limit the normal vasoconstriction by ET-1.

Also shown in Figure 5.9 is the effect of infusing 50μM SNP after the vasoconstriction of ET-1 had already commenced. SNP was able to immediately vasodilate against the constriction by ET-1, and although the vasoconstriction by ET-1 was able to continue, SNP was able to limit the normal response to ET-1 (Figure 5.9B). It is apparent that the SNP causes an immediate vasodilation.

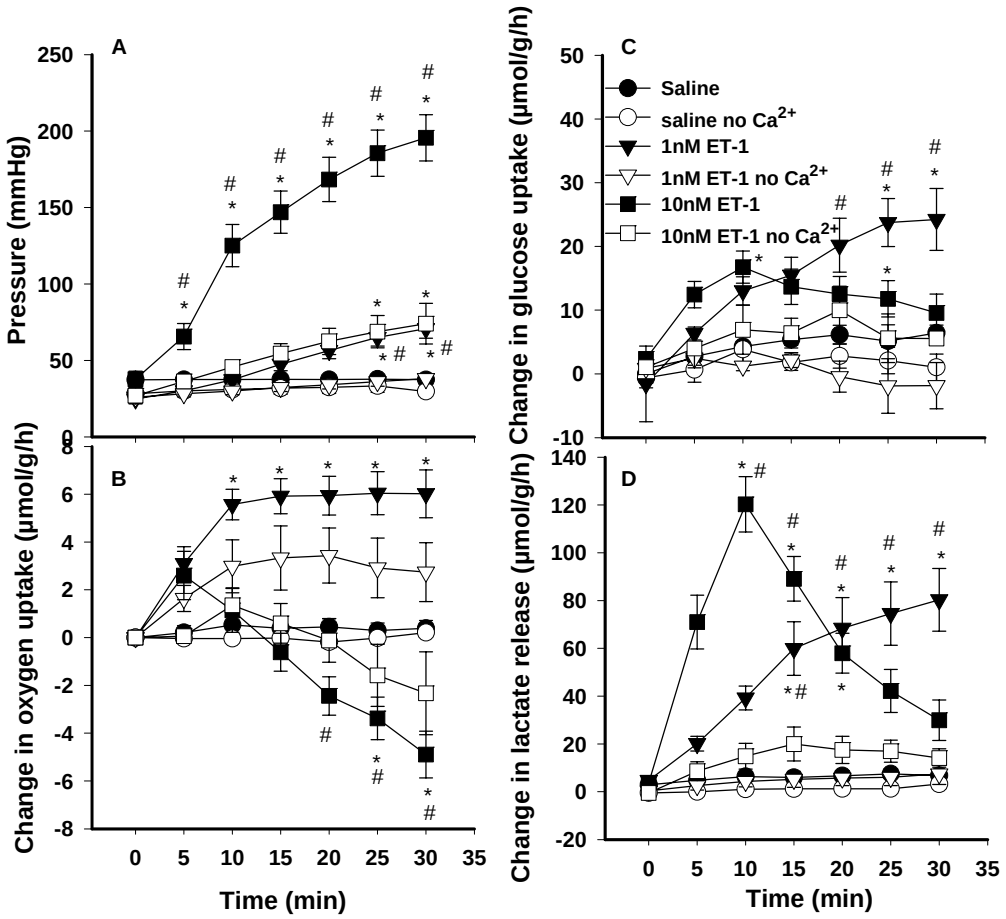

Figure 5.8. Impact of Ca^{2+} free Krebs buffer on ET-1 effects on perfusion pressure (A), oxygen uptake (B), glucose uptake (C) and lactate release (D). Perfusion medium was Ca^{2+} free for the entire perfusion; EGTA was added to the perfusion medium prior to performing surgery on each rat. ET-1 infusion commenced at 0 min and continued for 30 minutes. n = at least 3 for each point. *, significantly different to saline ($P<0.05$), and #, significantly different to corresponding dose of ET-1 alone ($P<0.05$) using two-way repeated measures ANOVA.

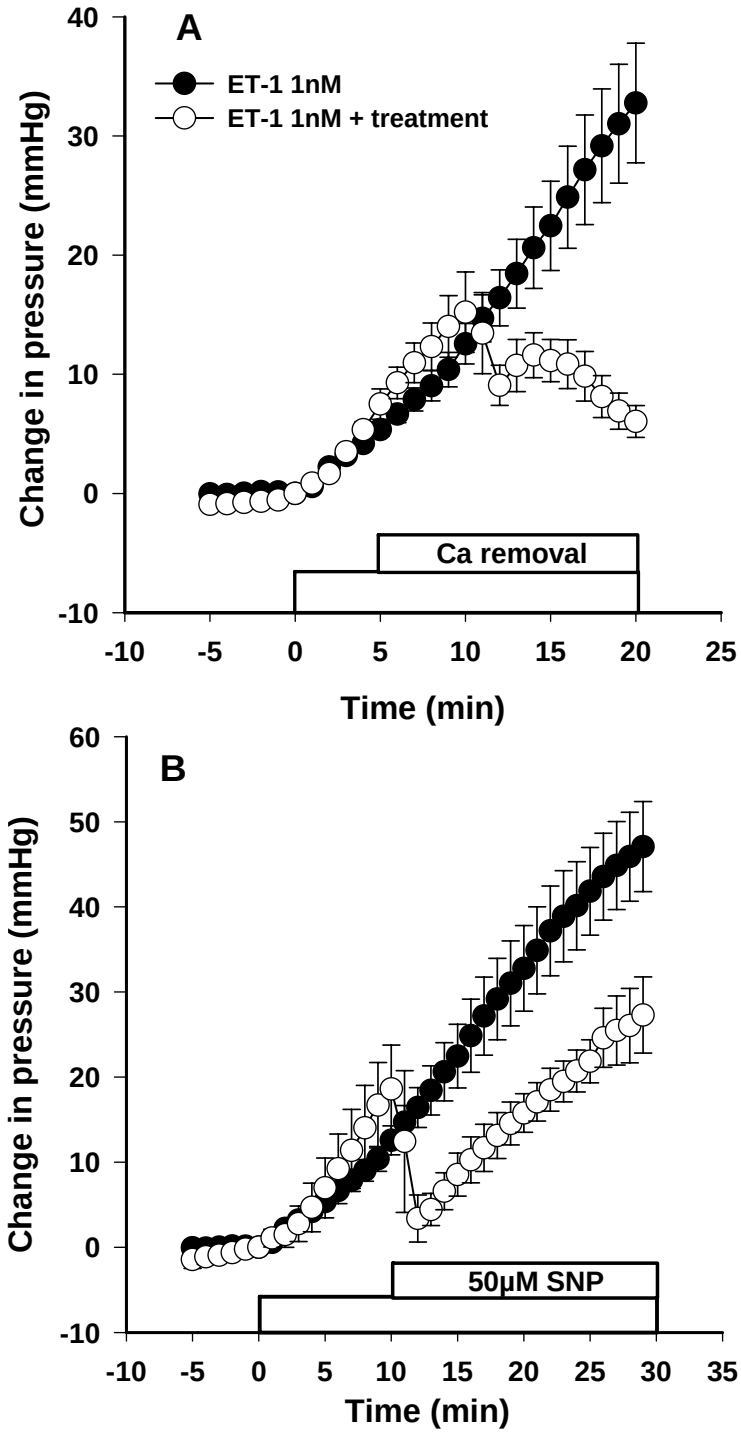

Figure 5.9. The effects of Ca^{2+} free buffer (A) and 50μM SNP (B) on the pressure due to 1nM ET-1 vasoconstriction. Protocols used are as shown in each graph, n = at least 4 for each group.

ET-1 and insulin in insulin-resistant animals

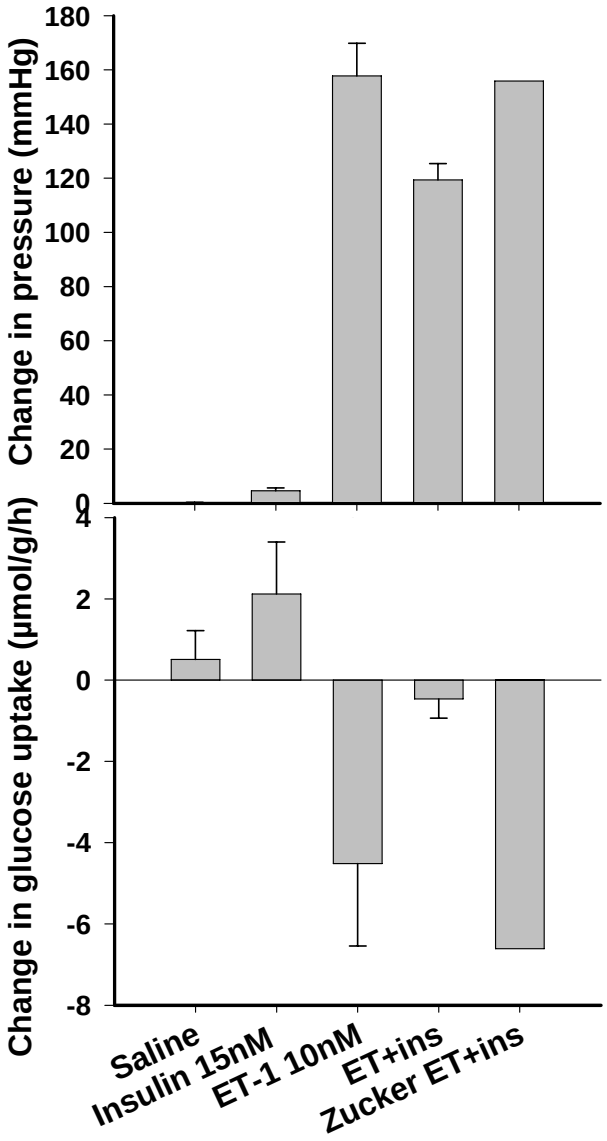

Figure 5.10. Comparison of the effects of insulin to oppose 10nM ET-1 in normal rats and a Zucker rat using protocol A in Figure 5.1. Only one Zucker has so far been used in this experiment, n = at least 6 for all other groups.

One Zucker rat was used with the same protocol as shown in Figure 5.1A, and the results are shown in Figure 5.10. The effect of insulin to lower the pressure increase due to ET-1 in lean Hooded Wistar rats appeared to be lost in the Zucker obese rat (Figure 5.10A). Also, while insulin was able to block ET-1-mediated inhibitions of oxygen uptake in Hooded Wistars, the Zucker obese rat showed no effect of insulin to block this effect (Figure 5.10B).

5.4 Discussion

The main conclusion arising from this chapter is that insulin is able to vasodilate against ET-1 in the perfused rat hindlimb. In spite of well-documented effects of insulin to cause vasodilation *in vivo*, this ability has previously not been observed against any other vasoconstrictor in the perfused rat hindlimb (data not shown). ET-1 is apparently unable to modify the effects of insulin on glucose uptake, although results vary depending on the method used. Also shown are results indicating that Ca^{2+} is essential for development of both vascular and metabolic effects of ET-1, once again suggesting that the metabolic actions of ET-1 are closely associated with, and likely dependent on, vasoconstriction.

Insulin was able to limit the effects of 1nM ET-1 on all parameters except for glucose uptake when infused for 20 minutes prior to ET-1. However insulin was unable to significantly affect the pressure due to 10nM ET-1, even when infused for 20 minutes prior to ET-1; unless a higher concentration of 45nM insulin was used. A previous study has shown that insulin can prevent vasoconstriction due to ET-1 (203) through an endothelium-independent mechanism.

Glucose uptake results varied with the different measurement methods used; A-V glucose showed no effect of ET-1 to block insulin-mediated glucose uptake, while 2-deoxyglucose results demonstrated a dose-dependent inhibition of insulin-mediated glucose uptake by ET-1. The inconsistency between the methods may be explained by the fact that the radiolabelled assay focuses on specific muscles, whereas the A-V determination is an indication of what is happening over the entire limb, including other tissues. Also, the A-V determination gives a time-point, while the 2-deoxyglucose results provide an average over the time of radiolabel infusion, in this case 10 minutes. It is possible that muscle is highly sensitive to the non-nutritive effects of high dose ET-1, and is the only tissue in the perfused hindlimb to undergo an ET-1-mediated redistribution of flow to the non-nutritive route, and thus show a decrease in insulin-mediated 2-deoxyglucose uptake. As A-V glucose difference samples all tissues within the hindlimb, there may be no overall insulin resistance observed with this method, although the majority of the tissue perfused should be muscle, and by this theory should also exhibit insulin resistance. There is other evidence that ET-1 leads to insulin resistance directly, as ET-1 infused into healthy

humans caused insulin resistance as measured by whole body and leg glucose uptake (174); this supports the radiolabelled glucose uptake data found herein.

Despite the fact that large concentrations of both insulin and ET-1 have been used in this study, the results suggest that a balance between the levels of insulin and ET-1 is critical for an outcome. The results shown above do not suggest that the metabolic effects of insulin may be inhibited by high ET-1 concentrations; however it is important to note that this study does not use physiological levels of either insulin or ET-1 (although physiological levels of ET-1 are not known), and that the hindlimb preparation would be likely to show substantially different effects to *in vivo* situations; the consequences of this will be discussed later.

Calcium is important for the development of ET-1-mediated vasoconstriction – the response to lower concentrations of ET-1 is rapid and transient, and is due to Ca^{2+} release from inositol triphosphate-sensitive intracellular stores, while the higher concentrations of ET-1 cause Ca^{2+} influx through voltage-gated Ca^{2+} channels, and involves the sarcoplasmic reticulum (204). Experiments that do not include Ca^{2+} in the perfusion medium therefore exhibit only limited vasoconstriction to ET-1. Also, when Ca^{2+} was removed after ET-1-mediated vasoconstriction had already occurred, the pressure dropped. The pressure drop was not as immediate as that observed with SNP due partly to the delay in the fresh buffer reaching the rat hindlimb, as the buffer must pass through the silastic tubing of the lung, the heat exchanger column and the pump, with a total volume of approximately 45 ml. The dilation was also not as rapid, possibly due to mixing of buffers within the perfusion apparatus, such that the Ca^{2+} removal is not immediate, but likely follows a gradient over time. Also evident from the results in this chapter is that SNP is able to dilate instantly against ET-1-mediated vasoconstriction, although vasoconstriction still progresses with time. Higher levels of SNP could not be used due to undesirable effects from low levels of cyanide in the SNP.

Data shown in earlier chapters have suggested that the metabolic effects of ET-1 are due to the vascular effects to redistribute flow, as SNP was able to block both vascular and metabolic effects, while having no direct effect of its own. It is interesting that the results presented in this chapter show that the effect of insulin to block ET-1-mediated metabolic effects, primarily oxygen consumption, does not coincide with a

significant inhibition of pressure development. However, insulin causes some metabolic effects itself, and, as shown here for the first time in the perfused rat hindlimb, vasodilatory effects against ET-1 as well. Because of this, it is difficult to separate the vascular and metabolic effects of ET-1 from those of insulin. The results using low Ca^{2+} concentration in the perfusion medium also exhibit some uncoupling of the metabolic and vascular actions, as 1nM ET-1 causes no significant increase in pressure in the absence of Ca^{2+}, while a small effect on oxygen uptake can still be observed. In this case, there is a separation between the vascular and metabolic effects of ET-1 that may detract from the proposal that all the metabolic effects of ET-1 are due to the vascular effects to redistribute flow. It is possible that while there is no significant vasoconstriction observed, there may be effects to redistribute flow, which could cause the changes in metabolic effects, although techniques to measure this would need to be extremely sensitive in the perfused hindlimb preparation.

As discussed, the vascular actions of insulin are most likely the net result of the vasodilatory action of NO and the vasoconstrictor activity of ET-1 (88-90). Eringa et al (88) have shown, with isolated blood vessels from muscle, that in the presence of a nitric oxide synthase inhibitor there is an insulin-mediated vasoconstriction that is blocked by an ET receptor antagonist. This observation combined with the results from previous chapters using low doses of ET-1 alone might suggest that insulin-mediated release of ET-1 is intended to aid in the recruitment of capillary blood flow in muscle. Data from previous chapters using 10nM ET-1 support the concept that high levels of ET-1 reported in type 2 diabetics(198), obese patients (160) and hyperinsulinemic states in hypertensive individuals (198) may be antagonistic of insulin's metabolic and vascular effects and a significant contributor to the elevated blood pressure. However, the results shown above where insulin was present before and during ET-1 addition show clearly that the metabolic effects of ET-1 at both low and high dose are opposed. Thus, insulin itself limits the effects of ET-1 (particularly low dose) on pressure and oxygen uptake. The stimulatory effect of low dose ET-1 is no longer present and the high dose effect takes on a character of an intermediate dose of ET-1. Therefore, in this study high concentrations of ET-1 do not cause insulin resistance, although others have suggested that elevated ET-1 levels may be the cause of insulin resistance in disease states (160, 174). However, this study uses doses of insulin that are much greater than those found *in vivo*, and as such, it is possible that elevated ET-1 levels may cause insulin resistance against physiological insulin

concentrations. Also, our study focuses only on the muscles of the hindlimb, and does not take into account whole body effects that may involve other tissues and/or changes in sympathetic nervous system effects that may be associated with such conditions. The concentration of insulin used is much greater than is found *in vivo*, and so may have non-specific actions on other receptors such as IGF-1 receptors. Also, if the level of insulin is already well above the concentration required to cause maximal glucose uptake, small changes in the distribution of flow through the muscle may not have a significant effect to alter insulin-mediated glucose uptake. Potentially the metabolic responses of lower doses of insulin may be more susceptible to ET-1-mediated flow changes, as in physiological situations the blood flow through the muscle is probably such that small alterations in blood flow are enough to have large effects on metabolism.

Further studies *in vivo* would be required to investigate that ET-1-mediated insulin resistance (174) could be attributed to flow redistribution. If this is the case, in hypertensive patients that have elevated levels of ET-1 (160), physiologic insulin may be unable to vasodilate against this to cause the normal redistribution of blood flow observed in healthy patients. This may limit the metabolic effects that are usually supported by insulin-mediated capillary recruitment. Also, if insulin is unable to limit the vasoconstrictor effects of high dose ET-1, the vascular and metabolic effects of ET-1 can continue unopposed. In this way, hyperinsulinemia associated with insulin resistance may lead to an increased release of ET-1, and the insulin resistance may exacerbate the apparent non-nutritive flow caused by high doses of ET-1. This hypothesis is partly supported by the results from the perfusion of the Zucker obese rat in this chapter, where insulin was unable to block the normal effects of ET-1 in an insulin-resistant state as efficiently as in other healthy rats within this study. Of course this experiment requires verification by repeating this experiment before any conclusions can be drawn.

The present study shows that insulin appears to behave essentially as SNP, decreasing the effect of 1 nM ET-1 on pressure and lactate release, and causing a net stimulation of oxygen uptake, glucose uptake and lactate release over basal with 10 nM ET-1. This may be explained similarly to the SNP data in earlier chapters, where vasodilation moderates the inhibitory effect of the high dose to cause a net stimulation. These data, together with the pressure results, indicate that insulin may

be able to behave like a vasodilator in the pump-perfused rat hindlimb against ET-1, a property that has not been demonstrated against any other vasoconstrictor in this preparation previously.

