Microvascular Dysfunction and the Development of Muscle Insulin Resistance

By

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STATEMENT

The work in the present thesis has exclusively been for the use of a Ph.D. in the area of biomedical research. The data in this thesis has not been used for any other higher degree or graduate diploma in any other university. All experimental and written work is my own, except which has been referenced accordingly and all experimental work abides by the Australian ethical conduct codes regarding animal experimentation.

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ABSTRACT

Defects in microvascular perfusion, which is important for nutrient exchange in skeletal muscle, contribute to insulin resistance associated with obesity and hypertension. One factor that may link this microvascular dysfunction in these conditions is the renin angiotensin system (RAS). The primary objective of this thesis was to address these issues using rat models of obesity and altered RAS activity.

Two different models of insulin resistance were analysed for the involvement of microvascular dysfunction. Rats were fed either a high fat or a high salt diet for 4 weeks to develop insulin resistance. The hyperinsulinaemic, euglycaemic clamp and 1-methyl xanthine techniques were used to investigate insulin sensitivity and microvascular recruitment, respectively. Despite different origins of insulin resistance, both animal models had markedly impaired insulin-mediated microvascular recruitment in muscle.

The contribution of increased angiotensin II (AngII) activity to microvascular dysfunction in both animal models was examined. Hindleg vascular AngII sensitivity (assessed using both the perfused rat hindlimb technique and locally infused AngII *in vivo*) was enhanced in the high salt but not the high fat-fed rat. Thus, although both models developed microvascular insulin resistance, only the high salt-fed rats exhibited increased vascular AngII activity.

There is conflicting evidence as to whether RAS dysregulation also contributes to insulin resistance or type 2 diabetes associated with obesity. The obese Zucker rat (a model of type 2 diabetes) has impaired insulin-mediated microvascular recruitment, and has been reported to have a dysregulated RAS, but represents a later stage of insulin resistance than the high fat-fed rat. To determine whether RAS inhibition enhances insulin sensitivity, high salt-fed and obese Zucker rats were treated with an angiotensin converting enzyme inhibitor (quinapril) for 4 weeks. Inhibition of ACE significantly improved insulin sensitivity and augmented insulin-stimulated microvascular perfusion in high salt-fed rats, but not in obese Zucker rats.

The data from this thesis demonstrate that the origin of microvascular insulin resistance is multifactorial. Dysregulation of the RAS is important in some but not all forms of microvascular insulin resistance. Therefore inhibition of RAS may not always be effective at treating insulin resistance and may only be beneficial during earlier stages of insulin resistance rather than later stages such as type 2 diabetes.

ABBREVIATIONS

1-MU	1-Methylurate
1-MX	1-Methylxanthine
2-DG	^{14}C-2-deoxyglucose
5%F	5% Fat
9%F	9% Fat
ACE	Angiotensin Converting Enzyme
ADaPT	ACE Inhibitor-Based Versus Diuretic-Based Antihypertensive Primary Treatment in Patients with Pre-Diabetes
ARB	Angiotensin Receptor Blocker
AMPK	5' Adenosine Monophosphate-Activated Protein Kinase
AngII	Angiotensin II
Ang-(1-7)	Angiotensin 1-7 Peptide
ANBP-2	The Second Australian National Blood Pressure Study
ANOVA	Analysis Of Variance
AS160	Akt Substrate 160
AT1R	Angiotensin Type I Receptor
AT2R	Angiotensin Type II Receptor
A-V	Arteriovenous
BMI	Body Mass Index
Ca^{2+}	Calcium
CAPPP	Captopril Prevention Project

CEU	Contrast Enhanced Ultrasound
CTRL	Control
DREAM	Diabetes REduction Assessment with Ramipril and Rosiglitazone Medication
eNOS	Endothelial Nitric Oxide Synthase
ET-1	Endothelin-1
FBF	Femoral Artery Blood Flow
FFA	Free Fatty Acid
FVR	Femoral Artery Vascular Resistance
GIR	Glucose Infusion Rate
GLUT-4	Glucose Transporter 4
HGU	Hindleg Glucose Uptake
HOPE	Heart Outcomes Prevention Evaluation Study
HPLC	High Performance Liquid Chromatography
HS	High Salt
HS+Q	Quinapril treated High Salt Rats
INS	Insulin
IRS	Insulin Receptor Substrate
JNK	c-Jun NH2-Terminal Kinase
L-NAME	Nω-Nitro-L-Arginine-Methyl Ester
LZ	Lean Zucker Rat
mmHG	Millimetres of Mercury
NADPH	Nicotinamide Adenine Dinucleotide Phosphate

NEP	Neutralendopeptidase 24-11
NO	Nitric Oxide
OZ	Obese Zucker Rat
OZ+Q	Quinapril treated Obese Zucker rat
PCA	Perchloric Acid
PEP	Prolyl-Endopeptidase
PI3K	Phosphatidylinositol-3-Kinase
R`g	Rate of Glucose Uptake
RAS	Renin Angiotensin System
Ra	Rate of Glucose Appearance
Rd	Rate of Glucose Disappearance
ROS	Reactive Oxygen Species
SAL	Saline
SEM	Standard Error of the Mean
SOLVD	Studies of Left Ventricular Dysfunction
TNFα	Tumour Necrosis Factor Alpha
T2D	Type 2 Diabetes
VALUE	Valsartan Antihypertensive Long-term Use Evaluation Study
VENRIKO	Vascular Endothelial Insulin Receptor Knockout
Vs.	Versus
Wt.	Weight
XO	Xanthine Oxidase
ZDF	Zucker Diabetic Fatty Rat

PREFACE

Some of the data obtained in the present thesis has been presented at scientific meetings and are listed below.

Posters at conferences

American Society for Biochemistry and Molecular Biology. Anaheim, USA. April 2010. Carol T. Bussey, **Dino Premilovac**, Michelle A. Keske, Stephen Rattigan and Stephen M. Richards. Low molecular weight adiponectin infusion does not alter the hemodynamic and metabolic actions of insulin in skeletal muscle in anesthetised rats.

Australian Diabetes Society/Australian Diabetes Educators Association Annual Scientific Meeting. Sydney, NSW, Australia. September, 2010. **Dino Premilovac**, Stephen M. Richards, Stephen Rattigan and Michelle A. Keske. Angiotensin Converting Enzyme Inhibition Does Not Improve Insulin-Mediated Microvascular Perfusion in Muscle or Insulin Sensitivity In The Female Obese Zucker Rat.

71st Scientific Sessions – American Diabetes Association Annual Meeting. San Diego, CA, USA. June 2011. **Dino Premilovac**, Stephen Rattigan, Stephen Richards, Michelle Keske. High Salt Feeding Reduces Insulin Stimulated Glucose Disposal and Impairs Skeletal Muscle Microvascular Recruitment.

71st Scientific Sessions – American Diabetes Association Annual Meeting. San Diego, CA, USA. June 2011. **Dino Premilovac**, Stephen Rattigan, Stephen Richards, Michelle Keske. Failure of Angiotensin Converting Enzyme Inhibition to Reverse Insulin Resistance in Female Obese Zucker Rats.

Experimental Biology. San Diego, CA, USA. April 2012. Stephen M. Richards, **Dino Premilovac**, Huei L.H. Ng, Eloise A. Bradley, Stephen Rattigan and Michelle A. Keske. Moderate increases in dietary fat impair microvascular but not myocyte actions of insulin in skeletal muscle of Sprague Dawley rats.

Oral presentations at conferences

Invited talk at the "International Conference on Clinical Research". San Francisco, CA, USA. July 2011. Michelle A. Keske, **Dino Premilovac**, Huei L.H. Ng and Stephen Rattigan. Imaging skeletal muscle microcirculation with contrast-enhanced ultrasound: diagnostic and therapeutic approaches to insulin resistance.

Australian Diabetes Society/Australian Diabetes Educators Association Annual Scientific Meeting. Perth, WA, Australia. September 2011. **Dino Premilovac**, Huei L.H. Ng, Stephen M. Richards, Eloise A. Bradley, Stephen Rattigan and Michelle A. Keske. Moderate increases in dietary fat impair microvascular and metabolic actions of insulin in skeletal muscle of Sprague Dawley rats.

CHAPTER 1

INTRODUCTION

1.1. Insulin Action

1.1.1. Metabolic Actions of Insulin

Insulin is an important hormone that is primarily responsible for regulating glucose and fat metabolism within the body. Following a meal, insulin is secreted into the circulation by pancreatic β-cells and acts on a number of tissues to regulate circulating blood glucose and fatty acid concentrations. The three main sites of insulin's metabolic actions are the liver, adipose tissue and skeletal muscle.

Insulin binding to the cell membrane-bound insulin receptor results in the phosphorylation of the insulin receptor substrate (IRS, Fig. 1.1) [1, 2] and subsequent activation of phosphatidylinositol-3-Kinase (PI3K) [3]. PI3K then activates protein kinase B (Akt) [4]. Akt is an important intracellular signalling molecule known for its regulation of many downstream proteins that in turn mediate insulin action [5]. In adipocytes insulin signalling via Akt culminates in the uptake of glucose and lipid [6]. In liver and skeletal muscle insulin signalling via Akt results in the inhibition of glycogen synthase kinase thus activating glycogen synthase and promoting glycogen synthesis/storage [6]. Insulin also opposes the effects of glucagon and acts to supress liver gluconeogenesis by phosphorylating and thus inactivating FOXO1 [7]. In skeletal muscle, insulin, via Akt, phosphorylates and activates AS160 [8, 9], which in turn recruits the transmembrane glucose transporter GLUT-4 to the plasma membrane and increases myocyte glucose uptake [8-10]. This is an important action of insulin and skeletal muscle accounts for approximately 75-90% of insulin-stimulated glucose uptake following a meal [11]. As well as glucose uptake, insulin has been shown to possess vascular actions in muscle [12].

Insulin exerts important actions on the vascular endothelium in addition to its classical action to increase glucose disposal into skeletal muscle. The vascular endothelium directly responds to insulin by increasing production of nitric oxide (NO). Quon and colleagues have elucidated a complete insulin signalling pathway in vascular endothelial cells leading to NO production [13-16]. This pathway requires activation of the

following signalling proteins: IRS-1/PI3K /PDK-1/Akt which leads to eNOS-mediated NO production. Interestingly, this endothelial pathway parallels the metabolic insulin signalling pathway (at least to the levels of Akt) in muscle and adipose tissue which implies an important link between insulin's vascular and metabolic actions.

Another important signalling pathway activated by insulin in the vasculature is the Ras/MAPK signalling pathway. Insulin stimulation of this pathway mediates insulin's effects on cell growth, cell differentiation, protein synthesis and gene expression [17]. Furthermore, in states of insulin resistance and type 2 diabetes (T2D), where there is compensatory hyperinsulinaemia, signalling through the Ras/MAPK pathway has been reported to be increased [18, 19]. This has been proposed to further reduce signalling through the PI3K/Akt pathway by phosphorylating the inhibitory serine residues on IRS [20, 21]. The Ras/MAPK pathway also leads to an increase in endothelin-1 (ET-1) release, which has vasoconstrictor effects in the vasculature [22, 23]. Thus, increased activation of the Ras/MAPK would also lead to increased production of ET-1 and reduced production of NO, thus favouring a state of vasoconstriction.