It is not clear from this study whether the effect of insulin to limit ET-1-mediated vasoconstriction results from vasodilation by NO (or another vasoactive substance), or some sort of signal interference. An acute vasodilation, where the pressure dropped below the point at which insulin infusion began, has only been shown at low doses of ET-1 (400pM) and has not been reproduced, although insulin did prevent the vasoconstriction from developing further. Insulin has been used at concentrations of up to 180nM against 1nM ET-1 without showing acute vasodilation (data not shown). SNP caused acute vasodilation against ET-1, while insulin did not, although NO has been proposed to be the mediator of each. However, there is a difference in the availability of NO in each case: SNP is an endothelium-independent vasodilator, and so the availability of NO is high, while insulin requires the endothelium for vasodilation. It may be that either the endothelium is damaged in the perfused rat hindlimb, so insulin is unable to release NO, or the release of NO by the endothelium even in a healthy system is not at a rate high enough to cause a significant vasodilation. Also, any NO that is released by insulin may be flushed away before it is able to cause any significant vasodilation due to the high flow rate in perfusion. A more ideal comparison to insulin would be an endothelium-dependent vasodilator, such as methacholine, which would potentially release NO at a similar rate to insulin, and therefore have a similar result. Preliminary results with methacholine have revealed that endothelium-dependent vasodilation is possible, and caused a vasodilation similar to that observed with SNP. As such, it appears that insulin does not vasodilate, but actually limits signal transduction by ET-1, or interferes with ET-1 binding to its receptor.

Previous studies by others have suggested insulin causes signal interference rather than vasodilation (203, 205). For example, Dick and Sturek (205) have shown that insulin can attenuate the Ca^{2+} response of dissociated vascular smooth muscle cells to ET-1. As this inhibition can occur in isolated cells, it is unlikely that vasodilation is directly responsible. However, that study used 10nM ET-1, which was the lowest concentration tested to elicit a rapid release of Ca^{2+}, assessed using microfluorometry of Fura-2, a fluorescent Ca^{2+} indicator. So while insulin was shown to have a

significant effect to block 10nM ET-1 increases in Ca^{2+}, effects on lower concentrations are not clear. Such evidence would tend to suggest that all the responses observed by insulin against ET-1 may be due to Ca^{2+} interference. However another study has shown that insulin also attenuates vasopressin responses (206), yet this interference has never been observed in perfusion; this implies that there is some divergent results based on whether perfusion or cell culture is used. While insulin can vasodilate against other vasoconstrictors *in vivo*, and also block their Ca^{2+} signalling in isolated cell studies, no effect of insulin to do either of these has been observed in the perfused rat hindlimb. Therefore, insulin appears unlikely to be able to vasodilate or affect intracellular Ca^{2+} signalling of all other vasoconstrictors, yet is effective against ET-1. The reason for this difference in sensitivity of the perfused hindlimb to insulin against various vasoconstrictors is unclear. A more recent study suggests that the interaction between insulin and ET-1 is more specific, and is in fact due to interference with the ET_A receptor, discovered using the ET_B specific sarafarotoxin S6c (203). As the data stands, the effect of insulin to block ET-1 is likely due to interference in the Ca^{2+} signalling. A contribution to this result by vasodilation due to NO or another vasoactive agent cannot be ruled out, although it is unlikely to be the predominant effect.

There is still no certainty of the role that NO plays in insulin action in the local environment. While studies by Eringa et al in isolated vessels from skeletal muscle have shown that insulin causes the release of both ET-1 and NO (88), other studies using L-NAME have been unable to block the capillary recruitment effects (and therefore possibly vasodilation) in the local environment (118). However, capillary recruitment was blocked by systemic NOS inhibition (117). Therefore, the role of NO in the local environment of the muscle is uncertain. Conceptually, it is unlikely that NO release from blood vessels in response to insulin would cause the recruitment of whole new areas, as the short half life of NO means that only adjacent, and most likely smaller downstream vessels would be affected. If, as discussed, insulin causes the release of ET-1 and NO in first order arterioles of the rat (88), and it is known that NO can increase the diameter of arterioles (116) it is apparent that NO has some role in the local environment, although this may not involve capillary recruitment directly (118). A previous study by others found that insulin can limit ET-1 induced vasoconstriction through an endothelium-independent mechanism (203). Therefore, they suggest that insulin may affect the intracellular rise in calcium due to ET_A

receptors, although they acknowledge that, since insulin does not affect other calcium dependent constrictors, the interaction is likely to be more complicated (203).

In conclusion, insulin is able to limit the vasoconstriction caused by ET-1 – whether by vasodilation or interference in the signalling mechanism is as yet unknown. Insulin does not appear to limit the vasoconstrictor effects of ET-1 as effectively as SNP or removal of calcium ions from the medium. While in this study ET-1 did not cause insulin resistance as hypothesized, it is likely that a balance between insulin and ET-1 exists under physiological situations, as other studies have shown that ET-1 infusion into healthy humans causes insulin resistance. Further studies *in vivo* would need to be carried out to confirm that these conclusions are relevant in a physiological situation.

CHAPTER 6

METHACHOLINE-MEDIATED VASODILATION CAN AFFECT GLUCOSE UPTAKE

6.1 Introduction

Methacholine (MC) infusion has been observed to increase insulin-mediated glucose uptake, an effect that occurred simultaneously with an increase in femoral blood flow in lean healthy men (52, 59). In other studies that have attempted to increase insulin-mediated glucose uptake using both NO-dependent and –independent vasodilators, simply augmenting the flow did not change glucose uptake in either humans, using SNP (122), adenosine (123) and bradykinin (124), or in rats using epinephrine (31). Recently, Mahajan et al (121) have demonstrated that insulin-mediated glucose uptake could be increased by methacholine infusion in a euglycemic hyperinsulinemic clamp in rats, while another vasodilator, bradykinin, had no effect. Furthermore, while both vasodilators were able to increase femoral blood flow to a similar extent, only methacholine, not bradykinin, was able to augment the insulin-mediated capillary recruitment as measured using the 1-MX method (121). It is interesting that two vasodilators, both acting by NO-dependent mechanisms, cause different effects on insulin sensitivity. It has been suggested that the differences in the metabolic action of these vasodilators may be due to the specific site of action of each vasodilator (52), and their ability to increase the distribution of flow within the muscle (121).

Several studies using methacholine have demonstrated a significant increase in glucose uptake (52, 59, 120, 121), although whether this effect is due to the vasodilator abilities of methacholine or due to a direct effect of methacholine on glucose uptake is not yet certain, as studies have suggested that NO-donors are able to cause glucose uptake independently of any vascular effects (207). A study of methacholine can be undertaken to observe the hemodynamic (vasodilation) effects in the preconstricted isolated pump-perfused rat hindlimb, and to confirm that these effects are at least partially NO-dependent. Also the metabolic effects of

methacholine can be observed to clarify whether methacholine can increase glucose uptake in the fully vasodilated hindlimb by a direct effect, where methacholine should have no further vasodilatory activity, and whether this effect is also NO-dependent.

6.2 Methods

6.2.1 Solutions

Solutions were made up to be 200 times the required final concentration to allow for infusion at 1/200th of the flow into the infusion port. Therefore, at a perfusion of constant flow rate 8ml/min, substances were infused at 40µl/min unless otherwise indicated.

Methacholine

Methacholine is freely soluble in saline, so for vehicle infusions saline was infused at a flow rate similar to the maximum used for methacholine.

Serotonin and Norepinephrine

Serotonin and norepinephrine were infused prior to methacholine to observe the vasodilatory effect in this system. These were diluted in saline and infused at a maximum of 1/200th of the flow rate to give 300nM and 70nM respectively.

L-NAME

L-NAME is freely soluble in saline, and was diluted appropriately before infusion to give a known concentration in the perfusion medium. A dose of 10µM L-NAME was used, which caused a significant increase in pressure, but should have been sufficient to completely block any NO.

6.2.2 Perfusion conditions

Hindlimb perfusions involving only the right leg were performed at 32^{o}C, with 2.54mM $CaCl_2$ and 8.3mM glucose. The flow rate was maintained at 8ml/min by a pump, and the regular Krebs buffer was used (section 2.2.2).

6.2.3 Perfusion protocol

Two different perfusion protocols were used in this chapter. Firstly, dose dependent effects of methacholine were studied where the rat hindlimb was preconstricted with NE or 5HT prior to MC infusion to more clearly observe any vasodilation. After the vasodilation had occurred, L-NAME was infused at a dose of 10µM (Figure 6.1A). The second protocol looked at the direct effects of MC on metabolism, so no preconstriction was required. MC was infused in a stepwise manner, and L-NAME was included in some of these experiments to determine whether any effect on glucose uptake was NO-dependent (Figure 6.1B).

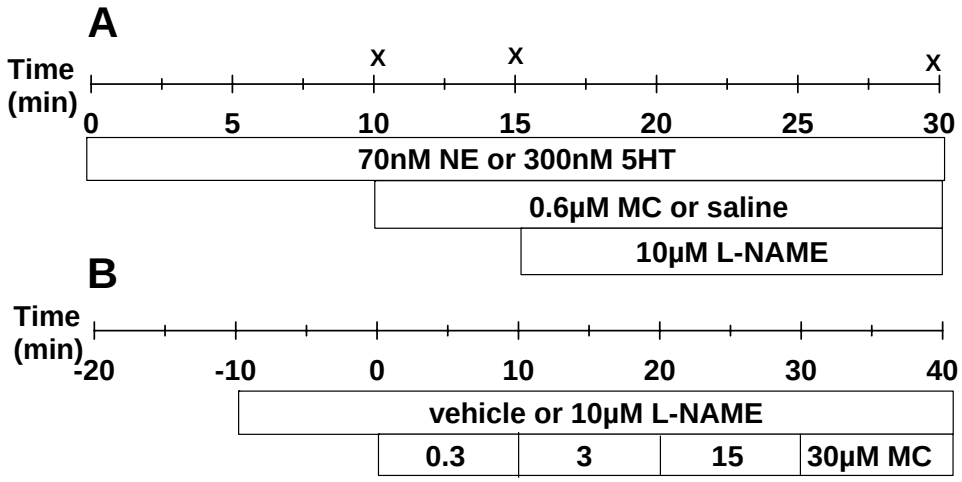

Figure 6.1. Protocol used in Chapter 6. The protocol was applied to the constant flow-pump-perfused rat hindlimb, with a flow of 8ml/min. The rat hindlimb was allowed to equilibrate for 30-40 minutes after surgery and hook up to the apparatus before the infusion of any test substances as indicated by the bars above. In protocol A, X marks the position of a pressure reading to be graphed later. In these experiments the vasodilation of MC against preconstriction was countered with L-NAME to ensure that MC is NO-dependent. In protocol B, an increasing dose of MC was infused against a background of either saline or 10µm L-NAME.

6.2.4 Radiolabelled glucose uptake

A ten minute 2-deoxyglucose protocol was used as described in Section 2.3. For experiments requiring 2-deoxyglucose, the appropriate radioactive glucose solution was infused for ten minutes immediately prior to the end of the experiment, and the muscle excised and frozen with liquid nitrogen, before being analysed as described in section 2.3.

6.2.5 Statistical Analysis

Two-way repeated measures ANOVA was performed using SigmaStat (SPSS Science; Chicago IL) for time course experiments, and one-way ANOVA or paired t-test for the histograms, with comparisons made between conditions using the Student-Newman-Keuls post hoc test. Significance was assumed at the level of $P<0.05$. Data are represented as means $\pm$ standard error.

6.3 Results

The first protocol shown in Figure 6.1A was used to assess whether MC was able to vasodilate against preconstriction by either NE or 5HT in the isolated pump-perfused rat hindlimb. Figure 6.2 demonstrates that MC is able to significantly dilate against both a type A (70nM NE) and type B (300nM 5HT) preconstriction. The dilation in each case was blocked by infusing L-NAME, suggesting that the vasodilation was NO-dependent. However, L-NAME when added to either vasoconstrictor in the absence of MC caused a significantly greater increase in pressure. This suggests that either there is a component of normal 5HT and NE vasoconstriction that involves a self-limiting NO vasodilation, or that MC is able to vasodilate against L-NAME.

Figure 6.3 shows again that L-NAME causes an increase in perfusion pressure alone, and that MC was able to vasodilate against this. This implies that either the L-NAME present is not sufficient to block MC-mediated NO-dependent vasodilation, or that MC is able to vasodilate using some NO-independent mechanism. There were no significant effects on oxygen uptake.

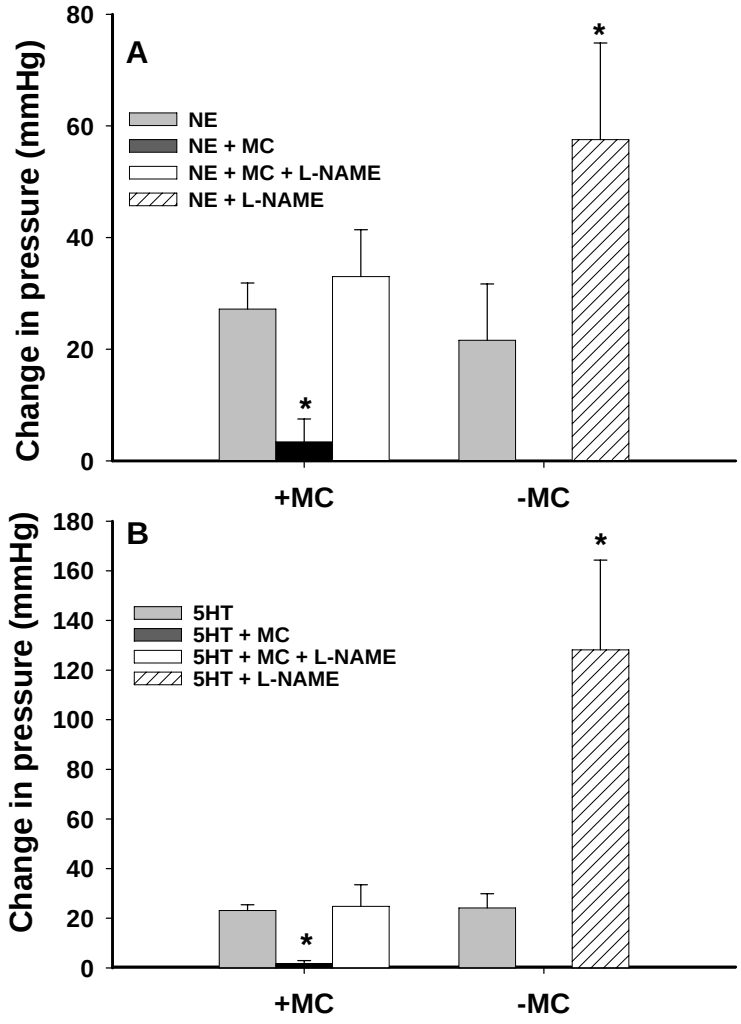

Figure 6.2. Effect of MC (0.6μM) on perfusion pressure of the constant flow pump-perfused rat hindlimb. The hindlimb was preconstricted by constant infusion of (A) 70nM NE or (B) 300nM 5HT using the protocol shown in Figure 6.1A. Infusion of MC (0.6μM) was commenced 10 minutes after beginning the infusion of either vasoconstrictor, and L-NAME infusion was initiated 5 minutes later. Perfusion pressure values for either vasoconstrictor alone were taken at 10 minutes, immediately prior to MC infusion; values for both vasoconstrictors with MC were taken at 15 minutes, immediately preceding L-NAME infusion, and values for the vasoconstrictor and L-NAME, $\pm$ MC were taken at 30 minutes, corresponding to the end of the experiment. The pressure at a time of 0 minutes was subtracted from all values to give an indication of change in pressure from basal. *, Significantly different from all other groups ($P<0.05$, one way repeated measures ANOVA for +MC group, paired t-test for –MC group).

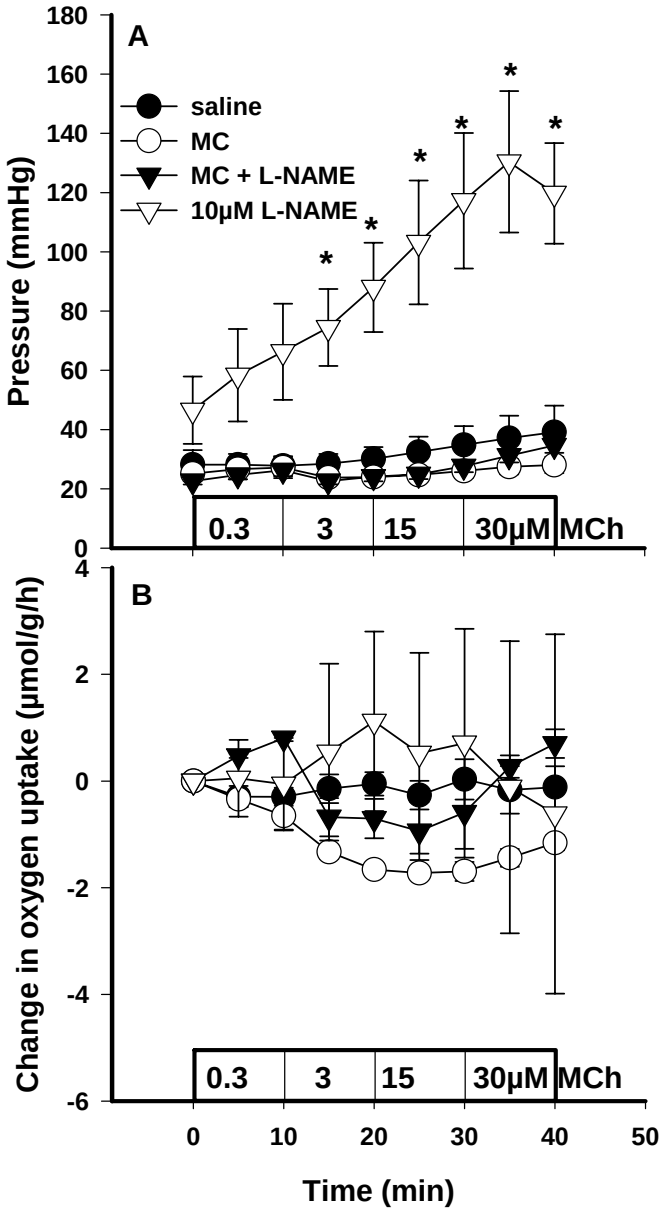

Figure 6.3. Effect of MC added in stepwise concentration increases as indicated on perfusion pressure (A) and on oxygen uptake (B) to the perfused rat hindlimb using the protocol described in Figure 6.1B. L-NAME when used was infused for 10 minutes prior to the infusion of the first dose of MC. *, significantly different from saline values ($P<0.05$, two-way repeated measures ANOVA).

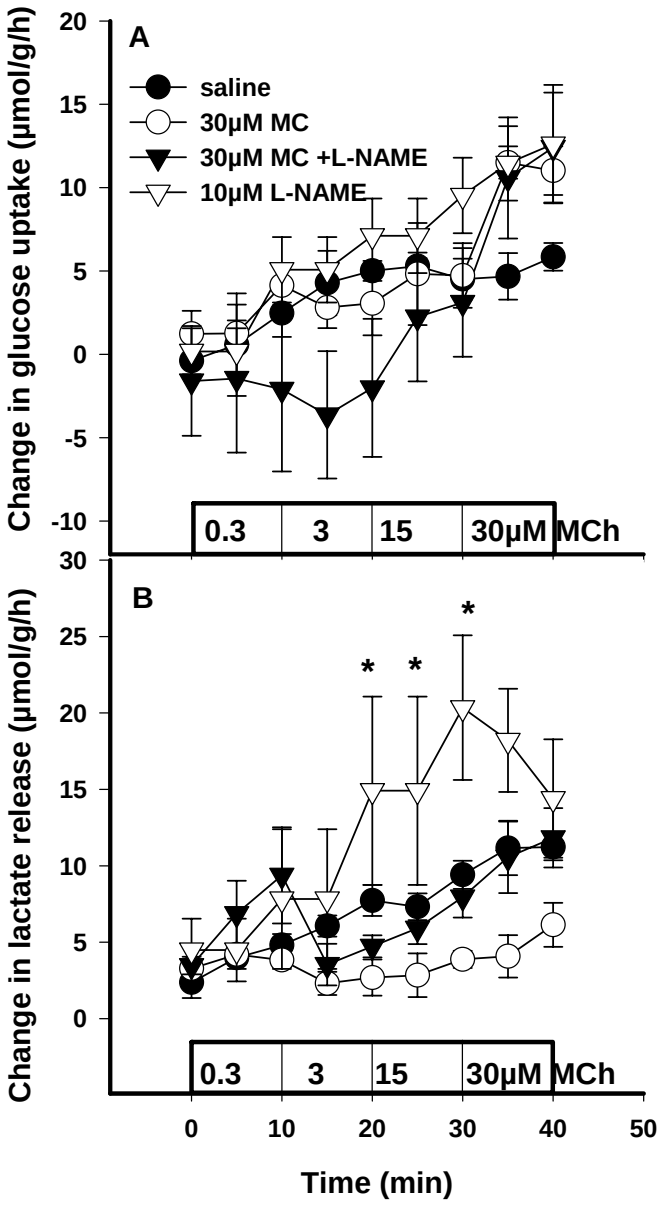

Figure 6.4. Effect of MC on glucose uptake (A) and lactate release (B) in the perfused rat hindlimb. Stepwise increases in the concentration of MC were used as indicated in the graphs, according to Protocol B in Figure 6.1. L-NAME when used was infused for 10 minutes prior to the infusion of the first dose of MC. *, significantly different from saline ($P<0.05$, two-way repeated measures ANOVA).

Figure 6.4 demonstrates all doses of MC used had no significant effect to increase glucose uptake when measured by A-V glucose difference. There was a trend for glucose uptake to increase over time throughout the experimental protocol. Lactate release is increased significantly over saline controls by 15 and 30μM MCh; this effect is no longer significant when L-NAME is infused.

Using 2-deoxyglucose uptake (R'g) by muscles of the lower leg (calf) a different outcome is apparent. While lower doses of MC were unable to stimulate R'g (Figure 6.5), 30μM was able to significantly increase R'g in soleus, plantarus and the red gastrocnemius muscle, as well as in the combined muscle; this effect was blocked by L-NAME. The fact that L-NAME caused an increase in R'g in the soleus may in some way confirm the results observed with the glucose uptake calculated from A-V glucose difference, where L-NAME appeared to cause glucose uptake alone. However, the R'g results differ from the A-V glucose difference calculations in regards to the effect of L-NAME to block MC-mediated glucose uptake.

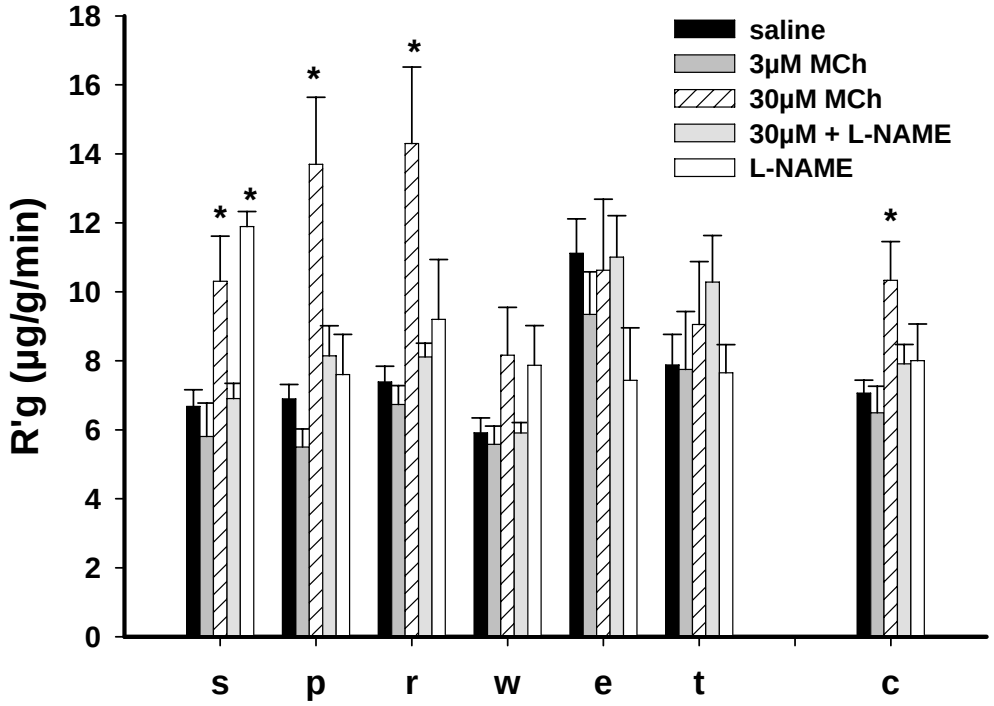

Figure 6.5. Rate of varying doses of MC on glucose uptake as determined using a 10 minute radiolabelled 2-deoxyglucose uptake protocol. The hindlimb was fully dilated and infusions of increasing doses of MC were made every ten minutes according to Protocol B in Figure 6.1. L-NAME (10μM) when used was infused 10 minutes prior to any infusion of MC. Muscles sampled for the determination of R'g at the end of the perfusion protocol were soleus (s), plantarus (p), gastrocnemius red (r) and white (w), the extensor digitorum longus (e) and tibialis (t). The combined value for all muscles assayed was also determined (c). *, significantly different from saline ($P<0.05$, one way ANOVA on each muscle).

6.4 Discussion

From the results of this chapter, it is apparent that the effect of MC to directly cause glucose uptake occurs only at doses much higher than those that cause vasodilation as seen in this study, and those that cause capillary recruitment (121). MC at concentrations less than 30μM was not able to stimulate glucose uptake, although a significant effect to vasodilate against preconstriction induced by either 5HT or NE was observed at considerably lower doses. Therefore, MC may be able to influence glucose metabolism by two mechanisms – at lower doses the vasodilation caused by MC *in vivo* would be able to increase nutrient and hormone delivery by increasing microvascular perfusion in a nutritive manner. At higher doses, a direct effect of MC to cause glucose uptake in the myocyte would probably occur simultaneously with the delivery effect.

It appears that at least a portion of both the hemodynamic and the metabolic effects is NO-dependent, although the L-NAME data alone also indicates that MC can dilate against this NOS inhibitor. The two methods used in this study to observe the effects on glucose uptake by MC did not appear to support each other, as AV- glucose difference showed no effect of MC to increase glucose uptake, however a significant effect was observed in some muscles using radiolabelled glucose uptake. Where a significant effect of MC-mediated glucose uptake was observed, L-NAME was able to block this increase, although L-NAME does appear to stimulate glucose uptake alone, in particular in the soleus muscle where a significant increase in glucose uptake was observed above basal. This may be due to hypoxia-mediated stimulation of glucose uptake in some muscles, as a previous study has indicated that L-NAME may reduce blood flow more in muscles composed of high-oxidative fibres compared to glycolytic fibres (208). In the R'g data, this theory is supported by a significant increase in the soleus mediated by L-NAME. This effect was not replicated in any other muscles measured. The stimulation of R'g that is observed with MC for the other muscles has been blocked by the inclusion of L-NAME, suggesting that in these muscles at least, glucose uptake by MC is NO dependent. The inconsistency between the radiolabelled and the A-V glucose determination is difficult to explain, but discussed in the previous chapter. R'g focuses on specific muscles, whereas the A-V determination is an indication of what is happening over the entire limb. The soleus R'g results may give an indication that there can be variable L-NAME effects

between different muscles. A change in the flow distribution within the limb (i.e. thigh to calf or vice versa) may also account for these apparently divergent results.

It is difficult to evaluate capillary recruitment directly in this system as the perfused hindlimb is considered to be fully vasodilated in the basal state, and due to the fact that both NE and 5HT used in the preconstriction studies have their own effects on flow distribution, it may be difficult to further analyse any effects of MC over and above the original vasoconstrictor effects. For these reasons we have assumed that vasodilation in the local environment is consistent with capillary recruitment *in vivo*, or at least occurs at a similar concentration of MC. From these results, it is therefore apparent that the ability of MC to augment insulin-mediated glucose uptake as shown by Mahajan et al (121) was due to the increase in capillary recruitment, as MC is unlikely to have any direct effect on glucose uptake at this dose. The doses used in the earlier study by Mahajan et al (121) are consistent with doses that in this study were able to cause only hemodynamic effects in the pump-perfused rat hindlimb, observed as vasodilation against another vasoconstrictor in the absence of any direct glucose uptake effects.

As discussed in the introduction, bulk blood flow to the limb had been thought to be a determinant of insulin action, however augmenting blood flow with various vasodilators such as adenosine (123), bradykinin (124), sodium nitroprusside (122), low doses of IGF-1 (209) and adrenaline (31) did not increase glucose uptake. This lead to the theory that the augmentation of blood flow only increases insulin action if it coincides with an increase in the perfusion of the muscle, or a greater level of nutritive perfusion. As such, MC is the only vasodilator to date that can augment insulin's metabolic effects during a hyperinsulinemic euglycemic clamp (1, 59, 120, 121), in theory through increasing the delivery of both insulin and glucose to the muscle by an increase in nutritive perfusion. Previous studies by others reported that MC increased flow, which was paralleled by an increase in glucose uptake; both of these effects were blocked by L-NMMA (59). These data did not fit with the Renkin equation for a fixed capillary surface area (210), so the authors concluded that capillary recruitment had occurred (59). Although a clear effect of MC to augment insulin-mediated capillary recruitment was observed (121), it is not clear that the increase in glucose uptake could be attributed purely to the greater delivery of glucose and insulin to the tissue, or whether possibly MC had a direct effect to cause glucose

uptake alone, as NO donors have previously been shown to cause glucose uptake (207, 211). Only the recent paper by this laboratory (121) included a direct measure of capillary recruitment (using 1-MX), and in so doing, indicated that MC augmented the capillary recruitment normally induced by insulin alone.

From these results it is concluded that MC can cause glucose uptake directly, but only at doses much higher than those that cause capillary recruitment: both of these processes are at least partly dependent on NO. The capillary recruitment observed with MC superimposed on insulin infusion by Mahajan et al (121) is therefore likely to be the cause of the increased insulin-mediated glucose uptake, as the dose used was much lower than that which caused glucose uptake directly in this study. As such, this supports the theory covered by this thesis; that glucose uptake by myocytes can be modified as a function of perfusion of the muscle.

CHAPTER 7

SGLT1 IS NOT INVOLVED IN INSULIN-MEDIATED GLUCOSE UPTAKE IN RAT MUSCLE

7.1 INTRODUCTION

The Na^+-D-glucose cotransporter SGLT1 was originally found in epithelial cells of the intestine and proximal renal tubule, where it absorbs glucose and galactose from food in the intestine, and reabsorbs glucose from the glomerular filtrate in the kidney (7). In both locations uptake of glucose is coupled to a Na^+/K^+ ATPase positioned on the serosal side of the cell to pump out the co-transported Na^+. This allows the intestinal and renal epithelial cells to expend energy to scavenge small concentrations of glucose against the concentration gradient (discussed in (8)). Phlorizin, an inhibitor of SGLT1-mediated glucose transport, has been used in experimental situations to normalize blood glucose in diabetic animals: it does this by preventing reabsorption of glucose in the kidney, particularly in type 1 diabetic animals, and also preventing glucose absorption in the intestine (212, 213), thus lowering plasma glucose and increasing glucose excretion.

A recent study proposed a new role for the SGLT1 in the control of glucose uptake from the blood into muscle interstitium. This was based on evidence for the expression of the SGLT1 in endothelial cells of rat skeletal muscle and heart (6) and on data from the constant flow perfused rat hindlimb system, where insulin stimulated glucose uptake by the muscle was totally blocked by phlorizin (6).

A control site for glucose uptake at this level could have considerable potential impact on muscle glucose metabolism under normal resting conditions, exercise, and diabetes, and make interpretation of the effects of microvascular flow changes on glucose uptake difficult. In that respect, previous studies have suggested that redistribution of flow may account for between 20 and 50% of the insulin-mediated glucose uptake (52, 62). In this thesis, previous chapters have attempted to clarify the

contribution of various vasomodulators to the metabolic action of insulin to increase glucose uptake. Earlier studies by, Rattigan et al (214) have demonstrated that serotonin infused into rats *in vivo* can inhibit insulin-mediated glucose uptake, probably by preventing normal insulin-mediated capillary recruitment. Redistribution of flow by insulin to cause capillary recruitment can therefore increase blood supply to insulin sensitive tissues such as skeletal muscle. As distribution of blood flow seems to be critical in the action of insulin (52, 62), as discussed throughout this thesis, a further control site involving an insulin sensitive Na^+-glucose co-transporter at the endothelial barrier could cloud the issue and make interpretation of changes in capillary recruitment complicated. Therefore, we have attempted to repeat and extend the findings of Elfeber et al (6) using the perfused hindlimb, measuring glucose uptake by 2-deoxyglucose uptake and by using low Na^+ buffer, where this has been used by others to assess the role of the SGLT1 in cellular events (215).

7.2 Methods

7.2.1 Solutions

Solutions were made up to be 200 times the required final concentration to allow for infusion at $1/200^{th}$ of the flow into the infusion port. Therefore, at a perfusion of constant flow rate 8ml/min, substances were infused at 40µl/min unless otherwise indicated.

The phlorizin and phloretin solutions were also used for direct analysis using the glucose analyser. Dilutions of these solutions were used to set up a standard curve.

Phlorizin and phloretin

Phlorizin was diluted in distilled water (300µl), then enough 1M NaOH was added to dissolve the phlorizin (no more than 700µl). This solution was made up to 10ml with saline, to give a final concentration of 40mM for infusion. Phloretin was diluted in the same way as phlorizin above, with a minimum amount of 1M NaOH to dissolve the phloretin.

Insulin

Humulin was diluted to 20mM insulin with saline, which when infused would give 100nM, as used in the study by Elfeber et al (6).

Vehicle

The vehicle infusion contained NaOH (70mM), equal to the maximum amount required to dissolve the phlorizin. When infused, the hindlimb was exposed to 350μM NaOH.

7.2.2 Perfusion conditions

This chapter uses both the regular Krebs, and the modified low sodium Krebs as listed in chapter 2.2.2; both buffers contain 2.54mM $CaCl_2$ and 8.3mM glucose. Hindlimb perfusions permitting flow through a single leg were performed at 32^{o}C. Flow rate was kept constant using a pump delivering 8ml/min.

7.2.3 Perfusion protocol

After hook-up to the perfusion apparatus, the hindlimb was allowed to equilibrate for 40 min before infusion of insulin (100nM) or phlorizin (200μM). The treatments included vehicle, insulin, phlorizin and insulin + phlorizin. Ten minutes after infusion of these treatments 2-deoxyglucose infusion was initiated, which continued for 30 minutes until the end of the perfusion, when the muscles were dissected out. The protocol is shown in Figure 7.1. Glucose and lactate samples were assayed on a glucose analyser (Yellow Springs). A second glucose assay using a spectrophotometer was also attempted.

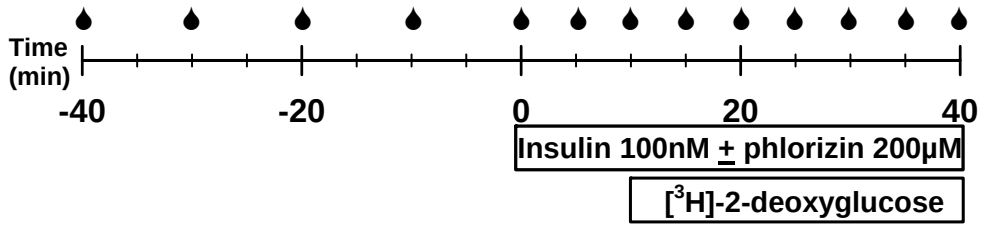

Figure 7.1. Experimental protocol for use in the surgically isolated constant flow perfused rat hindlimb. The rat was allowed to equilibrate at 8ml/min constant flow for 40 minutes prior to infusion of any substances. Venous samples were collected as indicated by the drops (⧫) for glucose and lactate analysis. A similar protocol was performed in the low sodium buffer experiments, although phlorizin was not included in experiments using this buffer.

7.2.4 Radiolabelled glucose uptake

The ^{3}H-2-deoxyglucose assay has been described in chapter 2.3. 2-Deoxyglucose was infused for 30 min prior to the end of perfusion, at which time muscles were dissected out and freeze-clamped. The muscles collected were soleus, plantaris, gastrocnemius (red and white muscle analysed separately), extensor digitorum longus, and tibialis. These were then powdered under liquid nitrogen and analysed as described previously (section 2.3)

7.2.5 Statistical Analysis

Two-way repeated-measures ANOVA was performed using SigmaStat (SPSS Science; Chicago, IL), with comparisons made between conditions using the Student-Newman-Keuls post hoc test. For radiolabelled glucose uptake, a one way ANOVA was used on each muscle. Significance was assumed at the level of $P < 0.05$. Data are presented as means ± SE.

7.3 Results

The protocol outlined in Figure 7.1 was used for all experiments. Figure 7.2 shows the effect of phlorizin on changes in oxygen uptake and perfusion pressure of the constant-flow pump-perfused rat hindlimb. Infusion of 200μM phlorizin alone for 30 min had no effect when compared to saline infusion over the same period. Insulin (100nM) alone increased oxygen uptake by approximately 2μmol/g/h ($P < 0.05$). Inclusion of the phlorizin with insulin had no significant effect on the stimulation by insulin (Figure 7.2). Pressure changes due to phlorizin, insulin, or insulin + phlorizin infusion did not exceed 6mmHg over the 40min infusion period and were not significant when compared to saline alone.

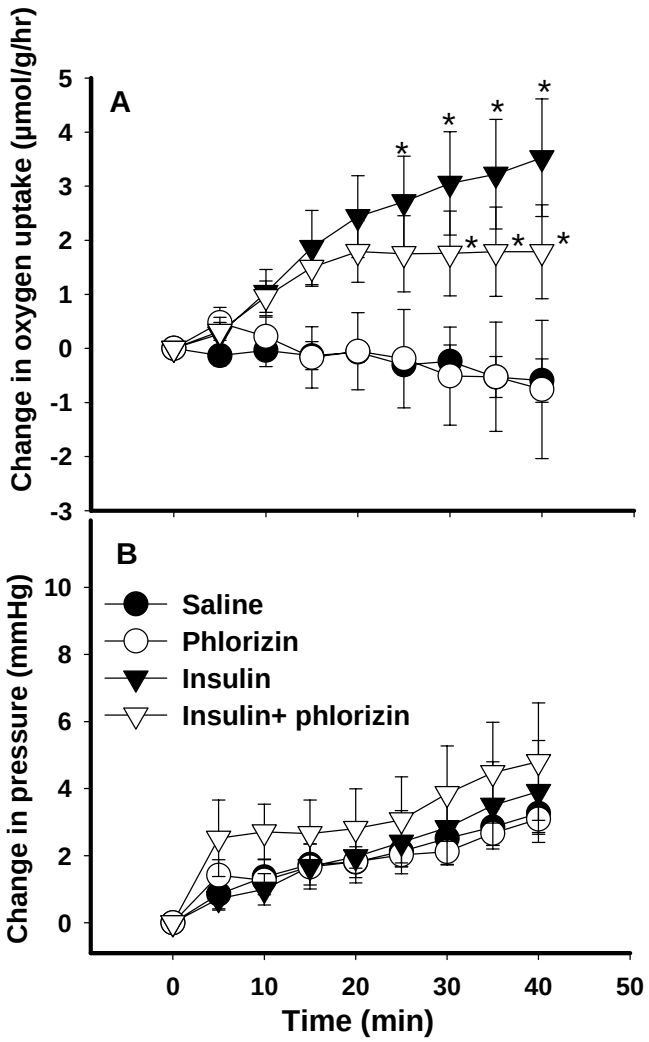

Figure 7.2. Effect of phlorizin on insulin-mediated oxygen uptake (A) and perfusion pressure (B) of the constant-flow perfused rat hindlimb. Insulin, saline, phlorizin, or insulin + phlorizin were infused for 40 min. Oxygen uptake was calculated from A-V difference and flow. Pressure was measured from an in-line arterial transducer. Values are means ± SE. *, Significantly different from saline and phlorizin ($P < 0.05$), $n = 6$ in each group.