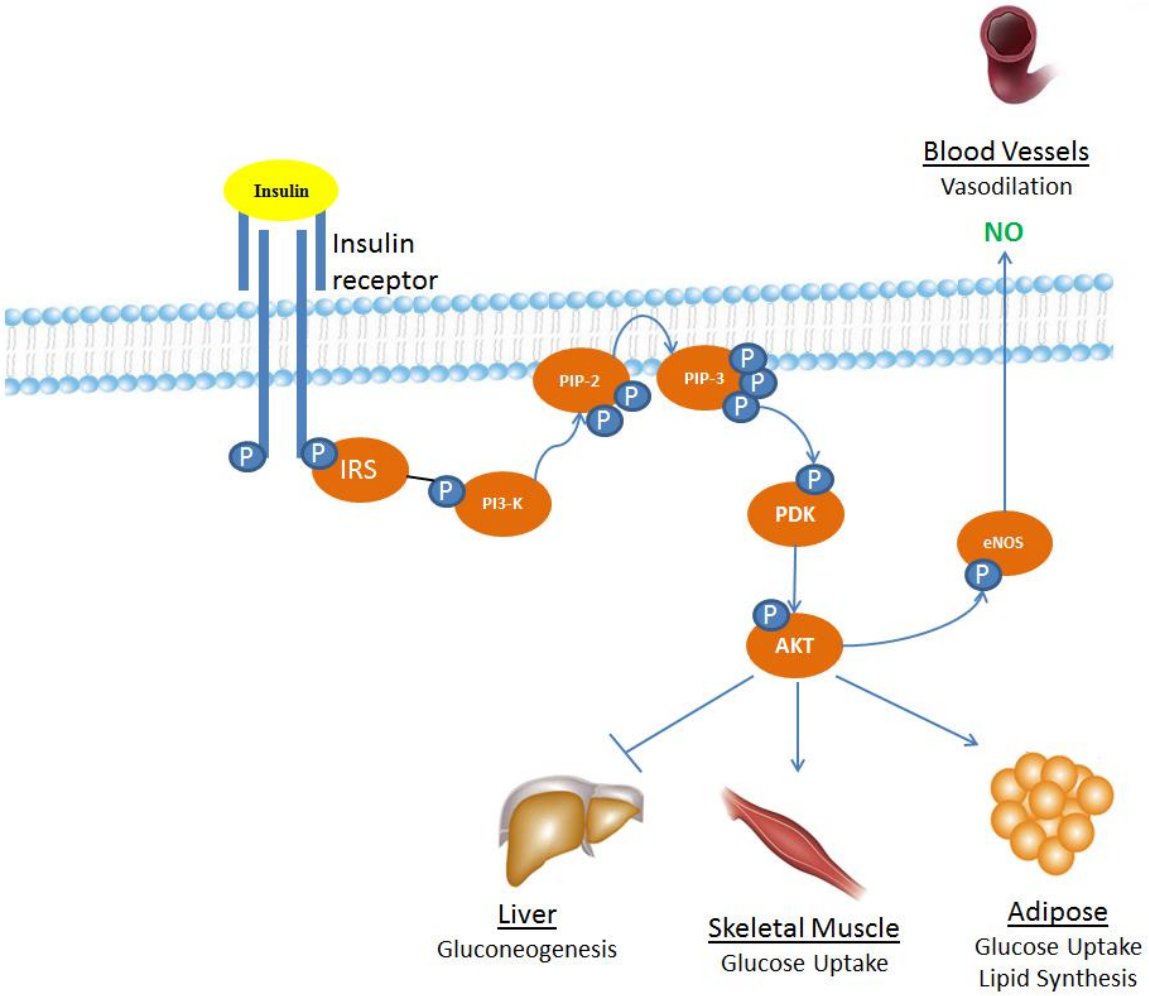

Figure 1.1. Insulin-IRS/PI3K/Akt signalling pathway.

Intracellular insulin signalling of the insulin IRS/PI3K/Akt pathway. Insulin activation of this pathway in liver causes inhibition of gluconeogenesis, in muscle insulin induces glucose uptake, and in adipose tissue insulin causes glucose and fatty acid uptake. In vascular endothelial cells insulin stimulates the production of nitric oxide (NO) by eNOS resulting in vasodilation. Modified from Muniyappa and colleagues (2007) [17].

1.1.2. Haemodynamic Action of Insulin

The vascular action of insulin was first documented by Abramson and colleagues in 1939. In their study the authors reported that infusion of pharmacological doses of insulin (40-280 Units) increased limb blood flow in humans [24]. These vascular effects of insulin were also reported in subsequent clinical studies by other investigators [25, 26]. However, because pharmacological doses of insulin were utilised without concomitant glucose infusion in these early studies, the increase in blood flow mediated by insulin was associated with significant hypoglycaemia. Thus, whether the increased blood flow was the result of insulin action or a response to severe hypoglycaemia, such as adrenaline release, could not be distinguished. It was not until the development of the hyperinsulinaemic euglycaemic clamp technique in 1975 by Norwich and colleagues that insulin's vascular actions could be dissociated from the effects of hypoglycaemia [27]. Subsequently, Liang and colleagues (1982) demonstrated that insulin infusion (4 and 8mU/kg/min) could significantly increase total limb blood flow independently of hypoglycaemia [28].

Studies by Baron and co-workers in the early 1990's subsequently showed that insulin could stimulate increases in total blood flow to skeletal muscle during normoglycaemic conditions in human subjects [12, 29]. In these reports the authors proposed that insulin-mediated increased flow could augment insulin's metabolic actions by increasing delivery of insulin and glucose to the myocyte [12, 29], thus facilitating increased insulin-mediated muscle glucose uptake. These studies were performed in human subjects during hyperinsulinaemic euglycaemic conditions, thus excluding the possible contribution of hypoglycaemia related adrenaline release [12, 29]. Since that time a number of investigations have produced results that support these findings by documenting insulin's ability to increase blood flow to skeletal muscle *in vivo* in both human [30-35] and animal studies [36-38]. However, considerable variations in the magnitude of insulin-mediated vasodilation in muscle were reported, and some authors failed to observe any increase in blood flow in response to insulin infusion [39-42]. One of the reasons for these discrepancies is related to the variety of methods used to investigate flow changes. Blood flow changes have been detected using techniques including thermodilution [12], plethysmography [30], dye dilution [43] and positron

emission tomography (PET) coupled with [^{15}O]H_2O [32]. Although each of these techniques measures total limb blood flow, they have different degrees of sensitivity.

What also appeared to be important in observing insulin-mediated vasodilation was subject selection and the experimental protocol utilised. Since the majority of these previous studies assessed insulin-mediated flow changes in humans, the large inter-individual response observed following insulin infusion has been attributed to the level of physical activity, muscularity and the level of capillarization within muscle [34, 44, 45]. Results also appear to differ based on the protocol used, since increases in blood flow are more prominent at higher doses of insulin and following prolonged infusions of insulin [46, 47]. However, the relationship between insulin's action on total muscle blood flow and its metabolic actions were initially controversial. In a number of studies Yki-Jarvinen and colleagues reported that insulin's metabolic effects on glucose uptake in muscle occur prior to the effects on total limb flow [48-50]. It should be noted, however, that this temporal discordance is due to the assumption that insulin's vascular action is restricted to only increases in total blood flow. These discrepancies led to considerable controversy and raised the question of whether increases in blood flow in response to insulin were physiologically relevant.

Although many of the studies that reported increases in total blood flow in response to insulin used supra-physiological/pharmacological concentrations of insulin [32, 51, 52], there are also reports of insulin-mediated vasodilation at physiological concentrations of insulin [29, 30, 35]. However, the latter studies have been criticized since prolonged infusions of insulin (several hours) were required before any changes in blood flow could be detected. It should be noted though that insulin levels typically remain elevated for 90-120 min following a meal [53, 54] and therefore whether these prolonged infusions of insulin are physiologically relevant is questionable. Nevertheless, for total blood flow to increase in skeletal muscle in response to insulin there must either be an increase in the cardiac output or redistribution of blood flow between organs.

Insulin infusion has been shown to increase cardiac output in healthy humans with little to no effects on mean arterial blood pressure [12, 34, 55, 56], suggesting that peripheral vascular resistance (calculated as the mean arterial pressure divided by the cardiac output or organ blood flow) may decline during insulin infusion. Indeed, there is evidence that different vascular beds respond differentially to increased circulating insulin concentrations. Using experimental animals it has been reported that hindleg and renal vascular beds dilate, whereas the superior mesenteric vascular bed constrict in response to hyperinsulinaemia [57, 58]. In healthy humans, hyperinsulinaemia was reported to induce a greater decrease in leg vascular resistance compared to systemic vascular resistance [55]. The consensus view appears to be that insulin can differentially regulate vascular resistance between different organs. Baron and colleagues (1994) proposed that insulin preferentially vasodilates the skeletal muscle vasculature to increase glucose and insulin deliver to the myocyte to facilitate increased muscle glucose uptake [55]. However, it has since been shown that increasing total limb blood flow alone is not necessarily associated with an increase in muscle glucose uptake [37, 48, 59].

This discrepancy between total limb blood flow and muscle glucose uptake led to the hypothesis that the distribution of blood flow within the muscle vasculature may be a more critical factor in determining muscle glucose uptake [60]. This action of insulin to increase microvascular blood flow and thus increase perfusion of muscle has since been supported by a number of investigations in humans and animal models [37, 38, 54, 61-65].

1.1.3. Insulin-mediated microvascular recruitment

While most studies have focused on insulin-mediated increases to total flow to skeletal muscle, what may be more physiologically relevant is the distribution of microvascular flow within muscle. Before discussing this aspect of insulin action it is necessary to give a description of the structure of skeletal muscle vasculature.

1.1.3.1. Structure of skeletal muscle microvasculature

The vasculature in skeletal muscle can be simply categorised into three types: arteries, capillaries and veins (Fig. 1.2). Blood enters the muscle through a feed artery which is generally classified as a 1^{st} order arteriole. Subsequent arteriolar branches are numbered in increasing order until the 5^{th} order arterioles, which lead to capillaries [66]. In muscle, 5^{th} order arterioles have been reported to give rise to groups of approximately 15 capillaries, which are collectively termed a module [67]. A capillary network is the term used to describe a group of modules arising from one 4^{th} order arteriole [67]. Total blood flow in skeletal muscle is initially controlled by constriction/dilation of the 1^{st}-3^{rd} order arterioles [67]. Flow distribution to muscle capillary networks is controlled by the 3^{rd}-5^{th} order arterioles [67]. It has been proposed that at any one time not all capillaries networks are perfused [66, 68]. Indeed a number of studies have shown that in humans and in animals that the microvascular perfusion in resting skeletal muscle is neither continuous nor uniform, but rather intermittent and heterogeneous in distribution [50, 69-71]. Thus, it has been suggested that vessels in muscle undergo vasomotion, that is, contraction and dilation at regular intervals to alternate blood flow through different capillary modules as needed for nutrient supply [72, 73]. However, it has also been proposed that the vascular system in muscle has a further level of complexity that involves modulation of blood flow between two distinct routes, termed nutritive and non-nutritive [74].

1.1.3.2. Nutritive and non-nutritive flow routes

The first reports to suggest the existence of two flow routes in muscle were performed in the 1940's by Pappenheimer [75]. In these studies Pappenheimer reported that oxygen consumption across the hindleg could be increased or decreased using different hormonal substances and proposed that this difference was the result of flow redistribution within the hindleg muscle vasculature [75]. Since that time Clark and associates have demonstrated that pharmacologically shifting flow between these two flow routes produces different metabolic consequences in the hindleg of rats [74, 76-78]. These two distinct flow routes were termed the nutritive and non-nutritive routes (Fig. 1.3). It was proposed that the non-nutritive flow route was made up of shorter, possibly larger diameter capillaries of lower resistance [79] some of which supply

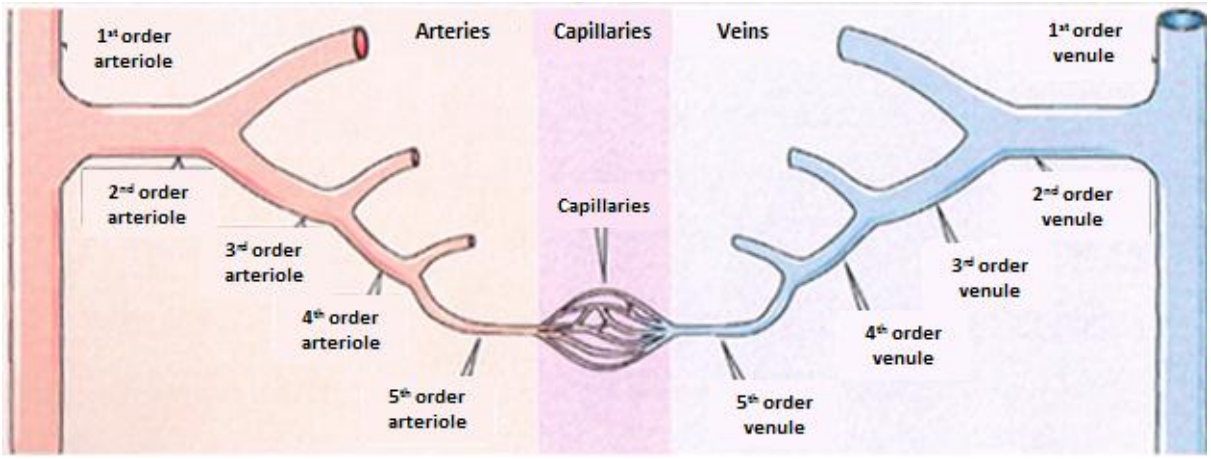

Figure 1.2. Microvascular network in skeletal muscle.