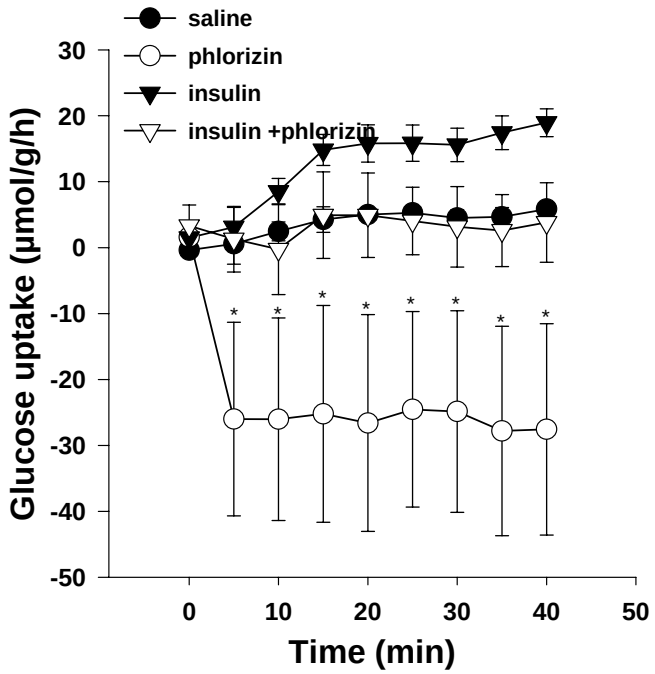

Figure 7.3. Effect of phlorizin on insulin-mediated changes in glucose uptake of the constant-flow perfused rat hindlimb. Insulin, saline, phlorizin, or insulin + phlorizin were infused for 40 min. Values are means ± SE. * $P<0.05$ vs saline, insulin and insulin + phlorizin.

Changes in glucose uptake using the raw data for venous glucose content are shown in Figure 7.3. Insulin alone did not significantly increase glucose uptake above basal, although a trend to increase was seen. Phlorizin appeared to inhibit this, as glucose uptake dropped below basal. However, phlorizin alone caused a negative glucose uptake that was significantly lower than all the other treatments. This indicates that more glucose is being released into the perfusate than is present in the arterial supply. As the perfusion system should only allow for the muscle bed and tissues of the leg to be perfused, the supply of glucose apparently entering the perfusion medium was unlikely to be biological. A sample of the phlorizin solution revealed that phlorizin was detected as glucose by the glucose analyser, suggesting that the apparent release of glucose was due to phlorizin in solution interfering with the assay. The interference of the glucose assay procedure by phlorizin was not due to the β-D-glucose moiety of phlorizin as an identical reaction was also evident with phloretin which has no glucose attached. The interference of phlorizin with the assay appeared

to be constant within the perfusion, although tended to vary between perfusions, despite constant infusion of the same concentration of phlorizin. It is possible that the phlorizin was interfering with the glucose detecting membrane of the glucose analyser in such a way as to change the sensitivity of the assay to phlorizin over time.

To overcome this interference, a second arterial sample containing the infused phlorizin was taken immediately proximal to the rat at the end of the perfusion. This value was then used as the arterial value for any samples that included phlorizin. Other methods for determining glucose uptake were also briefly considered, including a spectrophotometric method. Phlorizin once again interfered with this assay, significantly increasing the absorbance above that obtained with glucose alone.

Changes in glucose uptake and lactate output using corrected glucose values are shown in Figures 7.4 and 7.5. Insulin alone increased glucose uptake approx. 10μmol/g/h, although this was not significant. After correction it was apparent that phlorizin alone had no significant effect on basal or insulin-stimulated glucose uptake (Figure 7.4), although insulin alone did not appear to significantly affect glucose uptake measured using A-V glucose difference in this study.

Lactate uptake values are also shown in Figure 7.4. Phlorizin was not found to interfere with this assay and thus no corrections were necessary for these determinations. Whereas insulin increased lactate output approx. 15μmol/g/h ($P < 0.05$), this was not affected by 200μM phlorizin. In addition, phlorizin alone had no net effect on lactate output by the hindlimb.

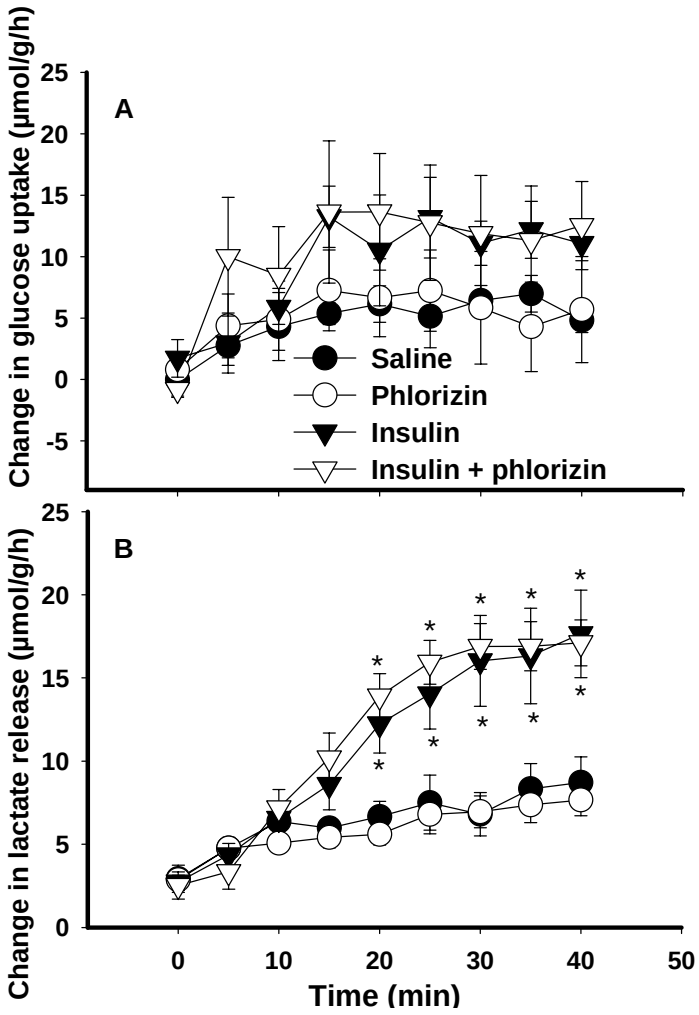

Figure 7.4. Effect of phlorizin on insulin-mediated changes in glucose uptake (A) and lactate output (B) of the constant-flow perfused rat hindlimb. Insulin, saline, phlorizin, or insulin + phlorizin were infused for 40 min. Experimental details are as given for Figure 7.1. Values are means ± SE. *, Significantly different from saline and phlorizin ($P < 0.05$).

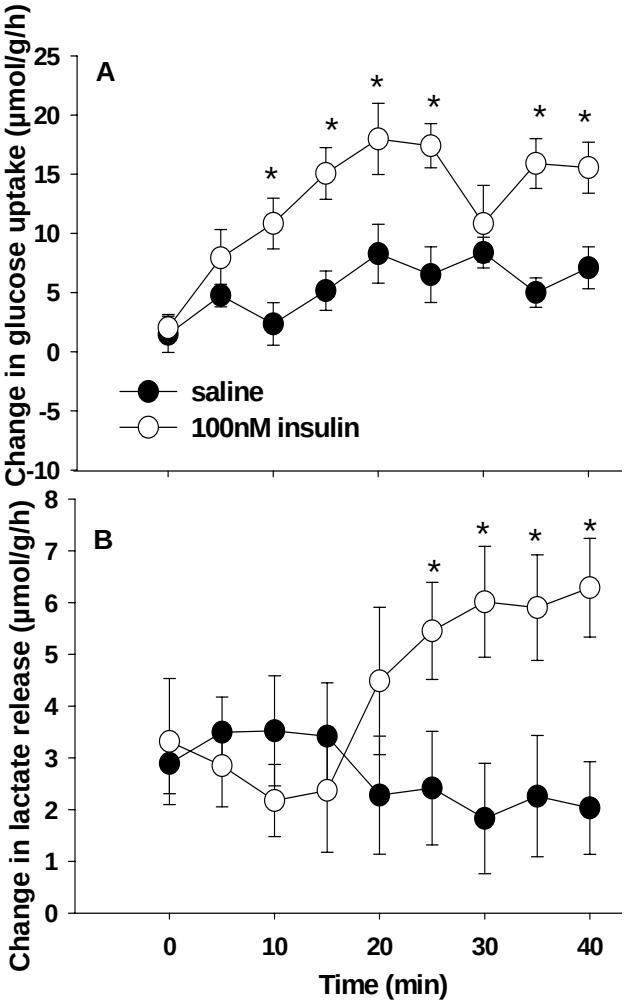

Figure 7.5. Effect of low Na^+ -buffer on insulin-mediated changes in glucose uptake (A) and lactate output (B) by the constant-flow perfused rat hindlimb. The hindlimb was equilibrated with the low Na+ -buffer for 30-40 min before the infusion of saline or insulin for 40 min as shown in Figure 7.1. Values are means ± SE. *, Significantly different from saline ($P < 0.05$).

Figure 7.5 shows the effect of insulin on changes in glucose uptake and lactate output conducted in hindlimbs equilibrated and perfused with low Na^+-buffer. The estimated sodium ion content was approx. 25mM and choline chloride replaced the NaCl component of the standard Krebs Henseleit-bicarbonate buffer. Using this buffer, 100nM insulin increased glucose uptake by approx. 15μmol/g/h ($P < 0.05$) and lactate output by approx. 8μmol/g/h ($P < 0.05$).

Values for 2-deoxyglucose uptake into individual muscles and for the combined group are shown in Figure 7.6. Despite the differences in fibre composition of the individual muscles, ranging for example, from the soleus at approx. 87% slow twitch and 13% fast oxidative glycolytic to the gastrocnemius white at approx. 16% fast oxidative glycolytic and 84% fast glycolytic, there was very little difference in the relative profile for the individual muscles. Insulin caused a significant increase in 2-deoxyglucose uptake in all muscles, and phlorizin did not alter this effect. Insulin-stimulated uptake of 2-deoxyglucose in the low Na^+ buffer tended to be greater than the corresponding rates in normal buffer, however, these differences were not significant. Low Na^+ buffer did not inhibit insulin-mediated uptake of 2-deoxyglucose.

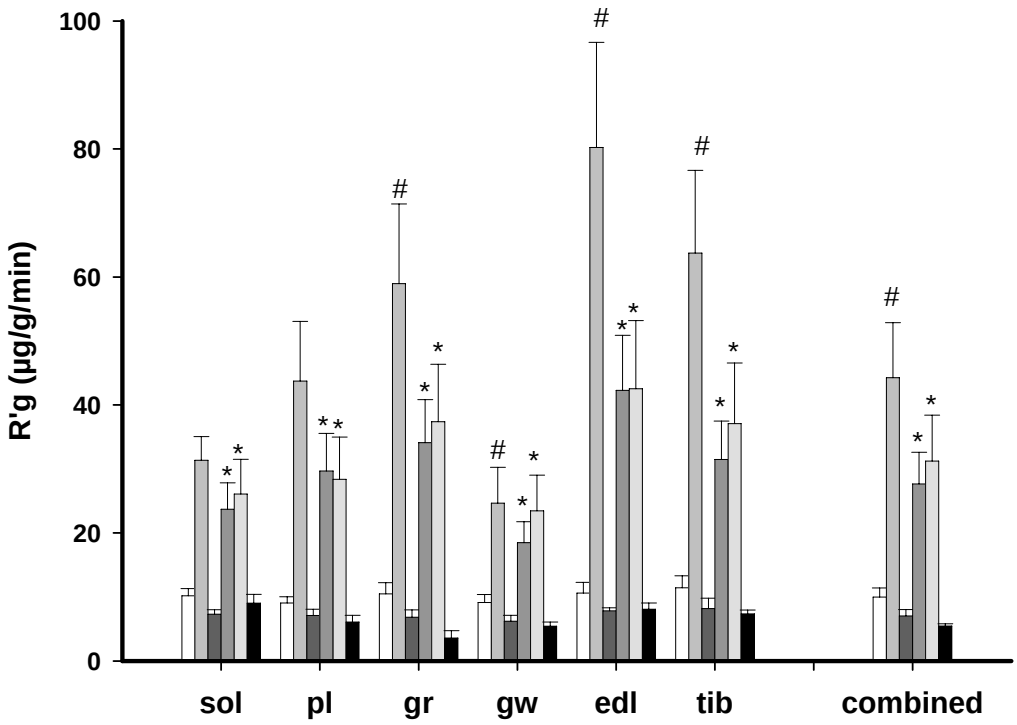

Figure 7.6. Effect of phlorizin or low Na^+ -buffer on insulin-mediated uptake of 2-deoxyglucose in muscles of the perfused rat hindlimb. Experimental details are as given for Figures 7.1 & 7.3. Bars from left to right are: saline at low Na^+, insulin at low Na^+, phlorizin, insulin, insulin + phlorizin, and saline. Muscles are: sol, soleus; pl, plantaris; gr, gastrocnemius red; gw, gastrocnemius white; edl, extensor digitorum longus; tib, tibialis. For 'combined' the product of uptake rate times wet weight for each muscle was summated and divided by the combined wet weight for all combined muscles. Values are means ± SE, n=5-6 for all groups. Statistics were carried out on low Na^+ treatments separately to other groups. *, Significantly different from saline and phlorizin ($P < 0.05$); #, significantly different from saline using low Na^+ buffer ($P < 0.05$).

7.4 Discussion

Contrary to the previous report by Elfeber et al (6) this study provided no evidence that the Na^+-D-glucose transporter is involved in insulin-mediated glucose uptake by the perfused rat hindlimb. Whereas evidence for the expression of this protein in the muscle capillary cells by those authors (6) is untested by our present study, we would now regard their perfusion results, on which a role in transendothelial glucose transport was based, as incomplete and lacking sufficient controls. In particular, it is evident in the present studies that phlorizin (as well as the non glucose-containing phloretin) substantially interferes with the glucose assay giving the impression that there is much higher glucose in the venous samples than is actually present. This interference was observed to be dose-dependent, and was constant within each experiment, although varied between experiments. As such, in some experiments insulin was observed to cause some glucose uptake using this assay even in the presence of phlorizin, although this appeared to be inhibited slightly by the phlorizin. As a result, the arterio-venous glucose difference is underestimated. The glucose analyser tended to overestimate the glucose in venous samples when phlorizin was present at 200μM, and this overestimation was matched by the increase in glucose removal due to insulin, giving the false impression that insulin-mediated glucose uptake was completely inhibited (Figure 7.3). Whereas corrections due to this interference have largely eliminated the apparent inhibition by phlorizin, glucose uptake was also assessed by the radioactive analogue, 2-deoxyglucose. Phlorizin had no inhibitory effect on insulin-mediated uptake of 2-deoxyglucose, although as 2-deoxyglucose is not thought to be a substrate for SGLT1 (11) it is not surprising that phlorizin was unable to block insulin-mediated 2-deoxyglucose uptake. However, the fact that a sugar analogue not transported by the SGLT1 protein is able to cross the endothelial cell barrier to cause insulin-mediated glucose uptake suggests that SGLT1-mediated transendothelial glucose transport is unlikely.

Phlorizin had no effect on the lactate assay. However, in the perfusion technique used by Elfelber et al (6) the perfusate contained 2mM L-lactate and 0.2mM pyruvate. This effectively masked the production of lactate that occurred with insulin addition and which can be useful as an alternate indicator of insulin's action. Thus in the present study, using lactate- and pyruvate-free buffer, it was noted that insulin increased lactate output and that this effect was not altered by phlorizin. This was a further

indicator that insulin-mediated glucose uptake was unlikely to have been inhibited by phlorizin.

Phlorizin is known to be an inhibitor of GLUT1 and GLUT4 transport proteins as well as SGLTs (216), however for this inhibition higher concentrations of phlorizin are normally required than those used in this study. Therefore it is unlikely that the phlorizin would have blocked the glucose uptake in muscle by these transporters. It is possible, although unlikely based on the dose used, that the results seen by Elfeber et al (6) regarding the ability of phlorizin to inhibit glucose uptake were due to inhibition of the facilitative glucose transporters GLUT1 and GLUT4. In terms of the results shown above regarding radiolabelled 2-deoxyglucose uptake, it is apparent that phlorizin had no effect on the GLUT1 and GLUT4 mediated glucose uptake, as the values for both insulin and vehicle in the presence of phlorizin were not significantly different from the controls.

When investigating the role of SGLT1 in cellular processes it is common to use low Na^+-buffer as an inhibitor (e.g. (215)). Thus in the present study low Na^+-buffer was used with and without insulin. There was no effect of the low Na^+ to inhibit insulin-mediated glucose or 2-deoxyglucose uptake.

The findings reported here show no role for SGLT1 during an insulin-induced increase in glucose consumption, so a role for this cotransporter in endothelial cell function is yet to be defined. A role of the SGLT1 in insulin action has previously been identified, although this study specifically looked at the small intestine (17). Another study has revealed that SGLT1 is able to cause NO release through altered Na^+ concentrations, which stimulates a Ca^{2+} efflux through the Na^+Ca^{2+} exchanger (5). While not causing significant relaxation alone, possibly due to a low magnitude of NO release, this effect increased the relaxation observed with insulin in porcine coronary conduit arteries. The role of SGLT1 in hypoxia has also been studied, whereby SGLT1 can transport glucose into the cell to stimulate glycolysis, in order to rectify the ATP deficiency seen in hypoxia (217). Elfeber et al (6) suggest that SGLT1-dependent D-glucose permeability exists to maintain adequate glucose supply when the plasma glucose concentration decreases, and a study in brain on an SGLT-like protein showed that the activity of the protein was enhanced in low-glucose conditions (9). In this way, the SGLT-like protein can continually carry glucose into

cells against the concentration gradient, and will help to preserve cell function even under stress conditions, including hypoxia and hypoglycemia.

Finally, it is noted that the specificity of the antibody used by Elfeber et al (6) for the immunostaining was not tested for cross-reactivity with the SGLT2 protein, therefore specific detection of the SGLT1 protein has not been shown. Also, the presence of SGLT1 in skeletal muscle was not observed using Western Blot. Elfeber et al (6) have suggested that the SGLT1 is located on the luminal surface of the endothelial cells, implying a role in the movement of glucose from plasma to the myocyte for insulin-mediated glucose uptake, although this localization was not specifically addressed. Furthermore, if insulin-mediated glucose uptake were to be controlled at the capillary by SGLT1, a continuous endothelial cell lining would be essential to this control. As the endothelial lining also contains intercellular clefts shown to be involved in passive diffusion of sugars (218), it is unlikely that the SGLT1 protein is involved in insulin-mediated glucose uptake.

In conclusion, as the data included in this chapter show no role for SGLT1 during increased glucose consumption, a role for this cotransporter in endothelial cell function is yet to be defined. It is possible, as argued by Elfeber et al (6), that SGLT1-dependent D-glucose permeability exists to maintain adequate glucose supply when the plasma glucose concentration decreases. However it is apparent that SGLT1 is not required for insulin-mediated glucose uptake in muscle.

CHAPTER 8

DISCUSSION

There are three main control points for glucose uptake into myocytes: these include the delivery of glucose by blood to the area, the transendothelial transport of glucose, and crossing the myocyte membrane to enter the cell. The studies contained within this thesis address the first two of these three points. The delivery of glucose can be altered by hemodynamic properties that change the distribution of blood through the muscle, including changes in blood flow and capillary recruitment. The transport of glucose across the endothelium has also been proposed to be a control point by a recent paper (6) whereby the sodium glucose contransporter (SGLT1) is responsible for all insulin-mediated glucose uptake, but not involved in basal glucose uptake. Specific studies separating the vascular delivery of glucose and transendothelial transport have not been performed, yet by using agents that are known to have specific vascular effects, such as sodium nitroprusside, conclusions can be made regarding the contribution of flow redistribution on glucose uptake.

8.1 Key findings

The work described in this thesis investigates the involvement of blood flow distribution on metabolism in the hindlimb, specifically glucose uptake. ET-1 was found to have dose-dependent effects on metabolism and aerobic contraction, most likely due to its vascular effects to redistribute blood flow within muscle. ET-1-mediated flow changes may therefore have profound effects on the action of insulin, however in this study it was found that insulin appeared to have vasodilator-like effects on ET-1-mediated vasoconstriction, not previously observed in the perfused hindlimb. Also in this thesis, methacholine was shown to have vasodilator effects that occur at much lower doses than any effects on glucose uptake, and thus the increase in insulin-mediated glucose uptake previously observed with methacholine (121) is likely to be due to its vasodilatory effects that amplify insulin-mediated capillary recruitment rather than direct effects to cause glucose uptake. And to emphasize the role of blood flow dispersion through muscle, the SGLT1 protein that has been

localized to the endothelial cells (6) was found not to be involved in insulin-mediated glucose uptake. Therefore, factors that alter blood flow in muscle can have profound effects on the metabolism of the tissue due to the change in delivery of nutrients to the area.

8.2 Limitations of the hindlimb perfusion – can ET-1 help?

As discussed, the perfused rat hindlimb is fully vasodilated, as even with a very high flow rate of 8ml/min as used throughout this study (compared to 1-2ml/min *in vivo*), the pressure is regularly only 20-30mmHg (compared to approximately 120mmHg *in vivo*). This may be because the buffer used in the perfusion does not contain hemodynamic factors found in plasma or blood cells that increase the perfusion pressure, and the hindlimb is without central nervous control. While the lack of nervous control of the hindlimb in some ways is an advantage as it permits studies to be undertaken without interference by homeostatic mechanisms, it may also be responsible for maintaining the vascular tone of the hindlimb. Various vasoconstrictors have been infused in the perfused rat hindlimb in an attempt to more accurately mimic the flow rate and pressure observed *in vivo*, although as yet with no great success. The observation here that insulin is able to dilate against ET-1 leads to the suggestion that ET-1 may be the tonic vasoconstriction that is required in perfusion to create a more physiological situation. There are several problems with this suggestion. The vasoconstriction by ET-1 may cause shear stress (219, 220), particularly if the flow rate remains at 8ml/min, which would lead to the release of more ET-1: this may be the reason that a steady plateau of pressure is never attained in this study with higher doses of ET-1. Therefore a careful balance is required between ET-1 concentration and flow rate as well as other factors in the perfused hindlimb to maintain a steady state. Obviously *in vivo* ET-1 is not solely responsible for the maintenance of vascular tone; in fact, if ET-1 were responsible for much of the basal vascular tone, it is unlikely that the pressure observed at the beginning of perfusion would be as low, due to the strong, long-lasting nature of ET-1-mediated vasoconstriction (135). However it may be a useful inclusion in experiments of this nature in the future, particularly for studying insulin-mediated vasodilation in the isolated pump-perfused rat hindlimb.

8.3 Mechanisms of dose-dependent effects of ET-1

A major question that has not been answered within the studies included in this thesis is the mechanism by which ET-1 is able to cause two different effects on metabolism (nutritive and non-nutritive) depending on the dose of ET-1 used.

Receptor-mediated differences in flow pattern

It is possible that two receptor subtypes may exist, one of which is engaged when ET-1 is present at low concentrations, which would manipulate blood flow to travel through the nutritive capillary network. At higher concentrations, ET-1 could bind to a second receptor subtype that would likely have a lower affinity, and thus need this higher concentration of ET-1 to be activated. The other possibility is that there may be some cross-reactivity with another receptor at such high concentrations. This receptor would probably be located so as to cause flow to enter the non-nutritive pathway, such as on the vessels of the nutritive network. So far there is no evidence of subtypes other than those discussed earlier, ET_A and ET_B. The two identified receptor types for ET-1 have different functions (136), and while the distribution of these receptors has been studied (221), as yet the specific distribution within the muscles relevant to this study has not been clarified. While previous studies tend to suggest that ET_A causes insulin resistance (179) and ET_B-mediated vasodilation limits these non-nutritive effects (222), no studies have observed receptor involvement at low ET-1 concentrations which may be nutritive, based on the results of this thesis. Furthermore, as yet the exact location of the non-nutritive pathway is unknown, although the subject of investigation within this laboratory (223). The confirmation of this mechanism of action would require proof of the existence of two receptor subtypes activated at different concentrations, and then verification that their localization patterns between nutritive and non-nutritive routes would support such a role. The receptor subtype mechanism of action for vasoconstrictors has previously been tested with norepinephrine, another vasoconstrictor that can cause both nutritive and non-nutritive perfusion depending on the dose used (224). While the nutritive flow pattern of norepinephrine appeared to be sensitive to Ca^{2+}, the non-nutritive flow pattern was unaffected by Ca^{2+} removal, which suggests two different mechanisms of causing the vasoconstriction. This previous result does not appear to apply to ET-1, as the removal of Ca^{2+} from the buffer blocked both stimulatory and inhibitory flow effects.

Progression of vasoconstriction through muscle

An alternative theory to explain the dose-dependent effects of ET-1 requires only one receptor subtype, and uses a kind of 'rolling' vasoconstriction of various capillary networks. As already discussed in chapter 3, and above (section 8.2), the hindlimb is thought to be fully vasodilated, as previous experiments have been unable to cause further vasodilation, and yet an increase in nutritive perfusion of the muscle can occur. This suggests that although the hindlimb may be fully vasodilated, it is not fully perfused, and as such the flow is probably passing through only a portion of the total capillary network. As such, only the perfused blood vessels would initially be exposed to ET-1, therefore the receptors responsible for vasoconstriction would only be activated in those areas receiving blood flow. As these vessels constrict to either decrease or cease flow (225), the flow must progress to a new area and recruit capillaries that were not previously perfused. These newly perfused blood vessels would then be subject to the vasoconstrictor effects of ET-1, and the process would continue to the next vascular bed. It is important to remember here that the vasoconstrictor effects of ET-1 are quite slow to begin (138) and long lasting (135), such that the gradual vasoconstriction would allow the flow to gradually decrease through one capillary bed over time, and thereby increase in another vascular bed. In this way, the initial vasoconstriction may wear off in the 'first' blood vessels, which may then eventually be perfused again. This would be accompanied by a plateau of pressure in the perfused hindlimb.

Higher concentrations of ET-1 have been shown within the studies contained in this thesis to cause a faster onset of increased pressure, consistent with a quicker development of vasoconstriction. In this case, the higher concentration of ET-1 would begin this 'rolling' activation of capillaries to occur at a faster rate, which may not allow time for the 'first' vascular bed to overcome the initial vasoconstriction. Eventually, every blood vessel would be constricted. In this case, the pressure would continue to increase, as observed within these studies, and the remaining flow may progress through the shunts of the non-nutritive pathway, or force its way through whichever vessels it may.

***In vivo* changes in flow pattern**

This above scenario appears to fit with the observations made in the pump-perfused rat hindlimb, although how these effects may manifest *in vivo* is not clear, particularly as flow is not necessarily constant *in vivo,* and rather than systemic administration of ET-1 as in perfusion, release from the local environment is what determines vasoconstriction (146). Although the involvement of ET-1 in basal vasoconstriction is controversial, as discussed earlier (Chapter 1), if local ET-1 vasoconstriction allowed a limited amount of flow to progress through the vascular bed, a general tonic vasoconstriction over the entire muscle could be maintained. This could possibly occur through a version of the 'rolling' progression discussed above, whereby areas receiving flow are somehow stimulated to release ET-1 in the local environment, eventually shutting down flow to that area (225) and forcing it to another. In this mechanism, elevated levels of ET-1 could cause vasoconstriction to increase in each area receiving flow, switching the tonic vasoconstriction by ET-1 from what may be nutritive perfusion to a more inhibitory flow pattern. Studies have shown that infusion of ET-1 into the healthy human forearm causes insulin resistance (174), and as such it seems that the infused ET-1 caused non-nutritive perfusion. It is possible that any further infusion of ET-1 into a whole body system may ensure an outcome of switching from a basal nutritive flow (imitated in these studies using 1nM) to non-nutritive (10nM), which would cause insulin resistance.

Determining the mechanism of ET-1-mediated flow changes

The exact mechanism of action of ET-1 *in vivo* will be difficult to clarify. As ET-1 is primarily a local, or paracrine, hormone (146), and due to the high affinity of ET-1 for its receptor (147), it will be difficult to effectively block either ET-1 receptor, yet this would be the most effective way of determining exactly the involvement of ET-1 receptors in nutritive and non-nutritive flow. In studies on porcine cerebellum membranes, Wu-Wong et al (147, 226) demonstrated that while ET receptor antagonists could be readily dissociated by washing, agonists were difficult to dissociate once bound due to their higher affinity. It was shown that the inhibitory effects of the antagonist on ET binding decreased with incubation time, probably due to differences in affinity for the receptor and dissociation times (147). Previous studies have used specific receptor blockers with greater success in perfusions in mouse hearts (138) and isolated vessels (204), where the antagonists could block up to 50% of the normal 10nM ET-1 response. ET receptor antagonists have also been used successfully *in vivo* in various animals (194, 222, 227-229) and humans (179).

Antagonists of both receptors used against ET-1 in the perfused rat hindlimb had no significant effect (our unpublished observations), although infusion of inhibitors was commenced at the same time as ET-1 infusion. For greater efficacy it may have been necessary to infuse the blockers prior to ET-1.

Clearly, in order to properly validate the studies within this thesis, the local concentration of ET-1 would need to be known. As yet, there is no accurate way to do this, as it is difficult to sample the immediate area of ET-1 release. Also, even though Wagner et al (146) have shown that 80% of ET-1 is released towards the vascular smooth muscle cells, they admit that the 20% on the luminal side may be from diffusion in their cell preparation, a value which may not be indicative of diffusion *in vivo*. Furthermore, even if the plasma levels could be used in this way to calculate the local concentration, this does not account for any degradation or binding of ET-1 before it reaches the plasma, and also does not include any recognition of the heterogeneity of ET-1 release through the body. As such, the plasma levels of ET-1 are likely to be a spill-over from the local environment, much lower than local levels, and the differences in plasma levels may only be small in spite of large changes in local release of ET-1.

Many studies have reported the ability of ET-1 to cause a transient vasodilation (132, 136, 230) before the typical vasoconstriction sets in. As discussed, ET_B receptors located on the endothelial cells are responsible for the vasodilatory effects of ET-1, while ET_A receptors (and in some places ET_B) located on vascular smooth muscle are responsible for the vasoconstriction. As vasodilation by ET-1 is not evident in the perfused rat hindlimb, even when preconstricted with AII or 5HT, it is unlikely that ET_B receptors have a significant presence or role in the hindlimb. Previous studies have emphasized this vasodilatory effect of ET-1 (148, 230); however it seems more likely that these receptors exist to limit the effect of ET-1 overflow from the local environment into the plasma. In the local environment, ET-1 release would initially bind the receptors on vascular smooth muscle to cause vasoconstriction; once the concentration increased and vasoconstriction became more augmented, the overflow of ET-1 would then bind the ET_B receptors on the endothelial cells to vasodilate, thus preventing any unnecessary increase in pressure. When given as a bolus, the first receptors that ET-1 would encounter are those on the endothelial cells, and as such vasodilation would be the first observable effect of ET-1. This would be overcome

once the ET-1 diffuses toward the vasoconstrictor receptors on the vascular smooth muscle cells. Therefore it would appear that ET-1 has its own control loop by which it can limit any excessive vasoconstriction ET-1 itself causes in the local area, and the apparent transient vasodilation observed with bolus ET-1 administration is due to the method of delivery rather than the typical *in vivo* physiological response.

8.4 Mechanisms of insulin's vascular effect – *in vivo* vs. perfusion

As discussed, the hemodynamic actions of insulin that have been observed as outlined in the introduction include increased blood flow, vasodilation, and capillary recruitment. These factors have been measured directly (blood flow) and with indirect methods (vasodilation is measured by pressure changes in perfusion and isolated vessels (88) and capillary recruitment has been measured *in vivo* using contrast enhanced ultrasound (36, 117), 1-methylxanthine (36, 117, 121, 223, 231) and laser Doppler flowmetry (35, 37, 232)). In perfusion, no hemodynamic action of insulin has yet been shown, although in isolated perfused arterioles, dilation has been observed (88). So the question remains, how does insulin exert its hemodynamic effects? Insulin causes nitric oxide (NO) release in the local environment which leads to vasodilation, as shown in isolated arterioles (88). Capillary recruitment has so far not been affected by NO synthase inhibition in the local environment (118), although systemic NO has been shown to be involved (117). Therefore, while both are dependent on NO, vasodilation seems to be a local effect, and capillary recruitment systemic. As yet it is not clear whether other factors are required for capillary recruitment – it is possible that there is some local regulation of recruitment by systemic NO. If, for example, ET-1 (and/or other vasoconstrictors and central input) were to maintain the basal vascular tone, the action of NO to vasodilate against this may cause capillary recruitment.

In perfusion, then, where there are no systemic effects or central input, NO would not cause capillary recruitment. It is possible that the apparent vasodilatory effects of insulin observed against ET-1 in the perfused rat hindlimb are not associated with capillary recruitment, but that local vasodilation can occur without capillary recruitment. There is also the possibility that insulin does not actually cause

vasodilation in this preparation, and in fact is blocking some signalling mechanism of ET-1, thus limiting the vasoconstrictor response. Observations from in Chapter 5 show that once insulin is infused the progression of the normal ET-1 response slows, although the vasoconstriction that has already occurred is unaffected. Dick and Sturek (205) have shown that insulin can attenuate the Ca^{2+} response of dissociated vascular smooth muscle cells to 10nM ET-1, and therefore that the effects of insulin against ET-1 are probably due to Ca^{2+} interference (205). Another study has shown that insulin also attenuates vasopressin responses due to Ca^{2+} interference (206), yet this has never been observed in perfusion. While insulin can vasodilate against other vasoconstrictors *in vivo*, and also block their Ca^{2+} signalling in isolated cell studies, no effect of insulin to do either of these has been observed in the perfused rat hindlimb. Therefore, insulin is unable to limit or vasodilate against the intracellular Ca^{2+} signalling of all other vasoconstrictors in the perfused rat hindlimb, yet is effective against ET-1. The reason for this difference in sensitivity of the perfused hindlimb to insulin against various vasoconstrictors is unclear. A more recent study suggests that the interaction between insulin and ET-1 is more specific, and is in fact due to interference with the ET_A receptor, discovered using the ET_B specific sarafarotoxin S6c (203). As the data stands, the effect of insulin to block ET-1 is likely due to interference in the Ca^{2+} signalling. A contribution by vasodilation, possibly by NO, cannot be ruled out yet, although it is unlikely to be the predominant effect.

8.5 ET-1 in disease

ET-1 levels are elevated in disease as discussed in chapter 1 (section 1.4.1.2), and have been associated with reduced glucose uptake and insulin resistance. Also, infusion of ET-1 into healthy humans causes insulin resistance as measured by reduced glucose uptake (174). In our study the effect of ET-1 to cause insulin resistance varied depending on the method used, however due to the very high, non-physiological concentrations of both ET-1 and insulin, this may not give an accurate picture of what happens in an *in vivo* situation (as discussed in chapter 5). As discussed, these high levels may be well above that needed for the maximal response, and so create an excessive, strong flow pattern which can not be modified. More apparent in the results of this thesis is the ability of insulin to limit the effects of ET-1, both vascular and metabolic.

This may have profound implications for the progression of various diseases. For example, as shown in this thesis, insulin can modulate the action of ET-1. However, in insulin resistant patients the ability of insulin to block ET-1 signalling may be decreased or absent, allowing ET-1 action to continue unopposed. This hypothesis is supported by the results from the perfusion of the Zucker obese rat, where insulin was unable to block the normal effects of ET-1 in this insulin-resistant state as efficiently as in other healthy rats within this thesis (Chapter 5), although this experiment obviously requires confirmation. This could lead to an increase in pressure, possibly hypertension, and eventually may cause a chronic non-nutritive flow pattern to develop. Once again, it is not known whether insulin affects ET-1 responses by vasodilation or signal interference; more work needs to be done to clarify the mechanism.

In hypertensive patients that have elevated plasma ET-1 levels, it is likely that insulin resistance could develop, as ET-1 infused into healthy patients causes insulin resistance (174). Higher levels of ET-1 in the perfused hindlimb have shown an inhibition of insulin-mediated radiolabelled 2-deoxyglucose uptake, thus suggesting that ET-1 may indeed cause insulin resistance *in vivo*.

Therefore it seems that the involvement of ET-1 in disease is complex, as is the interaction with insulin. Insulin resistance could possibly lead to hypertension through unregulated ET-1-mediated vasoconstriction, and high levels of ET-1 could lead to insulin resistance through flow redistribution.

8.6 Capillary Recruitment and Glucose Uptake

The introduction of this thesis outlined the relationship between capillary recruitment and glucose uptake (section 1.2.1), and it has been shown that increased substrate delivery in skeletal muscle can account for up to 20-30% of insulin-mediated glucose uptake (52, 62). As insulin interacts with the myocyte directly to cause GLUT4 translocation (18-20) and thereby increase glucose uptake (21), it is unlikely that there is another major control site for the delivery of glucose to muscle. However, a recent study by Elfeber et al (6) suggested that transport of glucose across the endothelium is essential for all insulin-mediated glucose uptake. As discussed in Chapter 7, this is

unlikely based on the results from our studies. Furthermore, work has been done previously to assess whether glucose uptake requires a receptor mediated mechanism across the endothelium (233), although more often insulin has been studied. As insulin is much larger than glucose, and there is no evidence for a receptor-mediated mechanism for insulin to traverse the endothelium (234-236) (although some authors have suggested otherwise (237)), it would seem unlikely that a glucose molecule would require such a receptor. Therefore the proposed role of the SGLT1 protein is likely to be as more of a scavenger, as in other tissues, whereby it can actively take up glucose against the concentration gradient using energy. In this way, capillary recruitment would be the main mechanism to deliver glucose to the muscle, as has repeatedly been shown by studies that can change glucose uptake by altering vascular perfusion of muscle (45, 52, 60, 62), while the direct effect of insulin on myocytes is still an essential element in insulin-mediated glucose uptake.

8.7 Conclusion

The work in this thesis collectively looks at vascular actions in perfused muscle of the rat, and the effect that varying the hemodynamic flow patterns can have on metabolic parameters such as glucose uptake. Many studies have found effects on glucose uptake by vasoactive agents such as ET-1 and methacholine, although often the vascular and metabolic effects have not been separated to observe whether one is dependent on the other. Throughout this thesis, we have shown that changes in flow distribution can influence metabolic effects, both basal and insulin-stimulated. Also, by disproving an earlier publication that suggested that trans-endothelial transport of glucose was necessary for insulin-mediated glucose uptake, we have further emphasized the role of blood flow dispersion within the muscle to affect the delivery and uptake of glucose.