The vasculature in muscle is composed of 3 types of vessels: arteries, capillaries and veins. Blood flow enters through the arteries which are responsible for oxygen and nutrient delivery. After a number of arteriolar branches, flow reaches the capillary network. Because capillaries have only one layer of endothelial cells they allow for efficient exchange of gas and other nutrients such as glucose between the blood and myocytes. As blood moves through the capillary network it enters the venous network and subsequently leaves the muscle. Modified from Boron and Boulpaep (2003) [80].

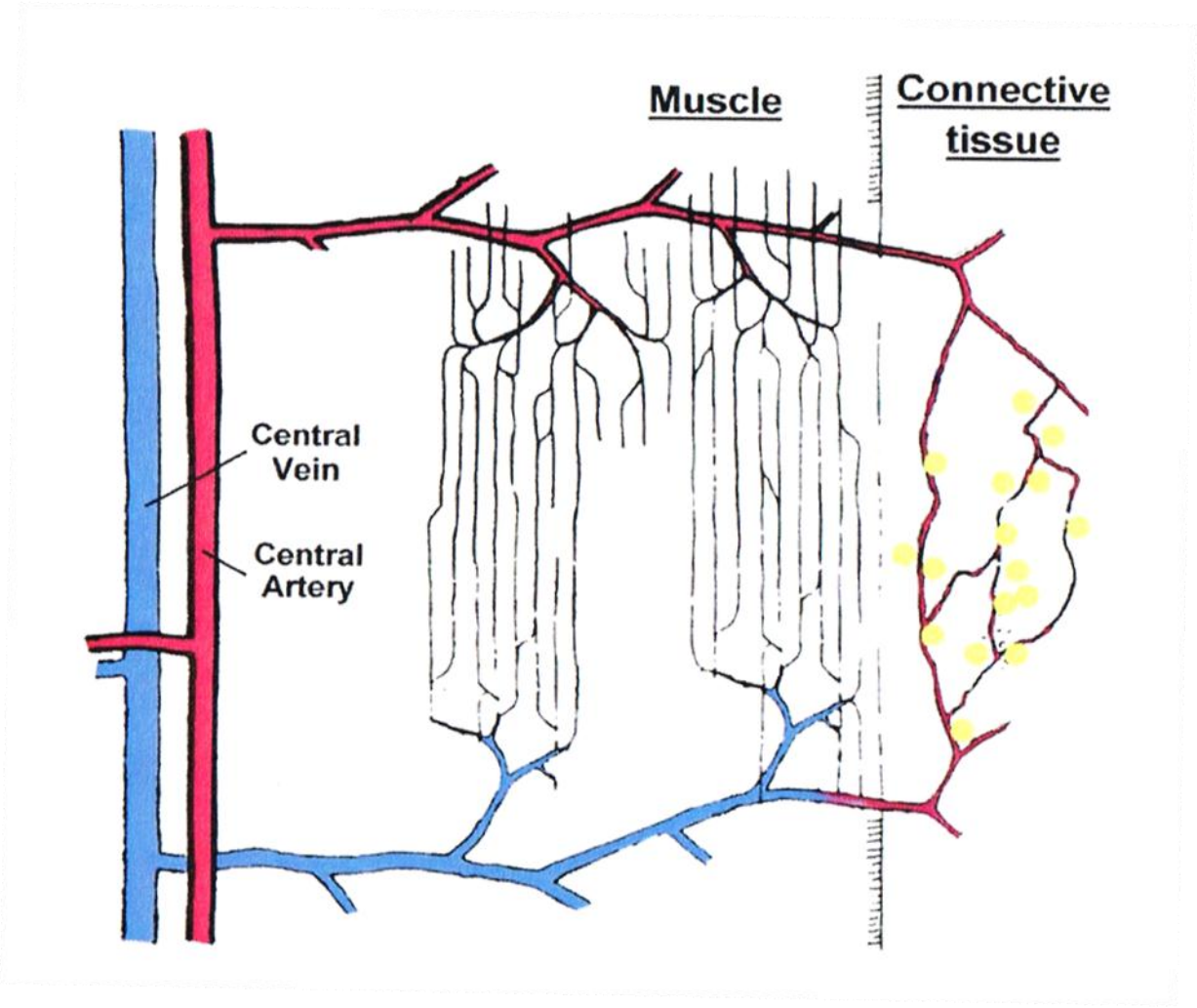

Figure 1.3. A schematic representation of the vascular networks in muscle.

Both the muscle and adjacent connective tissue are supplied by the transverse arteriole. However; as the diagram suggests, blood flow can be directed one of two ways, depending on the needs at the time. At rest, the flow bypasses the nutritive route and proceeds through the larger, but fewer, connective tissue vessels in contact with adipocytes (yellow). Conversely, during increased metabolic demand the flow is redistributed toward the nutritive vessels to allow an increase in myocyte metabolism. Adapted from Borgstrom 1988 [81].

connective tissue (septa and tendon)[82] and possibly some associated adipocytes [83]. Conversely, it was proposed that the nutritive flow route consisted of longer, more tortuous capillaries that wrap around muscle fibres to provide a large surface area, allowing for enhanced nutrient exchange between the blood and myocyte (reviewed in [74]). Clark and colleagues (2000) concluded that the non-nutritive flow route acted as a functional flow reserve that could be used to redistribute blood flow to the nutritive route during increased metabolic demand, such as exercise or following a meal to allow for increased myocyte glucose uptake [84]. This redistribution of flow within skeletal muscle has since been termed microvascular recruitment.

1.1.3.3. Microvascular recruitment

Microvascular recruitment is a term used to describe an increase in previously unperfused capillaries that results in increased skeletal muscle perfusion, thus increasing capillary surface area within muscle and leading to increased delivery of hormones and nutrients to the myocyte. Insulin has been reported to increase microvascular recruitment in a number of studies utilising various techniques. These include 1-methylxanthine (1-MX) metabolism [37], contrast enhanced ultrasound (CEU) [85], laser Doppler flowmetry [86], intravital microscopy [36] and others [32, 52]. For the purposes of this thesis only the 1-MX and CEU techniques will be discussed.

A novel method for assessing the extent of capillary exposure during hyperinsulinaemia was developed by Rattigan and colleagues in 1997 [37]. The technique depends on metabolism of the exogenous substrate 1-MX by the endothelial bound enzyme xanthine oxidase (XO). 1-MX is not vasoactive and is converted to a single product, 1-methylurate (1-MU), by XO. 1-MU is not metabolised further in the body and the metabolism of 1-MX to 1-MU is stoichiometric. Thus, both 1-MX and 1-MU can be readily separated and assessed using high performance liquid chromatography (HPLC) [77]. Studies have demonstrated that XO in bovine and human muscle is located in capillary endothelial cells but not in endothelial cells of large arteries and veins, or in myocytes [87, 88]. However, it should be noted that in human muscle, XO has also been found in smooth muscle cells of small arterioles and venules [87]. Nevertheless, since

XO is predominantly located in capillary endothelial cells, it is a useful marker for the extent of microvascular perfusion in muscle.

The constant-flow pump-perfused rat hindleg preparation is an *in vitro* technique that allows for examination of vascular perfusion in muscle in an intact hindleg under different conditions. Using this technique, infusion of serotonin was shown to increase vasoconstriction and shift flow away from capillaries supplying myocytes to favour the non-nutritive flow route, as indicated by inhibition of contraction or insulin-mediated muscle metabolism, dye kinetic studies and vascular casting [76, 78, 89-91]. Rattigan and colleagues (1997) demonstrated that serotonin-mediated vasoconstriction in the perfused rat hindleg significantly attenuated metabolism of 1-MX across the hindleg [77], thus implicating reduced nutritive muscle perfusion with reduced metabolism of 1-MX. Furthermore, using the anaesthetised rat technique, Rattigan and colleagues (1997) reported that metabolism of 1-MX increased during insulin infusion compared to saline [37], indicating an increase in the exposure of circulating 1-MX to XO. Using a 10mU/kg/min infusion of insulin, the authors demonstrated that as well as increasing microvascular recruitment (1-MX metabolism) insulin also stimulated an increase in femoral artery blood flow [37]. To distinguish the contribution of total flow to microvascular recruitment the authors utilised an infusion of epinephrine to increase femoral artery blood flow. The concentration of epinephrine was adjusted to achieve a similar increase in femoral artery blood flow to that seen with insulin, but was found not to increase the metabolism of 1-MX [37]. Thus, it was concluded that insulin possessed a specific mechanism that could redistribute flow to increase muscle perfusion and it was postulated that this leads to increased delivery of insulin and glucose to the myocyte. This ability of insulin to increase microvascular recruitment has since been demonstrated at a variety of physiological concentrations of insulin [65]. Interestingly Zhang and colleagues (2004) also reported that while the lower concentration of insulin increased microvascular recruitment, no effects were observed on femoral artery blood flow [65], suggesting differential control of the vessels controlling total flow and flow distribution by insulin.

There are, however, some limitations associated with the 1-MX technique. Firstly, the technique is dependent on obtaining a constant arterial concentration of 1-MX and therefore requires a prolonged infusion of 1-MX. Moreover, large blood samples are required for measurement of 1-MX metabolism across the limb. For these reasons it is not feasible to obtain multiple time point measures of 1-MX metabolism during the hyperinsulinaemic euglycaemic clamp technique. One advantage of this technique, however, is the ability to make measures of microvascular blood flow in both legs simultaneously. Furthermore, the technique is not suitable for human studies, since humans express a much lower level of XO compared to rats, thus making 1-MX A-V difference too small to be reliably measured [92]. For these reasons, other techniques (such as CEU) are preferred for assessing microvascular recruitment in human subjects.

CEU is a relatively new technique (last 15 years) that has been adapted for quantification of capillary perfusion in skeletal muscle. CEU has been used to quantify microvascular volume and perfusion in the myocardium [93-96], and more recently in skeletal muscle [38, 97, 98]. This technique employs infusion of albumin or phospholipid microbubbles filled with an inert, heavy gas (such as perflurocarbon) into the vasculature. The microbubbles are stable and possess similar rheology to red blood cells, and are exclusively intravascular [99]. When an ultrasound pulse is applied the microbubbles oscillate in size, and if the pulse is strong enough they burst and are destroyed. The oscillation and/or destruction of microbubbles results in the generation of an acoustic signal that can then be detected by the ultrasound transducer. With the contrast ultrasound settings, the strength of the signal received is dependent on the number of microbubbles in the field of view of the ultrasound transducer. Since the microbubbles are confined to the vasculature, the signal received is itself a reflection of the extent of tissue perfusion under the ultrasound transducer. Following destruction of microbubbles using a high intensity ultrasound pulse, the rate of reappearance of microbubbles is a reflection of blood velocity, while the capillary blood volume can be measured by the extent of tissue opacification.

Insulin-stimulated microvascular recruitment in skeletal muscle has been observed in both human and animal studies using CEU [38, 61, 98, 100, 101]. Reports have

demonstrated a strong correlation between the CEU and 1-MX methods for analysis of insulin-mediated microvascular recruitment in skeletal muscle [65, 101]. In these studies hyperinsulinaemic euglycaemic clamps using various concentrations of insulin (1.5, 3.0 and 10mU/kg/min) revealed that both CEU and 1-MX methods showed similar increases in in microvascular recruitment at each concentration of insulin [65, 101]. Furthermore, in both studies microvascular recruitment occurred at lower concentrations of insulin than changes in total flow [65, 101], supporting the notion that changes in microvascular recruitment and total flow in response to insulin are relatively independent of each other.