References

1. Baron, A. D. (1994) Hemodynamic actions of insulin. *American Journal of Physiology* 267, E187-E202

2. Defronzo, R. A., Ferrannini, E., Sato, Y., Felig, P., and Wahren, J. (1981) Synergistic interaction between exercise and insulin on peripheral glucose uptake. *Journal of Clinical Investigation* 68, 1468-1474

3. Zierler, K. (1999) Whole body glucose metabolism. *American Journal of Physiology* 276, E409-E426

4. Wood, I. A., and Trayhurn, P. (2003) Glucose transporters (GLUT and SGLT): expanded families of sugar transport proteins. *British Journal of Nutrition* 89, 3-9

5. Taubert, D., Rosenkranz, A., Berkels, R., Roesen, R., and Schomig, E. (2004) Acute effects of glucose and insulin on vascular endothelium. *Diabetologia* 47, 2059-2071

6. Elfeber, K., Stumpel, F., Gorboulev, V., Mattig, S., Deussen, A., Kaissling, B., and Keopsell, H. (2004) Na^+-D-glucose cotransporter in muscle capillaries increases glucose permeability. *Biochimica et biophysica Acta* 314, 301-305

7. Wright, E. M., Hager, K. M., and Turk, E. (1992) Sodium cotransport proteins. *Current Opinions in Cellular Biology* 4, 696-702

8. Larsen, E. H., Sorensen, J. B., and Sorensen, J. N. (2002) Analysis of the sodium recirculation theory of solute-coupled water transport in small intestine. *Journal of Physiology* 542, 33-50

9. Nishizaki, T., and Matsuoka, T. (1998) Low glucose enhances Na+/glucose transport in bovine brain artery endothelial cells. *Stroke* 29, 844-849

10. Zhou, L., Cryan, E. V., D'Andrea, M. R., Belkowski, S., Conway, B. R., and Demarest, K. T. (2003) Human cardiomyocytes express high level of

Na+/glucose cotransporter 1 (SGLT1). *Journal of Cellular Biochemistry* 90, 339-346

11. Poppe, R., Karbach, U., Gambaryan, S., Wiesinger, H., Lutzenburg, M., Kraemer, M., Witte, O. W., and Koepsell, H. (1997) Expression of the Na+-D-glucose cotransporter SGLT1 in neurons. *Journal of Neurochemistry* 69, 84-94

12. Diez-Sampedro, A., Hirayama, B. A., Osswald, C., Gorboulev, V., Baumgarten, K., Volk, C., Wright, E. M., and Koepsell, H. (2003) A glucose sensor hiding in a family of transporters. *Proceedings of the National Academy of Sciences of the United States of America* 100, 11753-11758

13. Clausen, T. (1975) The effect of insulin on glucose transport in muscle cells. *Current Topics in Membrane Transport* 6, 169-226

14. Bihler, I. (1968) The action of cardiotonic steroids on sugar-transport in muscle, in vitro. *Biochimica et biophysica Acta* 163, 401-410

15. Battaglia, F. C., Manchester, K. L., and Randle, P. J. (1960) Effects of insulin on monosaccharide transport and incorporation of amino acids into protein in diaphragm differentiated with phlorizin. *Biochimica et biophysica Acta* 43, 50-54

16. Kohn, P. G., and Clausen, T. (1971) The relationship between the transport of glucose and cations across cell membranes in isolated tissues. VI. The effect of insulin, ouabain, and metabolic inhibitors on the transport of 3-O-methylglucose and glucose in rat soleus muscles. *Biochimica et biophysica Acta* 225, 277-290

17. Kurokawa, T., Hashida, F., Kawabata, S., and Ishibashi, S. (1995) Evidence for the regulation of small intestinal Na+/glucose cotransporter by insulin. *Biochemistry and Molecular Biology International* 37, 33-38

18. Douen, A. G., Ramlal, T., Rastogi, S., Bilan, P., Cartee, G. D., Vranic, M., Holloszy, J., and Klip, A. (1990) Exercise induces recruitment of the "insulin-

responsive glucose tranporter". *Journal of Biological Chemistry* 265, 13427-13430

19. Bryant, N. J., Govers, R., and James, D. E. (2002) Regulated transport of the glucose transporter GLUT4. *Nature Reviews Molecular Cell Biology* 3, 267-277

20. Blok, J., Gibbs, E. M., Lienhard, G. E., Slot, J. W., and Geuze, H. J. (1988) Insulin-induced translocation of glucose transporters from post-Golgi compartments to the plasma membrane of 3T3-L1 adipocytes. *Journal of Cell Biology* 106, 69-76

21. Hansen, P. A., Nolte, L. A., Chen, M. M., and Holloszy, J. O. (1998) Increased GLUT-4 translocation mediates enhanced insulin sensitivity of muscle glucose transport after exercise. *Journal of Applied Physiology* 85, 1218-1222

22. Henriksen, E. J., Bourey, R. E., Rodnick, K. J., Koranyi, L., Permutt, M. A., and Holloszy, J. O. (1990) Glucose transporter protein content and glucose transport capacity in rat skeletal muscles. *American Journal of Physiology* 259, E593-E598

23. Lund, S., Holman, G. D., Schmitz, O., and Pedersen, O. (1995) Contraction stimulates translocation of glucose transporter GLUT4 in skeletal muscle through a mechanism distinct from that of insulin. *Proceedings of the National Academy of Sciences of the United States of America* 92, 5817-5821

24. Richter, E. A., Nielsen, J. N., Jorgensen, S. B., Frosig, C., and Wojtaszewski, J. F. (2003) Signalling to glucose transport in skeletal muscle during exercise. *Acta Physiologica Scandinvica* 178, 329-335

25. Laakso, M., Edelman, S. V., Brechtel, G., and Baron, A. D. (1990) Decreased effect of insulin to stimulate skeletal muscle blood flow in obese man. *Journal of Clinical Investigation* 85, 1844-1852

26. Abramson, D. I., Schkloven, N., Margolis, M. N., and Mirsky, I. A. (1939) Influence of massive doses of insulin on peripheral blood flow in man. *American Journal of Physiology* 128, 124-132

27. Tack, C. J. J., Schefman, A. E. P., Willems, J. L., Thien, T., Lutterman, J. A., and Smits, P. (1996) Direct vasodilator effects of physiological hyperinsulinaemia in human skeletal muscle. *European Journal of Clinical Investigation* 26, 772-778

28. Raitakari, M., Nuutila, P., Ruotsalainen, U., Laine, H., Teras, M., Iida, H., Makimattila, S., Utriainen, T., Oikonen, V., and Sipila, H. (1996) Evidence for dissociation of insulin stimulation of blood flow and glucose uptake in human skeletal muscle - Studies using [15O]H2O, [18F]fluoro-2-deoxy-D-glucose, and positron emission tomography. *Diabetes* 45, 1471-1477

29. Fugmann, A., Lind, L., Andersson, P. E., Millgard, J., Hanni, A., Berne, C., and Lithell, H. (1998) The effect of euglucaemic hyperinsulinaemia on forearm blood flow and glucose uptake in the human forearm. *Acta Diabetologica* 35, 203-206

30. Natali, A., Buzzigoli, G., Taddei, S., Santoro, D., Cerri, M., Pedrinelli, R., and Ferrannini, E. (1990) Effects of insulin on hemodynamics and metabolism in human forearm. *Diabetes* 39, 490-500

31. Rattigan, S., Clark, M. G., and Barrett, E. J. (1997) Hemodynamic actions of insulin in rat skeletal muscle: evidence for capillary recruitment. *Diabetes* 46, 1381-1388

32. Chaudhuri, A., Kanjwal, Y., Mohanty, P., Rao, S., Sung, B. H., Wilson, M. F., and Dandona, P. (1999) Insulin-induced vasodilatation of internal carotid artery. *Metabolism: Clinical and Experimental* 48, 1470-1473

33. Ueda, S., Petrie, J. R., Cleland, S. J., Elliott, H. L., and Connell, J. M. (1998) The vasodilating effect of insulin is dependent on local glucose uptake: a double blind, placebo-controlled study. *Journal of Clinical Endocrinology and Metabolism* 83, 2126-2131

34. Dawson, D., Vincent, M. A., Barrett, E. J., Kaul, S., Clark, A., Leong-Poi, H., and Lindner, J. R. (2002) Vascular recruitment in skeletal muscle during exercise and hyperinsulinemia assessed by contrast ultrasound. *American Journal of Physiology: Endocrinology and Metabolism* 282, E714-720

35. Clark, A. D., Barrett, E. J., Rattigan, S., Wallis, M. G., and Clark, M. G. (2001) Insulin stimulates laser Doppler signal by rat muscle in vivo consistent with nutritive flow recruitment. *Clinical Science* 100, 283-290

36. Vincent, M. A., Dawson, D., Clark, A. D., Lindner, J. R., Rattigan, S., Clark, M. G., and Barrett, E. J. (2002) Skeletal muscle microvascular recruitment by physiological hyperinsulinemia precedes increases in total blood flow. *Diabetes* 51, 42-48

37. Serne, E. H., Stehouwer, C. D., ter Maaten, J. C., ter Wee, P. M., Rauwerda, J. A., Donker, A. J., and Gans, R. O. (1999) Microvascular Function Relates to Insulin Sensitivity and Blood Pressure in Normal Subjects. *Circulation* 99, 896-902

38. Pappenheimer, J. R. (1941) Vasoconstrictor nerves and oxygen consumption in the isolated perfused hindlimb muscles of the dog. *Journal of Physiology* 99, 182-200

39. Zweifach, B. W., and Metz, D. B. (1955) Selective distribution of blood through the terminal vascular bed of mesenteric structures and skeletal muscle. *Angiology* 6, 282-289

40. Barlow, T. E., Haigh, A. L., and Walder, D. N. (1961) Evidence for two vascular pathways in skeletal muscle. *Clinical Science* 20, 367-385

41. Newman, J. M., Steen, J. T., and Clark, M. G. (1997) Vessels supplying septa and tendons as functional shunts in perfused rat hindlimb. *Microvascular Research* 54, 49-57

42. Clerk, L. H., Smith, M. E., Rattigan, S., and Clark, M. G. (2000) Increased chylomicron triglyceride hydrolysis by connective tissue flow in perfused rat

hindlimb. Implications for lipid storage. *Journal of Lipid Research* 41, 329-335

43. Clark, M. G., Rattigan, S., Newman, J. M., and Eldershaw, T. P. (1998) Vascular control of nutrient delivery by flow redistribution within muscle: implications for exercise and post-exercise muscle metabolism. *International Journal of Sports Medicine* 19, 391-400

44. Clark, M. G., Rattigan, S., Clerk, L. H., Vincent, M. A., Clark, A. D., Youd, J. M., and Newman, J. M. (2000) Nutritive and non-nutritive blood flow: rest and exercise. *Acta Physiologica Scandinvica* 168, 519-530

45. Rattigan, S., Dora, K. A., Colquhoun, E. Q., and Clark, M. G. (1993) Serotonin-mediated acute insulin resistance in the perfused rat hindlimb but not in incubated muscle: a role for the vascular system. *Life Sciences* 53, 1545-1555

46. Baron, A. D. (1996) The coupling of glucose metabolism and perfusion in human skeletal muscle - The potential role of endothelium-derived nitric oxide. *Diabetes* 45, S105-S109

47. Feldman, R. D., and Bierbrier, G. S. (1993) Insulin-mediated vasodilation: impairment with increased blood pressure and body mass. *Lancet* 342, 707-709

48. Baron, A. D., Laakso, M., Brechtel, G., and Edelman, S. V. (1991) Mechanism of insulin resistance in insulin-dependent diabetes mellitus: a major role for reduced skeletal muscle blood flow. *Journal of Clinical Endocrinology and Metabolism* 73, 637-643

49. Lillioja, S., Young, A. A., Culter, C. L., Ivy, J. L., Abbott, W. G., Zawadzki, J. K., Yki-Jarvinen, H., Christin, L., and Secomb, T. W. (1987) Skeletal muscle capillary density and fiber type are possible determinants of in vivo insulin resistance in man. *Journal of Clinical Investigation* 80, 415-424

50. Ohashi, T., Narimiya, M., Someya, Y., Kubokura, T., Kaburagi, M., Tajima, N., Ikeda, Y., and Isogai, Y. (1994) Effects of blood flow on glucose utilization and insulin sensitivity in rat skeletal muscle. *Jikeikai Medical Journal* 41, 99-104

51. Avogaro, A., Piarulli, F., Valerio, A., Miola, M., Calveri, M., Pavan, P., Vicini, P., Cobelli, C., Tiengo, A., Calo, L., and DelPrato, S. (1997) Forearm nitric oxide balance, vascular relaxation and glucose metabolism in NIDDM patients. *Diabetes* 46, 1040-1046

52. Baron, A. D., Steinberg, H., Brechtel, G., and Johnson, A. (1994) Skeletal muscle blood flow independently modulates insulin-mediated glucose uptake. *American Journal of Physiology* 266, E248-E253

53. Scherrer, U., Randin, D., Vollenweider, P., Vollenweider, L., and Nicod, P. (1994) Nitric oxide release accounts for insulin's vascular effects in humans. *Journal of Clinical Investigation* 94, 2511-2515

54. Laine, H., Yki-Jarvinen, H., Kirvela, O., Tolvanen, T., Raitakari, M., Solin, O., Haaparanta, M., Knuuti, J., and Nuutila, P. (1998) Insulin resistance of glucose uptake in skeletal muscle cannot be ameliorated by enhancing endothelium-dependent blood flow in obesity. *Journal of Clinical Investigation* 101, 1156-1162

55. Pendergrass, M., Fazioni, E., Collins, D., and Defronzo, R. A. (1995) Forearm blood flow is not a primary regulator of muscle glucose uptake. *Diabetes* 44 Suppl, 196A

56. Nuutila, P., Raitakari, M., Laine, H., Ruotsalainen, U., Kirvela, O., Knuuti, J., Teras, M., Sipila, H., Haaparanta, M., and Yki-Jarvinen, H. (1995) Insulin sensitivity of glucose uptake, blood flow and volume in IDDM as measured with 18FDG, H215O and C15O and positron emission tomography (PET). *Diabetes* 44 Suppl, 199A

57. Hernandez, M. A., and Jensen, M. D. (1995) Contribution of blood flow to leg glucose uptake during a mixed meal. *Diabetes* 44, 1165-1169

58. Taddei, S., and Salvetti, A. (1997) Insulin and vascular reactivity. *Nutrition, Metabolism, and Cardiovascular Diseases* 7, 117-123

59. Baron, A. D., Tarshoby, M., Hook, G., Lazaridis, E. N., Cronin, J., Johnson, A., and Steinberg, H. O. (2000) Interaction between insulin sensitivity and muscle perfusion on glucose uptake in human skeletal muscle: evidence for capillary recruitment. *Diabetes* 49, 768-774

60. Shankar, R., Zhu, J. S., Ladd, B., Henry, D., Shen, H. Q., and Baron, A. D. (1998) Central nervous system nitric oxide synthase activity regulates insulin secretion and insulin action. *Journal of Clinical Investigation* 102, 1403-1412

61. Baron, A. D., and Clark, M. G. (1997) Role of blood flow in the regulation of muscle glucose uptake. *Annual Review of Nutrition* 17:487-99, 487-499

62. Steinberg, H. O., Brechtel, G., Johnson, A., Sunblad, K., and Baron, A. D. (1995) Insulin mediated vasodilation is a determinant of in vivo insulin mediated glucose uptake. *Diabetes* 44 Suppl, 56A

63. Utriainen, T., Nuutila, P., Takala, T., Vicini, P., Ruotsalainen, U., Ronnemaa, T., Tolvanen, T., Raitakari, M., Haaparanta, M., Kirvela, O., Cobelli, C., and Yki-Jarvinen, H. (1997) Intact insulin stimulation of skeletal muscle blood flow, its heterogeneity and redistribution, but not of glucose uptake in non-insulin-dependent diabetes mellitus. *Journal of Clinical Investigation* 100, 777-785

64. Bradley, S. J., Kingwell, B. A., and McConell, G. K. (1999) Nitric oxide synthase inhibition reduces leg glucose uptake but not blood flow during dynamic exercise in humans. *Diabetes* 48, 1815-1821

65. Marette, A., Richardson, J. M., Ramlal, T., Balon, T. W., Vranic, M., Pessin, J. E., and Klip, A. (1992) Abundance, localization and insulin-induced translocation of glucose transporters in red and white muscle. *American Journal of Physiology* 263, C443-C452

66. Laakso, M., Edelman, S. V., Olefsky, J. M., Brechtel, G., Wallace, P., and Baron, A. D. (1990) Kinetics of in vivo muscle insulin-mediated glucose uptake in human obesity. *Diabetes* 39, 965-974

67. Cleland, S. J., Petrie, J. R., Ueda, S., Elliott, H. L., and Connell, J. M. (1999) Insulin-mediated vasodilation and glucose uptake are functionally linked in humans. *Hypertension* 33, 554-558

68. Friedman, J. E., Dudek, R. W., Whitehead, D. S., Downes, D. L., Frisell, W. R., Caro, J. F., and Dohm, G. L. (1991) Immunolocalization of glucose transporter GLUT4 within human skeletal muscle. *Diabetes* 40, 150-154

69. Petrie, J. R., Cleland, S. J., Ueda, S., Elliott, H. L., and Connell, J. M. (1999) Insulin-mediated vasodilation and forearm plethysmography: physiology not pharmacology. *Diabetologia* 42, 1376

70. Vollenweider, P., Tappy, L., Randin, D., Schneiter, P., Jequier, E., Nicod, P., and Scherrer, U. (1993) Differential effects of hyperinsulinemia and carbohydrate metabolism on sympathetic nerve activity and muscle blood flow in humans. *Journal of Clinical Investigation* 92, 147-154

71. Meneilly, G. S., Battistini, B., and Floras, J. S. (2001) Contrasting effects of L-arginine on insulin-mediated blood flow and glucose disposal in the elderly. *Metabolism: Clinical and Experimental* 50, 194-199

72. Vincent, M. A., Clerk, L. H., Lindner, J. R., Klibanov, A. L., Clark, M. G., Rattigan, S., and Barrett, E. J. (2004) Microvascular recruitment is an early insulin effect that regulates skeletal muscle glucose uptake in vivo. *Diabetes* 53, 1418-1423

73. Zhang, L., Vincent, M. A., Richards, S. M., Clerk, L. H., Rattigan, S., Clark, M. G., and Barrett, E. J. (2004) Insulin sensitivity of muscle capillary recruitment in vivo. *Diabetes* 53, 447-453

74. Bak, J. F. (1994) Insulin receptor function and glycogen synthase activity in human skeletal muscle. Physiology and pathophysiology. *Danish Medical Bulletin* 41, 179-192

75. Vehkavaara, S., Makimattila, S., Schlenzka, A., Vakkilainen, J., Westerbacka, J., and Yki-Jarvinen, H. (2000) Insulin therapy improves endothelial function in type 2 diabetes. *Arteriosclerosis, Thrombosis and Vascular Biology* 20, 545-550

76. Pieper, G. M., Meier, D. A., and Hager, S. R. (1995) Endothelial dysfunction in a model of hyperglycemia and hyperinsulinemia. *American Journal of Physiology* 269, H845-H850

77. Hsueh, W. A., and Anderson, P. W. (1992) Hypertension, the endothelial cell, and the vascular complications of diabetes mellitus. *Hypertension* 20, 253-263

78. Baumgartner-Parzer, S. M., and Waldhausl, W. K. (2001) The endothelium as a metabolic and endocrine organ: its relation with insulin resistance. *Experimental and Clinical Endocrinology and Diabetes* 109 Suppl 2:S166-79., S166-S179

79. Jorneskog, G., Kalani, M., Kuhl, J., Bavenholm, P., Katz, A., Allerstrand, G., Alvarsson, M., Efendic, S., Ostenson, C. G., Pernow, J., Wahren, J., and Brismar, K. (2005) Early microvascular dysfunction in healthy normal-weight males with heredity for type 2 diabetes. *Diabetes Care* 28, 1495-1497

80. McVeigh, G. E., Brennan, G. M., Johnston, G. D., McDermott, B. J., McGrath, L. T., HenryWR., Andrews, J. W., and Hayes, J. R. (1992) Impaired endothelium-dependent and independent vasodilation in patients with type 2 (non-insulin-dependent) diabetes mellitus. *Diabetologia* 35, 771-776