1.1.3.4. Mechanism of microvascular recruitment

Thus far, a number of studies have revealed that insulin can increase microvascular perfusion of skeletal muscle by redistributing flow to the capillary network associated with myocytes; however, the mechanisms responsible have not been fully investigated. While it appears that insulin's ability to increase total flow is dependent on dilation of 1^{st}-3^{rd} order arterioles, microvascular recruitment seems to be mediated by the 4^{th} and 5^{th} order arterioles [67, 102]. Insulin receptors are located on endothelial, vascular smooth muscle and skeletal muscle cells. Whether the mechanism responsible for increases in total flow and microvascular recruitment is dependent on insulin action on one or a combination of these cell types is unclear. However, various reports have implicated a role for NO in insulin-mediated vascular activity [100, 103].

Studies in primary cultures of endothelial cells have elucidated a complete insulin signalling pathway from the insulin receptor, to activation of eNOS and NO production by the IRS/PI3K/Akt pathway [13, 15, 104]. In turn NO diffuses into adjacent vascular smooth muscle cells to activate soluble guanylyl cyclase, resulting in vasodilation. This mechanism is consistent with previous studies by Vincent and colleagues (2003 and 2004) where it was demonstrated that insulin-mediated effects on total muscle flow and microvascular recruitment could be blocked using a systemic infusion of the nitric oxide synthase inhibitor, Nω-nitro-L-arginine-methyl ester (L-NAME) [100, 105].

A second possibility for insulin-mediated microvascular recruitment exits where insulin can by-pass the endothelial cell and act directly on vascular smooth muscle cells to induce vasodilation. Trovatti and colleagues (1995), using incubated human vascular smooth muscle cells, reported that insulin could stimulate a rapid increase in cyclic guanosine monophosphate (cGMP) and thus induce relaxation [106]. These authors also reported that this was also mediated by a NO-dependent mechanism since the insulin-mediated increase in cGMP in incubated vascular smooth muscle cells can be blocked by inhibiting nitric oxide synthase [106, 107].

In addition, there is still the possibility that other vasoactive substances are involved in insulin-mediated microvascular recruitment. Indeed Chai and colleagues (2009 and 2011) recently reported that angiotensin II (AngII) receptors also contribute to the control microvascular blood flow during basal and hyperinsulinaemic states (this will be discussed further in Section 1.4.4) [108, 109]. As well as mediating release of NO, insulin has also been reported to stimulate production of ET-1 via an MAPK-dependent pathway. Anfossi and colleagues (1994) have reported that insulin can stimulated ET-1 production in human vascular smooth muscle cells *in vitro* [22]. In addition, Misurski and colleagues (2001) reported that initial NO-mediated vasodilation can be overcome by generation of ET-1 when rat mesenteric vessels were exposed to high insulin concentrations [110]. This effect was blocked by addition of an ET-1 receptor antagonist [110]. Thus, it has been proposed that insulin causes generation of both NO and ET-1, and it is a balance between these two vasoactive agents that determines insulin's vascular effects in isolated rat skeletal muscle arterioles [111] and in the healthy human forearm [112].

It should be noted that the majority of investigations regarding insulin-mediated microvascular recruitment in muscle have involved systemic insulin infusion. Thus, there exits the possibility that a central insulin-mediated process may influence muscle metabolism by controlling microvascular perfusion of muscle. However, other reports would argue against this conclusion. Coggins and associates (2002) demonstrated that local infusion of insulin (0.05mU/kg/min), which did not increase systemic insulin concentrations, increased insulin-mediated microvascular recruitment and muscle

glucose uptake in the forearm of healthy human subjects [61]. The authors also found that these effects were in the absence of an increase in total forearm blood flow [61]. Additionally, Bradley and colleagues (2010) reported that central (intracerebroventricular) administration of insulin (135mU/min/kg of brain) in healthy rats had no effect on insulin's peripheral actions [113]. Indeed, the authors detected no effects on total femoral artery blood flow, microvascular recruitment in muscle or skeletal muscle glucose uptake during the central insulin infusion [113]. Together, these studies suggest that increased microvascular recruitment in muscle is most likely mediated by local rather than central effects of insulin.

In addition to the effects on NO release, direct actions on vascular smooth muscle cells, release of other vasoactive factors, changes in muscle metabolism and alterations in sympathetic nerve activity may contribute to insulin's vascular actions (reviewed by Muniyappa 2007)[17]. Thus, there may not be one single mechanism involved insulin-mediated vasodilation, but likely depends on a variety of factors, and modulation of these various processes could potentially alter overall insulin action and sensitivity.

1.1.4. Microvascular recruitment and muscle glucose uptake

Whilst it is generally accepted that insulin can increase blood flow to skeletal muscle, the physiological relevance of this in regulating glucose uptake and as a possible cause of insulin resistance remain controversial. Studies in both animals and humans have suggested that insulin-mediated microvascular recruitment in skeletal muscle plays an integral role in regulating muscle glucose uptake by augmenting delivery of insulin and glucose to the myocyte [38, 54, 61, 105, 114]. The delivery of glucose and insulin to skeletal muscle is dependent on blood flow, capillary surface area and permeability. Capillaries in muscle are the site of nutrient exchange, and according to the Fick principle, glucose uptake is equal to the product of the A-V glucose difference and flow [115]. Thus, during states in which capillary surface area and permeability are small, an increase in total blood flow to muscle would have minimal effect on glucose uptake *per se*, since the rate limiting step in this equation would be glucose extraction [116]. However, during states in which microvascular perfusion of muscle is increased, such as

following a glucose load or mixed meal, the resulting increase in both surface area and blood flow significantly increases insulin and glucose delivery to the myocyte [54, 117]. The end result is a significant increase in glucose disposal by muscle that correlates positively with increased microvascular recruitment. Indeed, reports have revealed that this effect of insulin on the microvasculature is both rapid in onset and highly sensitive to increased circulating insulin concentrations.

Vincent and colleagues (2004) performed a time-course study in rats and demonstrated that insulin increased microvascular perfusion of muscle within 5-10 min at physiological concentrations (3mU/kg/min) [105]. This increase in microvascular perfusion of muscle was found to precede not only the activation of the insulin signalling pathway and stimulation of glucose disposal in muscle but also insulin's effects on total blood flow [105]. In support of these findings, Zhang and colleagues (2004) performed an insulin dose-response study in rats and demonstrated that relatively low doses of insulin (1.0 and 1.5mU/kg/min) were capable of stimulating near-maximal microvascular recruitment in muscle without changes in total flow [65]. Together, these data suggest that insulin-stimulated microvascular recruitment in muscle is a highly sensitive, early event which likely plays an important role in facilitating enhancement of insulin-mediated glucose uptake in muscle. Thus, along with the direct PI3K/Akt dependent metabolic actions of insulin in skeletal muscle myocytes to promote GLUT-4 translocation and thus increase myocyte glucose uptake, insulin has a second action in muscle to enhance the access of insulin and glucose to myocytes by recruiting blood flow to the microvasculature.

The presence of insulin receptors on endothelial cells has been well documented [13, 15]. A number of reports have suggested that insulin-mediated vasodilation contributes to muscle glucose uptake by increasing delivery to skeletal muscle [118-120]. Indeed, insulin's haemodynamic effects have been in part attributed to insulin-mediated NO production via the IRS/PI3K/Akt pathway from vascular endothelial cells [107, 121-123]. In states of insulin resistance, insulin-mediated vasodilation has been shown to be attenuated and associated with reduced NO bioavailability [124]. In studies using incubated muscle, inhibition of nitric oxide synthase was reported not to affect insulin-

mediated glucose uptake [125, 126]. However, eNOS knockout mice display endothelial dysfunction, hypertension and marked attenuation in insulin-mediated glucose uptake *in vivo* [127], suggesting that NO contributes to insulin-stimulated glucose disposal *in vivo*. In support of this Vincent and colleagues (2003) utilised an acute infusion of L-NAME, a nitric oxide synthase inhibitor, to induce acute insulin resistance in otherwise healthy rats [100]. During hyperinsulinaemic euglycaemic clamp, the authors demonstrated that these rats displayed marked reduction in both insulin-mediated microvascular recruitment and muscle glucose uptake [100]. Thus, these latter studies imply that for maximal insulin-stimulated glucose uptake to occur *in vivo*, normal insulin-mediated NO signalling is required and suggests a role for NO in insulin-mediated microvascular recruitment.

1.2. Insulin resistance

Insulin resistance is defined as a reduced biological response to a given dose of insulin than expected and is a strongly associated with the pathogenesis of many metabolic disorders including obesity, dyslipidaemia and type 2 diabetes (T2D) [128, 129]. Together these diseases contribute to significantly increased risk of cardiovascular disease and T2D often leads to peripheral vascular disease. Incubated muscle preparations from insulin resistant, obese and T2D individuals exhibit substantial attenuation in insulin-mediated muscle glucose uptake and metabolism [130-132], suggesting a defect in insulin action in the myocyte. Despite considerable research, the primary cause of insulin resistance is not known. Because of the heterogeneous nature of the condition, it is likely that both genetic and environmental factors play a role in the development of insulin resistance. However, given the evidence that insulin also possesses haemodynamic actions that contribute to glucose metabolism *in vivo*, it is possible that the development of insulin resistance also has a haemodynamic origin.

1.2.1. Metabolic consequences of insulin resistance

It has been proposed that both the skeletal muscle and liver become resistant to the biological actions of insulin at a relatively early stage in the pathogenesis of insulin resistance. Insulin resistance results in dysfunction of insulin-mediated suppression of

liver glucose output and reduced muscle glucose uptake. With increased glucose output and reduced glucose uptake, blood glucose concentrations begin to rise. Increased blood glucose concentrations subsequently stimulate increased secretion of insulin from the pancreas and this is termed compensatory hyperinsulinaemia (Fig. 1.4). Initially, the increased insulin concentration is sufficient to maintain normoglycaemia, however, overtime the pancreatic insulin production and/or insulin action is diminished to such a point that uncontrolled hyperglycaemia ensues. The cause of insulin resistance appears to be heterogeneous in nature. Some reports have proposed that insulin resistance within the adipose tissue significantly contributes to insulin resistance via increased production of adipokines and inflammatory cytokines that are detrimental to normal insulin action (reviewed by Yudkin 2007) [133]. It has been proposed that adipocyte insulin resistance also leads to reduced lipogenesis within adipose tissue and results in the accumulation of lipids in non-adipose tissues, such as liver and muscle, which can interfere with the insulin signalling pathway to induce insulin resistance (reviewed by Kraegen and Cooney 2008 [134]).

Investigations have identified specific defects in the insulin receptor signalling pathway in skeletal muscle of insulin resistant subjects. Cusi and colleagues (2000) reported that the insulin signalling pathway in skeletal muscle of obese, insulin resistant individuals displayed selective resistance to the IRS/PI3K/Akt pathway and not the MAPK pathway [19]. The authors also reported that in subjects with T2D, insulin stimulation of the IRS/PI3K/Akt pathway was virtually absent while stimulation of the MAPK pathway was intact and normal [19]. The potential mechanisms contributing to the reduced insulin signalling and action include decreases in mitochondrial oxidative capacity [135], increased intramuscular lipid accumulation [136], increased reactive oxygen species (ROS) generation [137], and up-regulated inflammatory pathways [138]. In myocytes it has been reported that while mRNA and protein levels of GLUT-4 are unaffected [139], the insulin-mediated GLUT-4 translocation to the plasma membrane is markedly impaired in subjects with T2D [140]. In addition, hexokinase expression and activity are reported to be blunted during obesity and insulin resistance [141, 142], and insulin-stimulated activation of glycogen synthase decreased [141]. Similar to insulin action in skeletal muscle, Jiang and colleagues (1999) reported that the insulin signalling pathway in both aorta and microvessels of obese Zucker rats (a model of T2D) also displayed

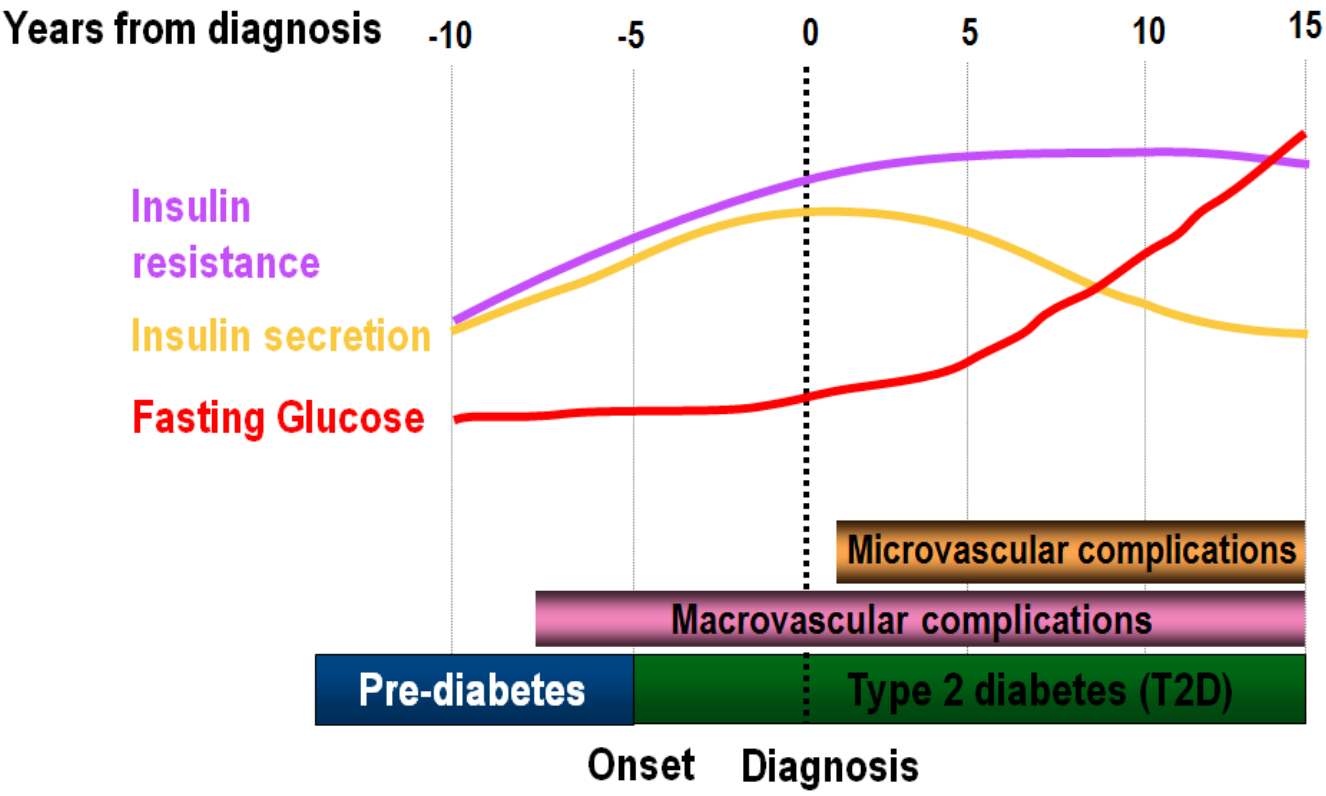

Figure 1.4. Aetiology of insulin resistance and type 2 diabetes.

Figure 1.4 is graphic representation of the progression of insulin resistance leading to the development of T2D in humans. The development of T2D can be separated into two distinct phases and generally takes many years to progress. The first is the pre-diabetic, insulin resistant stage often associated with obesity and macrovascular complications resulting in hypertension. In this stage increased pancreatic insulin secretion results in hyperinsulinaemia and is able maintain normoglycaemia. The second stage involves the development of T2D. This stage occurs when the pancreatic β-cells cannot cope with the persistently increased production of insulin and results in β-cell failure. At this stage, hyperinsulinaemia begins to reverse due to reduced insulin secretion and uncontrolled hyperglycaemia dominates, requiring exogenous insulin injections to prevent glucose toxicity. This end stage is generally associated with microvascular diseases such as nephropathy, neuropathy, retinopathy and peripheral vascular disease. Modified from the CADRE Lecture Kit [143].

selective resistance to the IRS/PI3K/Akt pathway and not the MAPK pathway [144]. Thus, while a number of metabolic defects significantly contribute to the pathogenesis of skeletal muscle insulin resistance, vascular dysfunction may also plays an important role in the development and progression of insulin resistance.

1.2.2. Insulin resistance and endothelial dysfunction

Vascular endothelial cells are capable of producing a variety of vasoactive molecules that can act on vascular smooth muscle cells and thus contribute to vascular tone. Endothelial dysfunction is primarily the result of decreased NO bioavailability, which can be due to impaired NO production by the endothelium and/or increased degradation of NO by ROS. A key feature of endothelial dysfunction is the inability of the vasculature to dilate in response to an appropriate stimulus. Since a number of studies have proposed that insulin-mediated vasodilation involves NO production by endothelial cells [59, 100, 103, 145, 146], it has been suggested that impairment in insulin-mediated vasodilation and endothelial dysfunction may have similar origins [147]. Indeed frequent association between endothelial dysfunction, hyperglycaemia, hyperinsulinaemia, dyslipidaemia and hypertension has led to the suggestion that endothelial dysfunction may be an early event that contributes to the development of insulin resistance [148-150].

In cross-sectional studies endothelial dysfunction is consistently present in patients with insulin resistance [124, 151, 152]. This also includes first degree relatives of patients with T2D and the individuals themselves [153, 154], suggesting a strong link between endothelial function and insulin sensitivity. Animal models provide additional support for the involvement of endothelial dysfunction in the development of insulin resistance. eNOS knockout mice display endothelial dysfunction, insulin resistance and hypertension [127]. These animals also have reduced capillary density and approximately a 40% reduction in insulin-mediated whole body and skeletal muscle glucose uptake [127, 155]. The eNOS knockout animals also display increased triglyceride and free fatty acid (FFA) concentrations, defective beta-oxidation and impaired mitochondrial function [156]. It has also been reported that animals with only

partial eNOS deficiency are normotensive and insulin sensitive, and only develop hypertension and insulin resistance when challenged with a high fat diet [157]. Similarly, vascular endothelial insulin receptor knockout (VENRIKO) also develop insulin resistance, but only when the mice are maintained on either low or high salt diets, suggesting that insulin signalling in the vascular endothelium contributes to insulin-mediated glucose disposal in certain contexts [158].

Recently, Kubota and colleagues (2011) reported that impairment of insulin signalling in vascular endothelial cells reduces insulin-mediated muscle glucose uptake *in vivo* [159]. The authors found that feeding control animals a high fat diet resulted in significant attenuation of microvascular recruitment, muscle glucose uptake and Akt phosphorylation in muscle during hyperinsulinaemic euglycaemic clamps [159]. In addition, vascular endothelial cells of these animals also exhibited significant reduction in the expression of IRS1 and IRS2 (the major IRS isoform in endothelial cells), and attenuation of eNOS activity [159], suggesting a role for impairment of vascular insulin action in these animals. The authors then went on to demonstrate that endothelial cell IRS2 knockout mice exhibited normal levels of IRS2 in skeletal muscle, liver and white adipose tissue while endothelial cell IRS2 was virtually absent [159]. This reduction in IRS2 was associated with reduced expression of both Akt and eNOS in endothelial cells [159]. During hyperinsulinaemic euglycaemic clamp studies the IRS2 knockout mice exhibited approximately a 50% reduction in whole body glucose disposal while liver glucose output was unaffected compared to control mice [159]. The authors found that the knockout mice exhibited almost complete attenuation of insulin-mediated microvascular recruitment and approximately a 50% reduction in muscle glucose uptake during hyperinsulinaemia associated with a marked reduction of insulin-stimulated phosphorylation of Akt within muscle cells [159]. Furthermore, when muscles from control and knockout animals were excised and incubated with insulin, no difference in insulin-mediated myocyte glucose uptake was detected [159]. These data suggest that impaired insulin signalling in endothelial cells, with reduction of IRS2 and insulin-induced eNOS phosphorylation, reduces insulin-mediated muscle glucose uptake via, at least in part, decreased insulin-mediated microvascular recruitment in skeletal muscle. In addition, these knockout animals seemingly simulate the pathogenesis of high fat diet-induced insulin resistance and implicate reduced insulin-mediated endothelial NO

production (via reduced eNOS activation) as a contributor to reduced insulin-mediated microvascular perfusion and muscle glucose uptake.

One of the major contributors to reduced NO bioavailability/activity (i.e. endothelial dysfunction) is increased oxidative stress resulting from increased ROS production in endothelial and vascular smooth muscle cells. Under normal conditions ROS possess physiological roles in regulating cell signalling, cell growth and proliferation. ROS can be produced by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, nitric oxide synthase, the mitochondrial electron transport chain and others mechanisms. Increased ROS production has been associated with the pathogenesis of both insulin resistance and hypertension (reviewed by Cersosimo 2006 [160]). For example, increased ROS production reduces NO bioavailability by converting NO into peroxynitrite, which itself is also detrimental to endothelial function. Increased production of ROS is reportedly mediated by a number of factors including increased FFA concentrations [161], increased salt intake [162], increased AngII activity [163] and hyperglycaemia [164]. These factors have also been shown to be associated with the development of endothelial dysfunction and insulin resistance. Therefore, by reducing NO bioavailability in the vasculature increased ROS production is likely to also result in the attenuation of insulin's vascular actions.

In addition to NO-dependent vasodilator actions of insulin in vascular endothelium, insulin also has other haemodynamic actions. For example, insulin stimulates production of ET-1 from the endothelium. This is evident because the vasodilator effects of insulin are augmented by ET-1 receptor blockade [165]. Furthermore, patients with T2D and obese subjects who have elevated insulin levels also have elevated levels of ET-1 and endothelial dysfunction can be ameliorated by infusion of an ET-1 receptor antagonist [166]. Thus, insulin has opposing haemodynamic actions with negligible effects of blood pressure in normal individuals. A shift in balance between vasoconstrictor and vasodilator actions of insulin may be an important factor in the vascular pathology of insulin resistance and T2D.

1.2.3. Acute models of microvascular insulin resistance

The findings that insulin has robust and rapid actions in the microvasculature suggest that microvascular recruitment by insulin is an important physiological response. The importance to insulin sensitivity is evident when the microvascular effect is acutely blunted. A number of studies have shown that acutely blocking insulin's vascular actions in muscle also impairs muscle glucose disposal. These studies include, but are not limited to the effects produced by: elevated FFAs, serotonin, tumour necrosis factor alpha (TNFα), glucosamine and ET-1 infusion.

1.2.3.1. Serotonin infusion

Using the perfused hindleg preparation Rattigan and colleagues (1993) demonstrated that vasoconstriction induced by serotonin, which favours non-nutritive flow [78], reduced insulin-mediated muscle glucose uptake [89]. However, incubating muscles with serotonin does not affect insulin-mediated muscle glucose uptake [89]. These data suggest that whilst serotonin does not directly inhibit insulin-mediated glucose uptake by the myocyte, by redistributing flow away from muscle, serotonin infusion attenuates insulin-mediated muscle glucose uptake. Using the anaesthetised rat preparation acute serotonin (in the form of α-methyl-serotonin) infusion was found to significantly reduce insulin-mediated microvascular recruitment in muscle and this was associated with a ~50% reduction in insulin-mediated muscle glucose uptake [167]. Taken together these data indicate that infusion of factors that inhibit the ability of insulin to increase microvascular recruitment in muscle also results in the development of acute muscle insulin resistance.