81. Watts, G. F., O'Brien, S. F., Silvester, W., and Millar, J. A. (1996) Impaired endothelium-dependent and independent dilatation of forearm resistance arteries in men with diet-treated non-insulin-dependent diabetes: role of dyslipidaemia. *Clinical Science* 91, 567-573

82. Julius, S., Gudbrandsson, T., Jamerson, K., Shahab, S. T., and Andersson, O. (1991) The hemodynamic link between insulin resistance and hypertension. *Journal of Hypertension* 9, 983-986

83. Cleland, S. J., Petrie, J. R., Small, M., Elliott, H. L., and Connell, J. M. (2000) Insulin action is associated with endothelial function in hypertension and type 2 diabetes. *Hypertension* 35, 507-511

84. Mather, K. J., Verma, S., and Anderson, T. J. (2001) Improved endothelial function with metformin in type 2 diabetes mellitus. *Journal of the American College of Cardiology* 37, 1344-1350

85. Natali, A., Baldeweg, S., Toschi, E., Capaldo, B., Barbaro, D., Gastaldelli, A., Yudkin, J. S., and Ferrannini, E. (2004) Vascular effects of improving metabolic control with metformin or rosiglitazone in type 2 diabetes. *Diabetes Care* 27, 1349-1357

86. Laakso, M., Edelman, S. V., Brechtel, G., and Baron, A. D. (1992) Impaired insulin-mediated skeletal muscle blood flow in patients with NIDDM. *Diabetes* 41, 1076-1083

87. Misurski, D. A., Wu, S. Q., McNeill, J. R., Wilson, T. W., and Gopalakrishnan, V. (2001) Insulin-induced biphasic responses in rat mesenteric vascular bed: role of endothelin. *Hypertension* 37, 1298-1302

88. Eringa, E. C., Stehouwer, C. D., Merlijn, T., Westerhof, N., and Sipkema, P. (2002) Physiological concentrations of insulin induce endothelin-mediated vasoconstriction during inhibition of NOS or PI3-kinase in skeletal muscle arterioles. *Cardiovascular Research* 56, 464-471

89. Verma, S., Yao, L., Stewart, D. J., Dumont, A. S., Anderson, T. J., and McNeill, J. H. (2001) Endothelin antagonism uncovers Insulin-mediated vasorelaxation in vitro and in vivo. *Hypertension* 37, 328-333

90. Cardillo, C., Nambi, S. S., Kilcoyne, C. M., Choucair, W. K., Katz, A., Quon, M. J., and Panza, J. A. (1999) Insulin stimulates both endothelin and nitric oxide activity in the human forearm. *Circulation* 100, 820-825

91. Ferri, C., Bellini, C., Desideri, G., De Mattia, G., and Santucci, A. (1996) Endogenous insulin modulates circulating endothelin-1 concentrations in humans. *Diabetes Care* 19, 504-506

92. Steinberg, H. O., Brechtel, G., Johnson, A., Fineberg, N., and Baron, A. D. (1994) Insulin-mediated skeletal muscle vasodilation is nitric oxide dependent. A novel action of insulin to increase nitric oxide release. *Journal of Clinical Investigation* 94, 1172-1179

93. Collier, J., and Vallance, P. (1991) Physiological importance of nitric oxide. An endogenous nitrovasodilator. *British Medical Journal* 302, 1289-1290

94. Palmer, R. M., Ashton, D. S., and Moncada, S. (1988) Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature* 333, 664-665

95. Palmer, R. M., Rees, D. D., Ashton, D. S., and Moncada, S. (1988) L-arginine is the physiological precursor for the formation of nitric oxide in endothelium-dependent relaxation. *Biochemical and Biophysical Research Communications* 153, 1251-1256

96. Gardiner, S. M., Compton, A. M., Bennett, T., Palmer, R. M., and Moncada, S. (1990) Control of regional blood flow by endothelium-derived nitric oxide. *Hypertension* 15, 486-492

97. Moncada, S., Radomski, M. W., and Palmer, R. M. (1988) Endothelium-derived relaxing factor: Identification as nitric oxide and role in the control of vascular tone and platelet function. *Biochemical Pharmacology* 37, 2495-2501

98. Ignarro, L. J., Buga, G. M., Wood, K. S., Byrns, R. E., and Chaudhuri, G. (1987) Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proceedings of the National Academy of Sciences of the United States of America* 84, 9265-9269

99. Persson, M. G., Gustafsson, L. E., Wiklund, N. P., Hedqvist, P., and Moncada, S. (1990) Endogenous nitric oxide as a modulator of rabbit skeletal muscle micro- circulation in vivo. *British Journal of Pharmacology* 100, 463-466

100. Braughler, J. M. (1983) Soluble guanylate cyclase activation by nitric oxide and its reversal. *Biochemical Pharmacology* 32, 811-818

101. Friebe, A., and Koesling, D. (2003) Regulation of nitric oxide-sensitive guanylyl cyclase. *Circulation Research* 93, 96-105

102. Pohl, U., and Lamontagne, D. (1991) Impaired tissue perfusion after inhibition of endothelium-derived nitric oxide. In *Endothelial Mechanisms of Vasomotor Control* (Drexler, H., Zeiher, A. M., Bassenge, E., and Just, H., eds) pp. 97-105, Steinkopff

103. Petrie, J. R., Ueda, S., Webb, D. J., Elliott, H. L., and Connell, J. M. (1996) Endothelial nitric oxide production and insulin sensitivity. A physiological link with implications for pathogenesis of cardiovascular disease. *Circulation* 93, 1331-1333

104. Schiffrin, E. L. (1994) The endothelium and control of blood vessel function in health and disease. *Clinical Investigations in Medicine* 17, 602-620

105. Baron, A. D. (2002) Insulin resistance and vascular function. *Journal of Diabetes and its Complications* 16, 92-102

106. Mather, K., Anderson, T. J., and Verma, S. (2001) Insulin action in the vasculature: physiology and pathophysiology. *Journal of Vascular Reseach* 38, 415-422

107. Steinberg, H. O., Tarshoby, M., Monestel, R., Hook, G., Cronin, J., Johnson, A., Bayazeed, B., and Baron, A. D. (1997) Elevated circulating free fatty acid levels impair endothelium-dependent vasodilation. *Journal of Clinical Investigation* 100, 1230-1239

108. Paradisi, G., Steinberg, H. O., Shepard, M. K., Hook, G., and Baron, A. D. (2003) Troglitazone therapy improves endothelial function to near normal levels in women with polycystic ovary syndrome. *Journal of Clinical Endocrinology and Metabolism* 88, 576-580

109. Lind, L., Fugmann, A., Millgard, J., Berne, C., and Lithell, H. (2002) Insulin-mediated vasodilatation, but not glucose uptake or endothelium-mediated vasodilatation, is enhanced in young females compared with males. *Clinical Science* 102, 241-246

110. Beckman, J. A., Goldfine, A. B., Gordon, M. B., Garrett, L. A., and Creager, M. A. (2002) Inhibition of protein kinase Cbeta prevents impaired endothelium-dependent vasodilation caused by hyperglycemia in humans. *Circulation Research* 90, 107-111

111. Steinberg, H. O., Bayazeed, B., Hook, G., Johnson, A., Cronin, J., and Baron, A. D. (1997) Endothelial dysfuncion is associated with cholesterol levels in the high normal range in humans. *Circulation* 96, 3287-3293

112. Steinberg, H. O., Chaker, H., Leaming, R., Johnson, A., Brechtel, G., and Baron, A. D. (1996) Obesity/insulin resistance is associated with endothelial dysfunction. Implications for the syndrome of insulin resistance. *Journal of Clinical Investigation* 97, 2601-2610

113. Baron, A. D., Laakso, M., Brechtel, G., and Edelman, S. V. (1991) Reduced capacity and affinity of skeletal muscle for insulin- mediated glucose uptake in noninsulin-dependent diabetic subjects. *Journal of Clinical Investigation* 87, 1186-1194

114. Zeng, G. Y., and Quon, M. J. (1996) Insulin-stimulated production of nitric oxide is inhibited by wortmannin - Direct measurement in vascular endothelial cells. *Journal of Clinical Investigation* 98, 894-898

115. Kahn, N. N., Acharya, K., Bhattacharya, S., Acharya, R., Mazumder, S., Bauman, W. A., and Sinha, A. K. (2000) Nitric oxide: the "second messenger" of insulin. *IUBMB Life* 49, 441-450

116. Chen, Y. L., and Messina, E. J. (1996) Dilation of isolated skeletal muscle arterioles by insulin is endothelium dependent and nitric oxide mediated. *American Journal of Physiology* 270, H2120-H2124

117. Vincent, M. A., Barrett, E. J., Lindner, J. R., Clark, M. G., and Rattigan, S. (2003) Inhibiting NOS blocks microvascular recruitment and blunts muscle glucose uptake in response to insulin. *American Journal of Physiology: Endocrinology and Metabolism* 285, E123-E129

118. Mahajan, H., Richards, S. M., Rattigan, S., and Clark, M. G. (2005) Local infusion of an inhibitor of Ca^{2+} dependent K^{+} channels (TEA), but not L-NAME blocks insulin-mediated capillary recruitment and glucose uptake in muscle *in vivo*. In 65th Scientific Sessions: ADA Annual Meeting, American Diabetes Association, San Diego

119. Roy, D., Perreault, M., and Marette, A. (1998) Insulin stimulation of glucose uptake in skeletal muscles and adipose tissues in vivo is NO dependent. *American Journal of Physiology* 274, E692-E699

120. Sarabi, M., Lind, L., Millgard, J., Hanni, A., Hagg, A., Berne, C., and Lithell, H. (1999) Local vasodilatation with metacholine, but not with nitroprusside, increases forearm glucose uptake. *Physiological Research* 48, 291-295

121. Mahajan, H., Richards, S. M., Rattigan, S., and Clark, M. G. (2004) Local methacholine but not bradykinin potentiates insulin-mediated glucose uptake in muscle in vivo by augmenting capillary recruitment. *Diabetologia* 47, 2226-2234

122. Natali, A., Quinones, G. A., Pecori, N., Sanna, G., Toschi, E., and Ferrannini, E. (1998) Vasodilation with sodium nitroprusside does not improve insulin action in essential hypertension. *Hypertension* 31, 632-636

123. Natali, A., Bonadonna, R., Santoro, D., Galvan, A. Q., Baldi, S., Frascerra, S., Palombo, C., Ghione, S., and Ferrannini, E. (1994) Insulin resistance and vasodilation in essential hypertension. Studies with adenosine. *Journal of Clinical Investigation* 94, 1570-1576

124. Nuutila, P., Raitakari, M., Laine, H., Kirvela, O., Takala, T., Utriainen, T., Makimattila, S., Pitkanen, O. P., Ruotsalainen, U., Iida, H., Knuuti, J., and Yki-Jarvinen, H. (1996) Role of blood flow in regulating insulin-stimulated glucose uptake in humans. Studies using bradykinin, [15O]water, and [18F]fluoro-deoxy- glucose and positron emission tomography. *Journal of Clinical Investigation* 97, 1741-1747

125. Williams, S. B., Cusco, J. A., Roddy, M. A., Johnstone, M. T., and Creager, M. A. (1996) Impaired nitric oxide-mediated vasodilation in patients with non-insulin-dependent diabetes mellitus. *Journal of the American College of Cardiology* 27, 567-574

126. Bagi, Z., and Koller, A. (2003) Lack of nitric oxide mediation of flow-dependent arteriolar dilation in type I diabetes is restored by sepiapterin. *Journal of Vascular Reseach* 40, 47-57

127. Salt, I. P., Morrow, V. A., Brandie, F. M., Connell, J. M., and Petrie, J. R. (2003) High Glucose Inhibits Insulin-stimulated Nitric Oxide Production without Reducing Endothelial Nitric-oxide Synthase Ser1177 Phosphorylation in Human Aortic Endothelial Cells. *Journal of Biological Chemistry* 278, 18791-18797

128. Ding, Q. F., Hayashi, T., Packiasamy, A. R., Miyazaki, A., Fukatsu, A., Shiraishi, H., Nomura, T., and Iguchi, A. (2004) The effect of high glucose on NO and O2- through endothelial GTPCH1 and NADPH oxidase. *Life Sciences* 75, 3185-3194

129. Mather, K. J., Lteif, A., Steinberg, H. O., and Baron, A. D. (2004) Interactions between endothelin and nitric oxide in the regulation of vascular tone in obesity and diabetes. *Diabetes* 53, 2060-2066

130. Newman, J. M., Dora, K. A., Rattigan, S., Edwards, S. J., Colquhoun, E. Q., and Clark, M. G. (1996) Norepinephrine and serotonin vasoconstriction in rat hindlimb control different vascular flow routes. *American Journal of Physiology* 270, E689-E699

131. Lüscher, T. F., and Barton, M. (2000) Endothelins and endothelin receptor antagonists: therapeutic considerations for a novel class of cardiovascular drugs. *Circulation* 102, 2434-2440

132. de Nucci, G., Thomas, R., D'Orleans-Juste, P., Antunes, E., Walder, C., Warner, T. D., and Vane, J. R. (1988) Pressor effects of circulating endothelin are limited by its removal in the pulmonary circulation and by the release of prostacyclin and endothelium-derived relaxing factor. *Proceedings of the National Academy of Sciences of the United States of America* 85, 9797-9800

133. Hergenroder, S., Munter, K., and Kirchengast, M. (1998) Effects of endothelin and endothelin receptor antagonism in arteriolar and venolar microcirculation. *VASA* 27, 216-219

134. Aaker, A., and Laughlin, M. H. (2002) Diaphragm arterioles are less responsive to a1-adrenergic constriction than gastrocnemius arterioles. *Journal of Applied Physiology* 92, 1808-1816

135. Pollock, D. M., Keith, T. L., and Highsmith, R. F. (1995) Endothelin receptors and calcium signaling. *FASEB Journal* 9, 1196-1204

136. D'Orleans-Juste, P., Claing, A., Warner, T. D., Yano, M., and Telemaque, S. (1993) Characterization of receptors for endothelins in the perfused arterial and venous mesenteric vasculatures of the rat. *British Journal of Pharmacology* 110, 687-692

137. Allcock, G. H., Warner, T. D., and Vane, J. R. (1995) Roles of endothelin receptors in the regional and systemic vascular responses to ET-1 in the anaesthetized ganglion-blocked rat: use of selective antagonists. *British Journal of Pharmacology* 116, 2482-2486

138. Piuhola, J., Makinen, M., Szokodi, I., and Ruskoaho, H. (2003) Dual role of endothelin-1 via ET_A and ET_B receptors in regulation of cardiac contractile function in mice. *American Journal of Physiology: Heart and Circulatory Physiology* 285, H112-H118

139. Porteri, E., Rizzoni, D., Guelfi, D., De Ciuceis, C., Castellano, M., Bettoni, G., Tiberio, G. A., Giulini, S. M., Sleiman, I., and Agabiti-rosei, E. (2002) Role of ET_A receptors in the vasoconstriction induced by endothelin-1 in subcutaneous small arteries of normotensive subjects and hypertensive patients. *Blood Pressure* 11, 6-12

140. Filep, J. G., Foldes-Filep, E., Rousseau, A., Sirois, P., and Fournier, A. (1993) Vascular responses to endothelin-1 following inhibition of nitric oxide synthesis in the conscious rat. *British Journal of Pharmacology* 110, 1213-1221

141. Roberts-Thomson, P., McRitchie, R. J., and Chalmers, J. P. (1996) Endothelin-1 produces heterogeneous regional haemodynamic effects in conscious rabbits. *Clinical Experiments in Hypertension* 18, 145-169

142. Warner, T. D., Mitchell, J. A., de Nucci, G., and Vane, J. R. (1989) Endothelin-1 and endothelin-3 release EDRF from isolated perfused arterial vessels of the rat and rabbit. *Journal of Cardiovascular Pharmacology* 13, S85-S88

143. Uchida, Y., Saotome, M., Nomura, A., Ninomiya, H., Ohse, H., Hirata, F., and Hasegawa, S. (1991) Endothelin-1-induced relaxation of guinea pig trachealis muscles. *Journal of Cardiovascular Pharmacology* 17 [suppl. 7], s212

144. Herrera, M., and Garvin, J. L. (2004) Endothelin stimulates endothelial nitric oxide synthase expression in the thick ascending limb. *American Journal of Renal Physiology*

145. Bentzer, P., Holbeck, S., and Grande, P.-O. (2002) Endothelin-1 reduces microvascular fluid permeability through secondary release of prostacyclin in cat skeletal muscle. *Microvascular research* 63, 50-60

146. Wagner, O. F., Christ, G., Wojta, J., Vierhapper, H., Parzer, S., Nowotny, P. J., Schneider, B., Waldhausl, W., and Binder, B. R. (1992) Polar secretion of endothelin-1 by cultured endothelial cells. *Journal of Biological Chemistry* 267, 16066-16068

147. Wu-Wong, J. R., Chiou, W. J., Naugles, K. E. J., and Opgenorth, T. J. (2003) Endothelin receptor antagonists exhibit diminishing potency following incubation with agonist. *Life Sciences* 54, 1727-1734

148. Kiowski, W., Lüscher, T. F., Linder, L., and Buhler, F. R. (1991) Endothelin-1-induced vasoconstriction in humans. *Circulation* 83, 469-475

149. Masaki, T. (1995) Possible role of endothelin in endothelial regulation of vascular tone. *Annual Reviews in Pharmacology and Toxicology* 35, 235-255

150. Merkus, D., Houweling, B., Mirza, A., Boomsma, F., van den Meiracker, A. H., and Duncker, D. J. (2003) Contribution of endothelin and its receptors to the regulation of vascular tone during exercise is different in the systemic, coronary and pulmonary circulation. *Cardiovascular Research* 59, 745-754

151. da Silva, A. A., Kuo, J. J., Tallam, L. S., and Hall, J. E. (2004) Role of endothelin-1 in blood pressure regulation in a rat model of visceral obesity and hypertension. *Hypertension* 43, 1-5

152. Ekelund, U., Adner, M., Edvinsson, L., and Mellander, S. (1995) Effects of the combined ET_A and ET_B receptor antagonist PD145065 on arteries, arterioles, and veins in the cat hindlimb. *Journal of Cardiovascular Pharmacology* 26 [suppl. 3], s211-s213

153. Cardillo, C., Kilcoyne, C. M., Waclawiw, M., Cannon, R. O. I., and Panza, J. A. (1999) Role of endothelin in the increased vascular tone of patients with essential hypertension. *Hypertension* 33, 753-758

154. Ahlborg, G., Weitzberg, E., and Lundberg, J. M. (1995) Circulating endothelin-1 reduces splanchnic and renal blood flow and splanchnic glucose production in humans. *Journal of Applied Physiology* 79, 141-145

155. King-VanVlack, C. E., Mewburn, J. D., and Chapler, C. K. (1999) Receptor-mediated actions of endothelin-1 in canine small intestine. *American Journal of Physiology: Gastrointestinal and Liver Physiology* 276, G1131-G1136

156. Ahn, Y. M., Gajdusek, C., London, S., Moon, C. T., Oh, C. W., and Mayberg, M. R. (2002) Sustained arterial narrowing after prolonged exposure to perivascular endothelin. *Neurosurgery* 50, 843-848

157. Iglarz, M., and Schiffrin, E. L. (2003) Role of endothelin-1 in hypertension. *Current Hypertension Reports* 5, 144-148

158. Galie, N., Manes, A., and Branzi, A. (2004) The endothelin system in pulmonary arterial hypertension. *Cardiovascular Research* 61, 227-237

159. Hopfner, R. L., and Gopalakrishnan, V. (1999) Endothelin: emerging role in diabetic vascular complications. *Diabetologia* 42, 1383-1394