1.2.3.2. Tumour necrosis factor α

Obesity is associated with chronic, low grade inflammation that often co-exists with insulin resistance [168-170]. Increased production of proinflammatory cytokines from adipose tissue, such as TNFα has been proposed as another factor that contributes to the development of insulin resistance [133]. TNFα has been reported to directly inhibit the insulin signalling pathway in various cell types [171-173], including vascular endothelial

cells [174]. Acute *in vivo* infusion of TNFα in both humans and animals has been shown to reduce whole body insulin sensitivity [175, 176]. Youd and colleagues (2000) demonstrated that infusion of TNFα was associated with complete attenuation in insulin-mediated microvascular recruitment [177]. This was associated with a ~50% reduction in insulin-mediated muscle glucose uptake [177]. However, it has been reported that acutely incubating muscles with TNFα does not significantly affect insulin-mediated muscle glucose uptake [178, 179]. Thus, as with serotonin these data indicate that TNFα does not directly affect myocyte insulin sensitivity, but inhibits insulin-mediated microvascular recruitment and subsequently reduces insulin-mediated muscle glucose uptake *in vivo*.

1.2.3.3. Free fatty acids

Obesity, insulin resistance and T2D have all been associated with increased circulating concentrations of FFAs [180]. Increased FFA concentrations have thus been proposed to contribute to the development of insulin resistance [180-182]. The increased FFA concentrations may induce insulin resistance by either alteration of the Randle cycle or through direct and indirect impairment of the insulin signalling pathway. The Randle cycle, or FFA cycle is a metabolic process involving competition of fatty acids and carbohydrates for substrates [183]. Elevated circulating FFAs result in increased oxidation of fatty acids and thus increased mitochondrial content of acetyl-CoA. This results in inhibition of pyruvate dehydrogenase activity [184] and leads to increased accumulation of citrate which in turn inhibits PFK-1 [185]. Inhibition of pyruvate dehydrogenase and PFK-1 results in an increase in the level of glucose-6-phosphate and thus inhibition of hexokinase, resulting in reduced glucose uptake. However, Boden and colleagues (1994) proposed that reduction in glucose oxidation was only responsible for approximately a third of fatty-acid induced inhibition of glucose uptake [186].

Indeed, other reports have suggested that FFA could induce insulin resistance by directly affecting insulin signalling in myocytes [187]. Increased FFAs concentrations have been shown to result in the accumulation of FFA metabolites fatty acyl-CoA, diacylglycerol and ceramides within non-adipose cells [188-190]. These metabolites have in turn been

shown to impair insulin signalling at the PI3K/Akt level via activation of PKC and result in inhibitory serine phosphorylation of the IRS [191, 192]. However, how much this direct inhibition of insulin signalling contributes to FFA induced insulin resistance is unclear and other reports have also uncovered a role for impairment in endothelial function following FFA infusion.

Steinberg and colleagues (1997) reported that acutely raising FFA in normal healthy subjects resulted in a 20% reduction in endothelium-dependent total leg blood flow in response to methacholine infusion [193]. In a subsequent study Steinberg and colleagues (2000) reported that long-term (8 hr) FFA infusion significantly attenuated limb NO production and reduced the insulin-stimulated increase in femoral artery blood flow in otherwise healthy subjects [194]. The authors showed that the reduction in insulin's effects on blood flow correlated strongly with reduced whole body glucose disposal [194]. Thus, these reports raised the possibility that increased FFA concentrations may result in the development of insulin resistance mediated by reduced insulin-mediated vasodilation and thus glucose uptake in muscle. Other investigations have confirmed this hypothesis in both animal and human studies. Subsequently, Clerk and colleagues (2002) found that intralipid and heparin infusion for 4 hrs resulted in attenuation of insulin-mediated microvascular recruitment in muscle associated with reduced insulin-mediated whole body and muscle glucose uptake [195]. Apparently this was associated with no significant effects on insulin-mediated changes in total blood flow [195]. Similarly, Liu and colleagues (2009) demonstrated that infusion of Intralipid and heparin in humans also resulted in marked attenuation of insulin-mediated microvascular recruitment in muscle associated with reduced whole body glucose disposal [196]. Interestingly, Han and colleagues (2009) recently reported that insulin sensitivity in muscle of FFA infused rats could be restored by incubating excised muscles in a modified Krebs-Heneseleit buffer for 2 hrs, allowing for FFA washout [197] suggesting that acute FFA infusion (2-4 hrs) does induce direct myocyte insulin resistance. Together, these studies raise the possibility that increasing FFA concentrations alone is sufficient to induce insulin resistance, which appears to be mediated by attenuation of insulin-mediated microvascular recruitment and not myocyte insulin resistance.

Interestingly a publication by Watanabe and colleagues (2005) suggests a link between FFA infusion, endothelial dysfunction and the renin angiotensin system (RAS). In their study the authors demonstrated that acutely elevating FFAs (1 hr) impaired endothelium-dependent, acetylcholine-mediated forearm vasodilation in otherwise healthy human subjects [198]. However, by giving the patients a single dose of either losartan (angiotensin receptor blocker) or perindopril (angiotensin converting enzyme inhibitor) the investigators were able to completely restore acetylcholine-mediated forearm vasodilation in these patients [198]. Moreover, an increase in FFAs did not affect the response to acetylcholine-mediated vasodilation when nitric oxide synthase was inhibited or when vitamin C was co-infused [198]. Together, these data would suggest that FFAs specifically reduce the availability of NO, presumably by the enhancement of oxidative stress possibly mediated by increased AngII activity. Since AngII has previously been reported to inhibit the insulin signalling pathway directly [199-202] or induce endothelial dysfunction by increasing oxidative stress [163, 203], increased FFA concentrations may be able to influence insulin sensitivity by increasing the activity of AngII in the vasculature.

1.2.3.4. Glucosamine infusion

Glucosamine, which increases flux through the hexosamine pathway, has also been shown to induce insulin resistance in a number of tissues including muscle, liver and adipose [204-207]. Whilst muscles incubated with glucosamine display reduced insulin-mediated glucose uptake, infusion of glucosamine has also been shown to induce muscle insulin resistance *in vivo* [205, 206, 208]. As well as metabolic effects, Holmang and colleagues (1999) demonstrated that glucosamine infusion was also associated with attenuation of insulin-stimulated total limb blood flow [209]. Wallis and colleagues (2005) found that acute glucosamine infusion resulted in attenuation of insulin-mediated effects on both total blood flow and microvascular recruitment [208]. This vascular insulin resistance was associated with marked attenuation of insulin-mediated muscle glucose uptake *in vivo* [208]. Together, these data suggest that as well as inducing myocyte insulin resistance, acute glucosamine infusion also attenuates insulin-mediated microvascular recruitment and thus further implicates a role for defects in microvascular insulin action as a contributor to insulin resistance.

1.2.3.5. Endothelin-1 infusion

Increased insulin-stimulation of ET-1 production in the endothelium has been proposed to contribute to the development of insulin resistance [210]. Acute infusion of ET-1 in humans and animal models increases mean arterial pressure and some studies have reported a decrease in blood flow to skeletal muscle, but findings are inconsistent [166, 211-213]. Kolka and colleagues (2005) reported that infusion of ET-1 in the perfused rat hindleg preparation increased vasoconstriction in a dose-dependent manner [214]. At lower doses ET-1 increased oxygen consumption across the hindleg, however, at higher doses oxygen consumption was significantly reduced, suggesting a biphasic effect on blood flow distribution in muscle [214]. Furthermore, infusion of insulin was able to overcome and reverse this ET-1-mediated effect on the vasculature, at both low and high doses, and ET-1 did not affect insulin-mediated muscle glucose uptake in this *in vitro* setting [214]. However, acute infusion of ET-1 in an in the anaesthetised rat preparation has been reported to reduce insulin sensitivity. Ross and colleagues (2007) found that a 30 min systemic infusion of ET-1, prior to hyperinsulinaemic euglycaemic clamp, attenuated insulin-mediated microvascular recruitment associated with reduced skeletal muscle glucose disposal [215]. Together, these studies suggest that while ET-1 may not affect insulin-mediated muscle glucose uptake when assessed independently of insulin's haemodynamic effects, elevated levels of ET-1 *in vivo* may contribute to reduced insulin-mediated microvascular recruitment and thus may limit insulin and glucose delivery to the muscle.

1.2.4. Chronic models of microvascular insulin resistance

While acute treatment with factors such as TNFα and FFAs allow for examination of how individual factors inhibit insulin action directly, a number of chronic models of insulin resistance have also been found to exhibit attenuation of insulin-mediated microvascular recruitment in muscle.

1.2.4.1. Human obesity

Obesity is becoming increasingly prevalent in society and is associated with the development of insulin resistance. It is now well recognised that obese individuals are at an increased risk of developing T2D and/or cardiovascular disease. However, not all obese subjects develop insulin resistance, and the relationship between these two conditions appears to be complex and poorly understood. One factor that has been proposed to link obesity and insulin resistance is impaired endothelial function. Indeed, Laakso and colleagues (1990) first reported that resistance arterioles of obese, insulin resistant subjects exhibited significantly decreased sensitivity to insulin-mediated dilation [12]. Subsequently, a number of studies have reported a relationship between obesity, insulin resistance and attenuation of endothelial function [124, 216, 217]. While these previous studies were limited to assessment of large vessels and thus total flow effects of insulin, more recent advances have identified that the microvascular response to insulin also appears to be reduced during obesity.

de Jongh and colleagues (2004) assessed insulin sensitivity and microvascular function in skin in healthy (BMI < 25kg/m^2) and obese individuals (BMI > 35kg/m^2) during hyperinsulinaemic euglycaemic clamps [218]. The authors reported that obese, insulin resistant subjects exhibited reduced insulin-mediated microvascular recruitment and this correlated strongly with decreased whole body insulin sensitivity [218]. Subsequently, Clerk and associates (2006) assessed insulin-mediated microvascular recruitment in skeletal muscle in healthy (BMI < 25kg/m2) and obese (BMI > 30kg/m2) subjects using the hyperinsulinaemic euglycaemic clamp technique [98]. The authors reported that hyperinsulinaemia increased skeletal muscle microvascular recruitment associated with increased whole body glucose disposal in healthy, lean subjects [98]. In contrast, obese subjects displayed significant attenuation of insulin-mediated microvascular recruitment in muscle and approximately a 50% reduction in insulin-mediated whole body glucose disposal compared to lean subjects [98]. These studies suggest that obesity is associated with impairment in insulin-mediated microvascular recruitment in muscle which may contribute to reduced muscle glucose disposal and thus worsen insulin resistance. However, while this microvascular action of insulin has been demonstrated during hyperinsulinaemic euglycaemic clamp studies, whether impairment of insulin-mediated

microvascular recruitment in muscle has physiological relevance in the development of insulin resistance has not been clear until relatively recently.

Keske and colleagues (2006 and 2009) reported that a mixed-meal stimulates an increase in microvascular perfusion in muscle following 120 min in lean, healthy subjects [54, 219]. The increase in microvascular perfusion in muscle was associated with a concomitant increase in circulating insulin-levels to facilitate post-prandial glucose disposal [54, 219]. Therefore, these data suggest that increased microvascular perfusion in response to a meal is a normal physiological response that occurs in healthy subjects and in part contributes to increased muscle glucose disposal. However, the authors reported that ingestion of the same mixed-meal in obese, insulin resistant subjects failed to increase microvascular perfusion of muscle [219]. Moreover, obese subjects exhibited a 100% higher post-prandial circulating insulin concentration (lean: ~230pmol/L vs. obese: ~450pmol/L) while the blood glucose concentrations were not different between lean and obese groups [219]. Together, these data suggest that obese individuals exhibit impaired insulin-mediated microvascular recruitment in skeletal muscle and therefore may contribute to the post-prandial hyperinsulinaemia, and thus may subsequently worsen insulin resistance.

1.2.4.2. High fat fed rat

For the last 50 years animal models have been used to examine the effects of increased caloric intake on the pathogenesis of obesity and insulin resistance [220-222]. Increasing dietary fat has been shown to induce whole body insulin resistance following just 4 weeks of intervention [223]. Kraegen and colleagues (1986) demonstrated that high fat fed rats exhibit increased body weight and especially fat mass, while only showing mild signs of hyperinsulinaemia and no apparent hyperglycaemia [223]. However, the animals exhibited significant attenuation in whole body insulin resistance and specifically reduced insulin-mediated glucose uptake in skeletal muscle [223]. The main mechanisms of high fat diet induced insulin resistance had been primarily attributed to one of two factors; (i) increased fatty acid metabolite build-up in non-adipose tissues such as muscle that inhibit insulin signalling (reviewed by Kraegen and colleagues 2008 [134]) or (ii)

increased inflammatory cytokine secretion (TNFα for example) by either adipocytes or infiltrating macrophages that can inhibit various actions of insulin (reviewed by Hotamisligil 2006 [169]). Others have proposed a role of the vasculature as another contributor to the development of insulin resistance following high fat feeding. Naderali and Williams (2001) reported that two days of high fat feeding was sufficient to reduce endothelium-dependent vasodilation of third order mesenteric arteries to carbamylcholine when compared to control fed animals [224]. This was present without alterations in body weight, fat mass or plasma glucose and insulin levels [224]. Indeed, the only apparent effect of the high fat diet was an increase in the circulating FFA concentration [224], again implicating increased FFAs as a mediating factor in the development of endothelial dysfunction.

Other studies have revealed that long-term high fat feeding also results in significant attenuation of endothelium-dependent vasodilation in skeletal muscle arterioles [225, 226]. Erdei and colleagues (2006) found that feeding rats a high fat diet (36% fat wt./wt.) for 10 weeks significantly reduced the response to acetylcholine-mediated vasodilation compared to control arteries [226]. The authors found that the reduced acetylcholine response was likely mediated by increased ROS production since the free radical scavenger Tiron was able to augment acetylcholine-mediated vasodilation in arteries from high fat fed, but not control rats [226]. St-Pierre and colleagues (2010) reported that feeding rats a high fat diet for 4 weeks resulted in increased adiposity, hyperinsulinaemia and in the development of whole body insulin resistance [227]. High fat fed rats exhibited impairments in insulin-mediated effects on both total leg blood flow and microvascular recruitment [227]. This vascular insulin resistance was associated with marked reduction of insulin-mediated skeletal muscle glucose uptake compared to control fed animals [227]. Together, these data indicate that defects in the insulin's vascular action may in part be responsible for the insulin resistant state that develops following high fat feeding. Whether this loss of insulin's vascular action is an early event that contributes to the development of high fat-induced insulin resistance is currently not known and requires further examination.

A number of reports have identified a role for the RAS in the development of insulin resistance in the high fat fed model. Treating high fat fed animals with an angiotensin converting enzyme inhibitor has been shown to prevent the development of insulin resistance in high fat fed animals [228-231]. The exact mechanisms responsible for the prevention of insulin resistance were not investigated in these studies but other reports have indicated that increased AngII signalling in the vasculature may contribute to the loss of endothelial function [232]. Visawanad and colleagues (2006) demonstrated that AngII sensitivity was increased in aorta of high fat fed insulin resistant rats [232]. The authors also found that treating these rats with tempol, a free radical scavenger, restored acetylcholine-mediated vasodilation and attenuated AngII-mediated vasoconstriction following high fat feeding [232]. Thus, increased ROS production, possibly due to increased AngII activity, may be an important factor in the development of endothelial dysfunction, contributing to the development of insulin resistance during high fat feeding.

1.2.4.3. Obese Zucker rat

The obese Zucker (OZ) rat is a model of severe obesity that develops T2D. Obesity in these rats is the result of a recessive mutation, designated *fa*, that inactivates the leptin receptor [233]. The leptin receptor controls satiety and as a result the OZ rats cannot respond to leptin and thus hyperphagia ensues. In addition to excess food intake, OZ (*fa*/*fa*) rats have also been shown to exhibit decreased energy expenditure [234]. As a result the OZ rats weigh significantly more that their lean littermates (*Fa*/?) by 4-5 weeks of age [235]. The OZ rats also exhibit dyslipidaemia, hyperglycaemia, hyperinsulinaemia and insulin resistance at an early age and by 12 weeks of age display severe glucose intolerance [236-238]. Furthermore, defects in muscle glucose uptake have been detected by 4 weeks of age in incubated muscle and *in vivo* [239, 240]. Some [241], but not all studies [242] have reported that these animals develop hypertension.

Along with the metabolic dysfunction, OZ rats also develop large and small vessel vascular alterations resulting from endothelial dysfunction [243, 244]. Studies in isolated vessels have revealed that the ability of insulin to attenuate vasoconstriction is impaired

in vessels from OZ rats compared to lean controls [245, 246]. During hyperinsulinaemic euglycaemic clamp, OZ rats exhibit attenuation of both insulin-stimulation of total leg flow and insulin-mediated microvascular recruitment in muscle compared to lean control rats [242]. This vascular dysfunction was associated with marked attenuation of insulin-mediated whole body and skeletal muscle glucose disposal [242]. While it has been reported that a number of factors may contribute to endothelial dysfunction in the OZ rats [247, 248], increased production of ROS appears to be one of the major protagonists [246, 249], possibly mediated by enhancement of vascular AngII activity [163, 250].

Indeed, endothelium dependent vasodilation in response to acetylcholine can be improved by treating vessels from OZ rats with free radical scavengers, ACE inhibitors and neural endopeptidase inhibitors [244, 251-253]. Siddiqui and Hussain (2007) demonstrated that aorta of OZ rats display significant enhancement of AngII vasoconstriction [250]. Since AngII has been reported to increase ROS production in the vasculature [163], the increased endothelial dysfunction in OZ rats may partly be the result of increased AngII-mediated ROS production. This may explain why inhibition of ACE in OZ rats is beneficial to endothelial function. Additionally, inhibition of ACE has also been reported to improve insulin-mediated muscle glucose uptake in OZ rats [254-256]. However, in these previous studies insulin-mediated muscle glucose uptake was assessed using *in vitro* muscle incubation. Whether this effect of ACE inhibitors in OZ rats translates to improved insulin-mediated vascular action and muscle glucose uptake *in vivo* is not known.

1.4. Insulin resistance and the renin angiotensin system

1.4.1. Major components of the RAS

The RAS is crucially involved in chronic regulation of systemic blood pressure [257], and convincing evidence exists that obesity, hypertension and the metabolic syndrome are associated with RAS dysregulation [258]. AngII, the major vasoconstrictor peptide of the RAS is partially responsible for maintaining blood pressure and water and salt homeostasis [257]. Biosynthesis of AngII occurs via the conversion of angiotensinogen

into the partially active angiotensin I by the renin (Fig. 1.5) [257]. Angiotensin I can subsequently be cleaved into the potent vasoconstrictor AngII by ACE, chymase or cathepsin A. ACE has a secondary function that involves the breakdown of bradykinin into its inactive products. AngII has a half-life of approximately 15 sec in the vasculature [259] and can bind to two receptor subtypes, the AT1R and AT2R [260], with the AT1R being the predominant vasoconstrictor receptor [261]. As well as formation of AngII, recent evidence has revealed a second pathway for the RAS that involves the generation of Ang-(1-7), which also has vasoactive properties mediated by the Mas receptor (Fig. 1.5). The formation of Ang-(1-7) involves cleavage of angiotensin I by ACE2 to form Ang-(1-9), and subsequent cleavage of Ang-(1-9) by ACE or neutralendopeptidase 24-11 (NEP). Ang-(1-7) can also be formed directly from cleavage of angiotensin I by prolyl-endopeptidase (PEP) and NEP or from cleavage of AngII by ACE2, PEP and prolyl-carboxypeptidase (PCP).

1.4.1.1 Angiotensin II signalling

AngII binding to either the AT1R or AT2R has demonstrated counter-regulatory roles in the cardiovascular system [262]. Briefly, AngII binding to the AT1R activates G protein–coupled phospholipase C and inositol-1, 4, 5-triphosphate, which increase the intracellular Ca^{2+} concentration, resulting in vasoconstriction (reviewed by Touyz and Schiffrin 2000 [263]). Conversely, AngII binding to the AT2R activates a counter-regulatory pathway to induce vasodilation via activation of the NO/cyclic guanosine-3´, 5´-monophosphate (cGMP) system [264, 265]. AngII–induced vasodilation in various resistance vessels has also been demonstrated to be mediated by AT2R activation of the NO/cGMP pathway and is not subject to desensitization, unlike AT1R–mediated vasoconstriction [266, 267], suggesting that AT1Rs are more reactive/adaptive to changes in disease states. Furthermore, in the presence of AT1R blockade, AngII has been reported to induce sustained vasodilation, which is abolished by the AT2R antagonist PD123319. AngII-mediated vasodilation appears to be mediated by the NO/cGMP pathway [268].

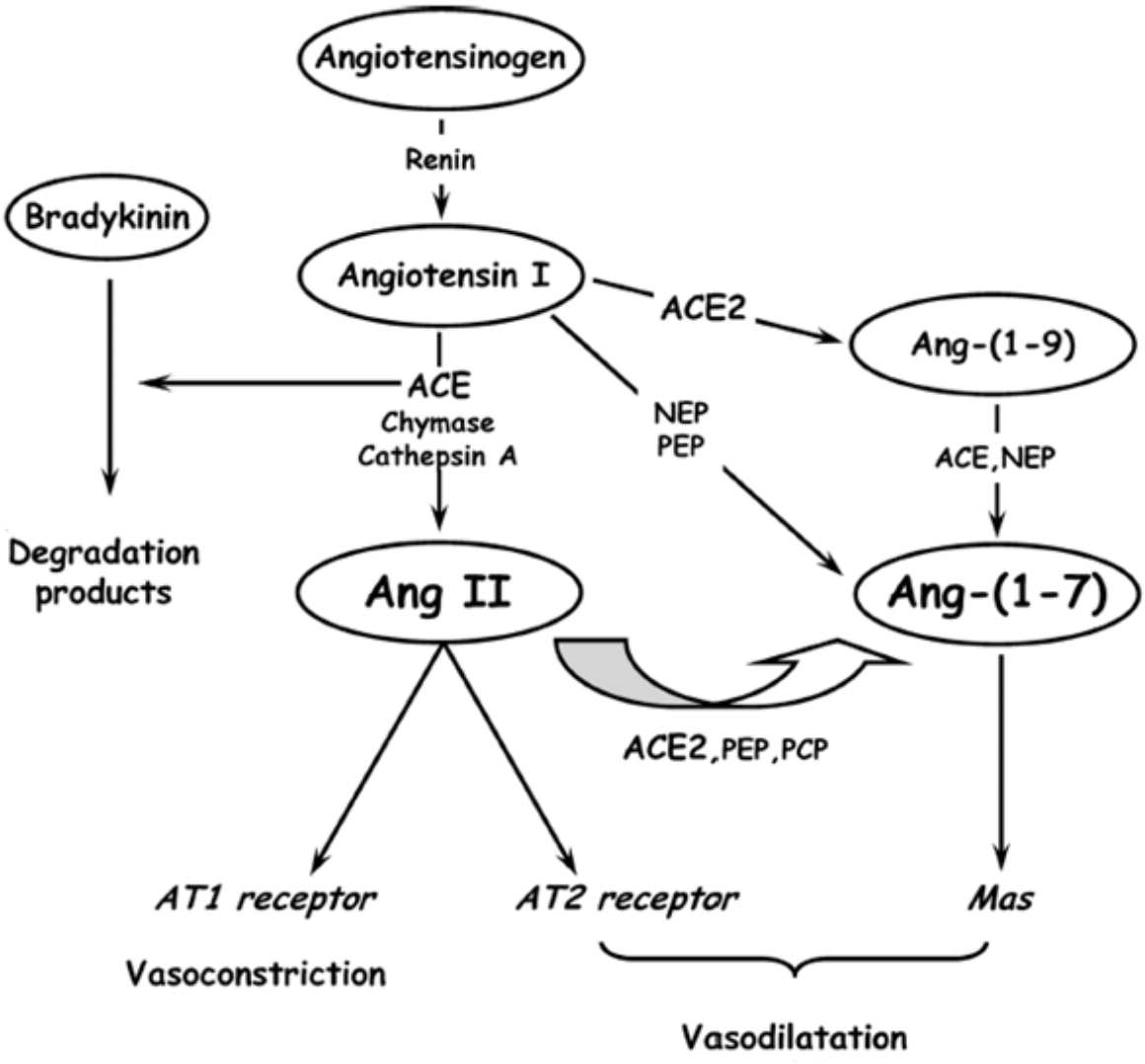

Figure 1.5. The RAS cascade resulting in production of AngII and Ang-(1-7) and subsequent vascular effects.