160. Ferri, C., Carlomagno, A., Coassin, S., Baldoncini, R., Faldetta, M. R. C., Laurenti, O., Properzi, G., Santucci, A., and De Mattia, G. (1995) Circulating endothelin-1 levels increase during euglycemic hyperinsulinemic clamp in lean NIDDM men. *Diabetes Care* 18, 226-233

161. Kiowski, W., Sutsch, G., Hunziker, P., Muller, P., Kim, J., Oechslin, E., Schmitt, R., Jones, R., and Bertel, O. (1995) Evidence for endothelin-1-mediated vasoconstriction in severe chronic heart failure. *Lancet* 346, 732-736

162. Frelin, C., and Guedin, D. (1994) Why are circulating concentrations of endothelin-1 so low? *Cardiovascular Research* 28, 1613-1622

163. Kjekshus, H., Smiseth, O. A., Klinge, R., Oie, E., Hystad, M. E., and Attramadal, H. (2000) Regulation of ET: pulmonary release of ET contributes to increased plasma ET levels and vasoconstriction in CHF. *American Journal of Physiology: Heart and Circulatory Physiology* 278, H1299-1310

164. Ruschitzka, F., Shaw, S., Gygi, D., Noll, G., Barton, M., and Luscher, T. F. (1999) Endothelial dysfunction in acute renal failure: role of circulating and tissue endothelin-1. *Journal of the American Society of Nephrology* 10, 953-962

165. Palma, B. D., Gabriel, A., Jr., Bignotto, M., and Tufik, S. (2002) Paradoxical sleep deprivation increases plasma endothelin levels. *Brazilian Journal of Medical Biological Research* 35, 75-79

166. Glowinska, B., Urban, M., Hryniewicz, A., Peczynska, J., Florys, B., and Al-Hwish, M. (2004) Endothelin-1 plasma concentration in children and adolescents with atherogenic risk factors. *Polish Heart Journal* 61, 329-338

167. Minami, S., Yamano, S., Yamamoto, Y., Sasaki, R., Nakashima, T., Takaoka, M., and Hashimoto, T. (2001) Associations of plasma endothelin concentration with carotid atherosclerosis and asymptomatic cerebrovascular lesions in patients with essential hypertension. *Hypertension Research* 24, 663-670

168. Cardillo, C., Campia, U., Iantorno, M., and Panza, J. A. (2004) Enhanced vascular activity of endogenous endothelin-1 in obese hypertensive patients. *Hypertension* 43, 36-40

169. Taddei, S., Virdis, A., Ghiadoni, L., Sudano, I., Notari, M., and Salvetti, A. (1999) Vasoconstriction to endogenous endothelin-1 is increased in the peripheral circulation of patients with essential hypertension. *Circulation* 100, 1680-1683

170. Taddei, S., Virdis, A., Ghiadoni, L., Sudano, I., Magagna, A., and Salvetti, A. (2001) Role of endothelin in the control of peripheral vascular tone in human hypertension. *Heart Failure Reviews* 6, 277-285

171. Hirata, Y., Hayakawa, H., Suzuki, E., Kimura, K., Kikuchi, K., Nagano, T., Hirobe, M., and Omata, M. (1995) Direct measurements of endothelium-derived nitric oxide release by stimulation of endothelin receptors in rat kidney and its alteration in salt-induced hypertension. *Circulation* 91, 1229-1235

172. Kakoki, M., Hirata, Y., Hayakawa, H., Tojo, A., Nagata, D., Suzuki, E., Kimura, K., Goto, A., Kikuchi, K., Nagano, T., and Omata, M. (1999) Effects of hypertension, diabetes mellitus, and hypercholesterolemia on endothelin

type B receptor-mediated nitric oxide release from rat kidney. *Circulation* 99, 1242-1248

173. Metsarinne, K., Saijonmaa, O., Yki-Järvinen, H., and Fyhrquist, F. (1994) Insulin increases the release of endothelin in endothelial cell cultures in vitro but not in vivo. *Metabolism* 43, 878-882

174. Ottosson-Seeberger, A., Lundberg, J. M., Alvestrand, A., and Ahlborg, G. (1997) Exogenous endothelin-1 causes peripheral insulin resistance in healthy humans. *Acta Physiologica Scandinvica* 161, 211-220

175. Balsiger, B., Rickenbacher, A., Boden, P. J., Biecker, E., Tsui, J., Dashwood, M. R., Reichen, J., and Shaw, S. G. (2002) Endothelin A-receptor blockade in experimental diabetes improves glucose balance and gastrointestinal function. *Clinical Science* 103 [suppl 48], 430s-433s

176. Miller, A. W., Tulbert, C., Puskar, M., and Busija, D. W. (2002) Enhanced endothelin activity prevents vasodilation to insulin in insulin resistance. *Hypertension* 40, 78-82

177. Wilkes, J. J., Hevener, A., and Olefsky, J. (2003) Chronic endothelin-1 treatment leads to insulin resistance in vivo. *Diabetes* 52, 1904-1909

178. Mather, K. J., Mirzamohammadi, B., Lteif, A., Steinberg, H. O., and Baron, A. D. (2002) Endothelin contributes to basal vascular tone and endothelial dysfunction in human obesity and type 2 diabetes. *Diabetes* 51, 3517-3523

179. Ahlborg, G., and Lindstrom, J. (2002) Insulin sensitivity and big ET-1 conversion to ET-1 after ET_A- or ET_B-receptor blockade in humans. *Journal of Applied Physiology* 93, 2112-2121

180. Idris, I., Patiag, D., Gray, S., and Donnelly, R. (2001) Tissue- and time-dependent effects of endothelin-1 on insulin-stimulated glucose uptake. *Biochemical Pharmacology* 62, 1705-1708

181. Ishibashi, K.-I., Imamura, T., Sharma, P. M., Huang, J., Ugi, S., and Olefsky, J. M. (2001) Chronic endothelin-1 treatment leads to heterologous desensitization of insulin signaling in 3T3-L1 adipocytes. *Journal of Clinical Investigation* 107, 1193-1202

182. Ruderman, N. B., Houghton, C. R., and Hems, R. (1971) Evaluation of the isolated perfused rat hindquarter for the study of muscle metabolism. *Biochemistry Journal* 124, 639-651

183. Colquhoun, E. Q., Hettiarachchi, M., Ye, J. M., Richter, E. A., Hniat, A. J., Rattigan, S., and Clark, M. G. (1988) Vasopressin and angiotensin II stimulate oxygen uptake in the perfused rat hindlimb. *Life Sciences* 43, 1747-1754

184. Ross, B. D. (1972) *Perfusion techniques in biochemistry:a laboratory manual*, Clarendon Press, Oxford

185. Greene, E. C. (1968) *Anatomy of the rat*, Hafner Publishing Co., U.S.A.

186. Richter, E. A., Ruderman, N. B., Gavras, H., Belur, E. R., and Galbo, H. (1982) Muscle glycogenolysis during exercise: dual control by epinephrine and contractions. *American Journal of Physiology* 242, E25-E32

187. Kraegen, E. W., James, D. E., Jenkins, A. B., and Chisholm, D. J. (1985) Dose-response curves for in vivo insulin sensitivity in individual tissues in rats. *American Journal of Physiology* 248, E353-E362

188. James, D. E., Jenkins, A. B., and Kraegen, E. W. (1985) Heterogeneity of insulin action in individual muscles in vivo: euglycemic clamp studies in rats. *American Journal of Physiology* 248, E567-E574

189. Newman, J. M., Rattigan, S., and Clark, M. G. (2002) Nutritive blood flow improves interstitial glucose and lactate exchange in perfused rat hindlimb. *American Journal of Physiology: Heart and Circulatory Physiology* 283, H186-H192

190. Clark, M. G., Colquhoun, E. Q., Rattigan, S., Dora, K. A., Eldershaw, T. P., Hall, J. L., and Ye, J. (1995) Vascular and endocrine control of muscle metabolism. *American Journal of Physiology* 268, E797-E812

191. Dora, K. A., Rattigan, S., Colquhoun, E. Q., and Clark, M. G. (1994) Aerobic muscle contraction impaired by serotonin-mediated vasoconstriction. *Journal of Applied Physiology* 77, 277-284

192. Kedzierski, R. M., and Yanagisawa, M. (2001) Endothelin system: the double-edged sword in health and disease. *Annual Reviews in Pharmacology and Toxicology* 41, 851-876

193. Maguire, J. J., and Davenport, A. P. (1995) ETA receptor-mediated constrictor responses to endothelin peptides in human blood vessels in vitro. *British Journal of Pharmacology* 115, 191-197

194. Ekelund, U., Albert, U., Edvinsson, L., and Mellander, S. (1993) In-vivo effects of endothelin-1 and ET_A receptor blockade on arterial, venous and capillary functions in skeletal muscle. *Acta Physiologica Scandinvica* 148, 273-283

195. Dahlof, B., Gustafsson, D., Hedner, T., Jern, S., and Hansson, L. (1990) Regional haemodynamic effects of endothelin-1 in rat and man: unexpected adverse reaction. *Journal of Hypertension* 8, 811-817

196. Lund-Johansen, P. (1967) Hemodynamics in early essential hypertension. *Acta Medica Scandinavica* 181 Suppl 482, 8-101

197. McEniery, C. M., Wilkinson, I. B., Jenkins, D. G., and Webb, D. J. (2002) Endogenous endothelin-1 limits exercise-induced vasodilation in hypertensive humans. *Hypertension* 40, 202-206

198. Cardillo, C., Campia, U., Bryant, M. B., and Panza, J. A. (2002) Increased activity of endogenous endothelin in patients with type II diabetes mellitus. *Circulation* 106, 1783-1787

199. Maeda, S., Miyauchi, T., Iemitsu, M., Tanabe, T., Irukayama-Tomobe, Y., Goto, K., Yamaguchi, I., and Matsuda, M. (2002) Involvement of endogenous endothelin-1 in exercise-induced redistribution of tissue blood flow: an endothelin receptor antagonist reduces the redistribution. *Circulation* 106, 2188-2193

200. Tanabe, K., Yamamoto, A., Suzuki, N., Yokoyama, Y., Osada, N., Nakayama, M., Akashi, Y., Seki, A., Samejima, H., Oya, M., Murabayashi, T., Omiya, K., Itoh, H., Miyake, F., and Murayama, M. (2000) Physiological role of endothelin-1 in non-working muscles during exercise in healthy subjects. *Japanese Circulation Journal* 64, 27-31

201. Maeda, S., Miyauchi, T., Sakane, M., Saito, M., Maki, S., Goto, K., and Matsuda, M. (1997) Does endothelin-1 participate in the exercise-induced changes in blood flow distribution of muscles in humans? *Journal of Applied Physiology* 82, 1107-1111

202. Rattigan, S., Dora, K. A., Tong, A. C. Y., and Clark, M. G. (1996) Perfused skeletal muscle contraction and metabolism improved by angiotensin II-mediated vasoconstriction. *American Journal of Physiology: Endocrinology and Metabolism* 271, E96-E103

203. Hasdai, D., Holmes, D. R., Jr., Richardson, D. M., Izhar, U., and Lerman, A. (1998) Insulin and IGF-I attenuate the coronary vasoconstrictor effects of endothelin-1 but not of sarafotoxin 6c. *Cardiovascular Research* 39, 644-650

204. Seo, B., Oemar, B. S., Siebenmann, R., von Segesser, L., and Lüscher, T. F. (1994) Both ET_A and ET_B receptors mediate contraction to endothelin-1 in human blood vessels. *Circulation* 89, 1203-1208

205. Dick, G. M., and Sturek, M. (1996) Effects of a physiological insulin concentration on the endothelin-sensitive Ca2+ store in porcine coronary artery smooth muscle. *Diabetes* 45, 876-880

206. Standley, P. R., Zhang, F., Ram, J. L., Zemel, M. B., and Sowers, J. R. (1991) Insulin attenuates vasopressin-induced calcium transients and a voltage-

dependent calcium response in rat vascular smooth muscle cells. *Journal of Clinical Investigation* 88, 1230-1236

207. Etgen, G. J., Jr, Fryburg, D. A., and Gibbs, E. M. (1997) Nitric oxide stimulates skeletal muscle glucose transport through a calcium/contraction- and phosphatidylinositol-3-kinase-independent pathway. *Diabetes* 46, 1915-1919

208. Hirai, T., Visneski, M. D., Kearns, K. J., Zelis, R., and Musch, T. I. (1994) Effects of NO synthase inhibition on the muscular blood flow response to treadmill exercise in rats. *Journal of Applied Physiology* 77, 1288-1293

209. Pendergrass, M., Fazioni, E., Collins, D., and Defronzo, R. A. (1998) IGF-I increases forearm blood flow without increasing forearm glucose uptake. *American Journal of Physiology* 275, E345-E350

210. Renkin, E. M. (1984) Control of microcirculation and blood-tissue exchange. In *Handbook of Physiology - The Cardiovascular System IV* (Renkin, E. M., Michel, C. C., and Geiger, S. R., eds) pp. 627-687, American Physiological Society Bethesda

211. Young, J. C., and Balon, T. W. (1997) Role of dihydropyridine sensitive calcium channels in glucose transport in skeletal muscle. *Life Sciences* 61, 335-342

212. Kahn, B. B., Shulman, G. I., DeFronzo, R. A., Cushman, S. W., and Rossetti, L. (1991) Normalization of blood glucose in diabetic rats with phlorizin treatment reverses insulin-resistant glucose transport in adipose cells without restoring glucose transporter gene expression. *Journal of Clinical Investigation* 87, 561-570

213. Arakawa, K., Ishihara, T., Oku, A., Nawano, M., Ueta, K., Kitamura, K., Matsumoto, M., and Saito, A. (2001) Improved diabetic syndrome in C57BL/KsJ-db/db mice by oral administration of the Na(+)-glucose cotransporter inhibitor T-1095. *British Journal of Pharmacology* 132, 578-586

214. Rattigan, S., Clark, M. G., and Barrett, E. J. (1999) Acute vasoconstriction-induced insulin resistance in rat muscle in vivo. *Diabetes* 48, 564-569

215. Loike, J. D., Hickman, S., Kuang, K., Xu, M., Cao, L., Vera, J. C., Silverstein, S. C., and Fischbarg, J. (1996) Sodium-glucose cotransporters display sodium- and phlorizin-dependent water permeability. *American Journal of Physiology* 271, C1774-C1779

216. Kasahara, T., and Kasahara, M. (1997) Characterization of rat Glut4 glucose transporter expressed in the yeast Saccharomyces cerevisiae: comparison with Glut1 glucose transporter. *Biochimica et biophysica Acta* 1324, 111-119

217. Berna, N., Arnould, T., Remacle, J., and Michiels, C. (2001) Hypoxia-induced increase in intracellular calcium concentration in endothelial cells: role of the Na(+)-glucose cotransporter. *Journal of Cellular Biochemistry* 84, 115-131

218. Vock, R., Weibel, E. R., Hoppeler, H., Ordway, G., Weber, J. M., and Taylor, C. R. (1996) Design of the oxygen and substrate pathways. V. Structural basis of vascular substrate supply to muscle cells. *Journal of Experimental Biology* 199, 1675-1688

219. Kuchan, M. J., and Frangos, J. A. (1993) Shear stress regulates endothelin-1 release via protein kinase C and cGMP in cultured endothelial cells. *American Journal of Physiology* 264, H150-156

220. Malek, A. M., Greene, A. L., and Izumo, S. (1993) Regulation of endothelin 1 gene by fluid shear stress is transcriptionally mediated and independent of protein kinase C and cAMP. *Proceedings of the National Academy of Sciences of the United States of America* 90, 5999-6003

221. Loesch, A. (2005) Localisation of endothelin-1 and its receptors in vascular tissue as seen at the electron microscopic level. *Current Vascular Pharmacology* 3, 381-392

222. Just, A., Olson, A. J., and Arendshorst, W. J. (2004) Dual constrictor and dilator actions of ET(B) receptors in the rat renal microcirculation: interactions

with ET(A) receptors. *American Journal of Physiology: Renal Physiology* 286, F660-F668

223. Clerk, L. H., Smith, M. E., Rattigan, S., and Clark, M. G. (2003) Nonnutritive flow impairs uptake of fatty acid by white muscles of the perfused rat hindlimb. *American Journal of Physiology: Endocrinology and Metabolism* 284, E611-E617

224. Dora, K. A., Richards, S. M., Rattigan, S., Colquhoun, E. Q., and Clark, M. G. (1992) Serotonin and norepinephrine vasoconstriction in rat hindlimb have different oxygen requirements. *American Journal of Physiology* 262, H698-H703

225. Fortes, Z. B., de Nucci, G., and Garcia-Leme, J. (1989) Effect of endothelin-1 on arterioles and venules in vivo. *Journal of Cardiovascular Pharmacology* 13 [suppl 5], s200-s201

226. Wu-Wong, J. R., Chiou, W. J., Magnuson, S. R., and Opgenorth, T. J. (1994) Endothelin receptor agonists and antagonists exhibit different dissociation characteristics. *Biochimica et biophysica Acta* 1224, 288-294

227. Ekelund, U. (1994) *In vivo* effects of endothelin-2, endothelin-3 and ET_A receptor blockade on arterial, venous and capillary functions in cat skeletal muscle. *Acta Physiologica Scandinvica* 150, 47-56

228. Takamura, M., Parent, R., Cernacek, P., and Lavallee, M. (2000) Influence of dual ET_A/ET_B-receptor blockade on coronary responses to treadmill exercise in dogs. *Journal of Applied Physiology* 89, 2041-2048

229. Wolfard, A., Csaszar, J., Gera, L., Petri, A., Simonka, J. A., Baloghi, A., and Boros, M. (2002) Endothelin-A receptor antagonist treatment improves the periosteal microcirculation after hindlimb ischemia and reperfusion in the rat. *Microcirculation* 9, 471-476

230. Roberts-Thomson, P., McRitchie, R. J., and Chalmers, J. P. (1994) Endothelin-1 causes a biphasic response in systemic vasculature and increases

myocardial contractility in conscious rabbits. *Journal of Cardiovascular Pharmacology* 24, 100-107

231. Mahajan, H., Richards, S. M., Rattigan, S., and Clark, M. G. (2003) T-1032, a cyclic GMP phosphodiesterase-5 inhibitor, acutely blocks physiologic insulin-mediated muscle haemodynamic effects and glucose uptake in vivo. *British Journal of Pharmacology* 140, 1283-1291

232. Serne, E. H., IJzerman, R. G., Gans, R. O., Nijveldt, R., De Vries, G., Evertz, R., Donker, A. J., and Stehouwer, C. D. (2002) Direct evidence for insulin-induced capillary recruitment in skin of healthy subjects during physiological hyperinsulinemia. *Diabetes* 51, 1515-1522

233. Slot, J. W., Moxley, R., Geuze, H. J., and James, D. E. (1990) No evidence for expression of the insulin-regulatable glucose transporter in endothelial cells. *Nature* 346, 369-371

234. Steil, G. M., Ader, M., Moore, D. M., Rebrin, K., and Bergman, R. N. (1996) Transendothelial insulin transport is not saturable in vivo - No evidence for a receptor-mediated process. *Journal of Clinical Investigation* 97, 1497-1503

235. Steil, G. M., Rebrin, K., and Moore, D. M. (1995) Receptor independent transport of insulin across capillary endothelial cells. *Diabetes* 44 Suppl, 84A

236. Hamilton-Wessler, M., Ader, M., Dea, M. K., Moore, D., Loftager, M., Markussen, J., and Bergman, R. N. (2002) Mode of transcapillary transport of insulin and insulin analog NN304 in dog hindlimb: evidence for passive diffusion. *Diabetes* 51, 574-582

237. Yang, Y. J., Hope, I. D., Ader, M., and Bergman, R. N. (1989) Insulin transport across capillaries is rate limiting for insulin action in dogs. *Journal of Clinical Investigation* 84, 1620-1628