Angiotensinogen is cleaved by renin, resulting in the formation of angiotensin I. Angiotensin I can be cleaved by ACE, chymase or cathepsin A resulting in the formation of AngII. Formation of AngII leads to the classical actions of the RAS to regulated vascular tone via activation of both AT1 and AT2 receptors. ACE also acts to break down and inactivate bradykinin. ACE2 can breakdown angiotensin I to form Ang-(1-9), which can subsequently be metabolised to form Ang-(1-7) by ACE or NEP. ACE-(1-7) can also be directly formed from either angiotensin I (via NEP or PEP) or AngII (via ACE2 or PEP or PCP). Formation of Ang-(1-7) results in vasodilation via activation of the Mas receptor. Modified from Santos and colleagues 2008 [269].

ACE, angiotensin converting enzyme; AngII, angiotensin II; AT1, AngII type 1 receptor; AT2, AngII type 2 receptor; Mas, Ang (1–7) receptor Mas; PCP, prolyl-carboxypeptidase; PEP, prolyl-endopeptidase; and NEP, neutralendopeptidase 24-11.

Besides regulating intracellular Ca^{2+} concentrations and thus vasoconstriction, AngII binding to the AT1R is intimately involved in activation of tyrosine kinases (extensively reviewed by Mehta and Griendling 2007)[260]. Briefly, AngII can activate both non-receptor-type tyrosine kinases (Src, Fyn, STAT/JAK and focal adhesion kinase), receptor-type kinases (epidermal growth factor, platelet-derived growth factor and insulin receptors) and MAP kinases (ERK 1 and 2, JNK) via activation of the AT1R [260]. Many of these are involved in regulating signalling cascades of a number of hormones, growth factors and cytokines and thus play important roles for distinct molecular cross-talk by AngII. For this reason, increased activation/dysregulation of AngII signalling has been implicated in the pathogenesis of a number of disease states including hypertension, cardiovascular disease, insulin resistance and T2D.

1.4.2. Inhibition of RAS does/does not improve insulin resistance

Angiotensin receptor blockers (ARBs) and ACE inhibitors are commonly prescribed blood pressure lowering drugs that work by inhibiting AngII activity. A number of clinical trials have investigated the effects of these drugs on the development of T2D in hypertensive individuals and produced varying results.

Several clinical trials have reported that treatment with either ACE inhibitors or ARBs improves insulin sensitivity in patients with cardiovascular disease and insulin resistance. Torlone and colleagues (1993) reported that three month treatment with the ACE inhibitor captopril significantly increased muscle glucose disposal during a hyperinsulinaemic euglycaemic clamp in patients with arterial hypertension and T2D [270]. Similarly, Yavuz and colleagues (2003) reported that subjects with essential hypertension and insulin resistance treated with an ACE inhibitor or ARB for 6 months displayed improved flow-mediated dilation and enhanced insulin sensitivity compared to placebo [271]. Aksnes and associates (2006) assessed insulin sensitivity in patients with hypertension and/or cardiovascular disease following 8 week treatment with ARB plus amlodipine (5mg; calcium channel blocker) or amlodipine alone (10mg)[272]. Following the treatment period, insulin sensitivity was assessed by hyperinsulinaemic euglycaemic clamps and patients receiving the combination of ARB plus amlodipine displayed

moderately improved whole body glucose disposal compared to amlodipine alone [272]. Since blood pressure was similarly reduced in both groups, the authors proposed that ARBs improve glucose metabolism at the cellular level beyond what can be expected by the reduced blood pressure lowering effects alone [272].

One of the earliest, long-term clinical trials to report a potential role for inhibition of AngII in improving or delaying new-onset diabetes was the Captopril Prevention Project (CAPPP) trial [273]. In this study the authors found that captopril (ACE inhibitor) treatment for 5 years reduced new-onset diabetes by 30% compared to placebo in patients with hypertension [273]. This potential anti-diabetic effect of ACE inhibition was confirmed in the Heart Outcomes Prevention Evaluation (HOPE) study [274]. In this study, 5 year ramipril (ACE inhibitor) treatment was associated with a 30% decrease in new-onset diabetes and improved metabolic control, assessed by lowed HbA1C levels in patients with cardiovascular disease or T2D plus one other risk factor such as hypertension [274]. The investigators of the Valsartan Antihypertensive Long-term Use Evaluation (VALUE) trial demonstrated no difference in the cardiac outcomes between 4 year of valsartan (ARB) and amlodipine (calcium channel blocker) treatment despite better blood pressure control in the amlodipine group [275]. However, there was a 23% lower incidence of new-onset diabetes in patients treated with valsartan compared to amlodipine [275]. Thus, the authors suggested that the reduction in the incidence of new-onset diabetes observed with ACE inhibitors and ARBs is probably related to blockade of AngII activity rather than a blood pressure lowering effect alone. Similar beneficial effects of ACE inhibitors and ARBs have been reported in the Second Australian National Blood Pressure Study (ANBP-2) study [276], the Studies of Left Ventricular Dysfunction (SOLVD) study [277] and the ACE inhibitor-based versus Diuretic-based antihypertensive Primary Treatment in patients with pre-diabetes (ADaPT) [278].

In contrast to the above studies, the Diabetes REduction Assessment with ramipril and rosiglitazone Medication (DREAM) clinical trial reported that in patients with impaired glucose tolerance, ACE inhibition did not significantly affect the incidence of T2D (18.1%) after three years of follow-up compared with placebo (19.5%) [279]. Because patients with cardiovascular disease or uncontrolled hypertension were excluded from

the DREAM trial, one possible explanation for the discrepancy between the DREAM trial and other clinical trials may be the difference between the patient populations studied. However, it should be noted that participants receiving ramipril were more likely to have regression to relative normoglycaemia than those patients receiving placebo [279]. In addition, at the end of the study, plasma glucose levels two hours after an oral glucose load were significantly lower in the ramipril group, indicative of improved glucose handling [279].

Other smaller clinical trials have also failed to detect improvement in insulin sensitivity following ACE inhibition or ARB treatment. Petrie and colleagues (2000) reported that 4 week trandolapril (ACE inhibitor) treatment did not affect whole body glucose disposal as assessed by the hyperinsulinaemic euglycaemic clamps in hypertensive patients with T2D [280]. Similarly, New and associates (2000) found no effect of ACE inhibition for 19 days on insulin sensitivity during hyperinsulinaemic euglycaemic clamp studies in hypertensive patients with T2D [281]. While it could be argued that the treatment length in these previous studies was not long enough to produce beneficial effects, more recent reports have revealed similar results following longer treatment interventions. Perlstein and associates (2012) assessed insulin sensitivity and endothelial function in abdominally obese, mildly-hypertensive patients with impaired fasting glucose following 8 weeks of ARB treatment [282]. The authors reported that ARB treatment did not affect whole body glucose disposal or endothelial function, measured by hyperinsulinaemic euglycaemic clamp and reactive hyperaemia-peripheral arterial tonometry, respectively [282]. However, the authors did note a significant improvement in β-cell function following ARB treatment compared to placebo [282]. Similarly, Lteif and colleagues (2012) assessed endothelial function and insulin sensitivity in obese, insulin resistant patients without hypertension following 12 weeks of ARB treatment [283]. The authors reported no significant effects of ARB on whole body glucose disposal, leg blood flow or leg vascular conductance during a hyperinsulinaemic euglycaemic clamp compared to the placebo group [283]. However, the authors did note a moderate increase in leg glucose uptake following ARB treatment, suggesting a possible improvement in glucose extraction by muscle [283].

Thus, there is considerable debate regarding whether ACE inhibitors and ARBs prevent the development of T2D in people without cardiovascular disease. Although ACE inhibitors and ARBs show considerable promise as anti-diabetic agents, their effects under a variety of metabolic conditions have yet to be fully elucidated. The discrepancies between these previous findings may be partly attributable to the underlying bias regarding the selection of participants in these various studies. Indeed, most reports where AngII inhibition improved insulin sensitivity were in subjects that exhibited insulin resistance and hypertension or cardiovascular disease [270-272]. In contrast, reports where no improvement in insulin sensitivity was detected following RAS inhibition have largely been in individuals with either severe hypertension and T2D [280, 281] or obese, non-hypertensive insulin resistant subjects [282, 283]. Taken together these data suggest that ACE inhibition or ARB treatment may only be of benefit in insulin resistant individuals that also exhibit cardiovascular disease. Indeed, this proposition would be supported by the results of large clinical trials, such as HOPE and VALUE, where prevention of new-onset diabetes was the primary outcome following long-term inhibition of ACE or ARB treatment in hypertensive, insulin resistant individuals. Thus, it would seem that improving insulin sensitivity by inhibiting AngII in humans requires a specific time-frame where hypertension and insulin resistance co-exit, but without T2D.

1.4.3. RAS components in insulin resistance

1.4.3.1. Angiotensin II and insulin resistance

Increased activity of AngII, specifically via the AT1R, has been implicated in the development of insulin resistance and T2D [284, 285]. Previous studies have indicated that, like insulin, AngII can stimulate tyrosine phosphorylation of the IRS subunits [286]. For this reason studies have demonstrated that acute AngII infusion with insulin generally results in an apparent increase in insulin sensitivity in normal healthy subjects [287, 288]. However, in newly diagnosed T2D patients, Fliser and colleagues (1997) found that acute infusion of AngII attenuated insulin sensitivity [289], suggesting that the insulin sensitising effect of AngII may be limited to states in which the RAS is not dysregulated. Furthermore, this insulin sensitising effect of AngII appears to be limited

to acute infusions only, with many studies identifying that chronic increases in AngII culminate in the development of hypertension and insulin resistance [199, 290, 291].

Chronically increasing AngII activity via the AT1R has been shown to be detrimental to normal insulin signalling in skeletal muscle, endothelial and smooth muscle cells [163, 203, 292-294]. A number of mechanisms appear to be involved in the development of AngII-mediated insulin resistance in these tissues. These include, but are not limited to; (i) increased inhibitory (serine) phosphorylation of the IRS substrate [202] and PI3K [295] likely mediated by AngII activation of JNK and MAP kinase; (ii) induction of NAPDH oxidase [200] resulting in increased ROS production and thus inhibition of insulin signalling and insulin-mediated NO release; (iii) inhibition of GLUT-4 translocation likely mediated by AngII altering either Akt [199] and/or adenosine monophosphate-activated protein kinase (AMPK) [296] activity; and (iv) AngII stimulation of NF-kappaβ which in turn stimulates production of proinflammatory cytokines including TNFα [291] and interleukin-6 [297] that can negatively impact insulin signalling. As well as inhibiting the insulin signalling pathway directly through these various mechanisms, increased AngII activity via the AT1R has also been implicated in the development of endothelial dysfunction. By acting through the AT1R, which itself results in vasoconstriction, AngII can also result in the up-regulation of NAPDH oxidase-derived ROS [200], resulting in reduced NO bioavailability. Together these effects of AngII have previously been proposed to contribute to the development of endothelial dysfunction resulting in enhanced vascular tone and the development of hypertension and vascular insulin resistance [150, 163].

1.4.3.2. Bradykinin and insulin resistance

Bradykinin is degraded by ACE into its inactive products (Fig. 1.5). Bradykinin is a short-lived molecule that participates in vascular dilation via the bradykinin B2 receptor, which is a constitutively expressed G-protein-coupled receptor [298]. ACE inhibition has been proposed to improve insulin sensitivity in muscle via a bradykinin-mediated mechanism [299, 300]. However, there are conflicting reports whether this insulin-sensitising effect also occurs *in vivo*.

In vitro, bradykinin has also been proposed to augment the insulin signalling cascade by mimicking insulin activation of IRS and PI3K in both skeletal and cardiac muscle [301, 302]. This is supported by a report by Henriksen and colleagues (1998) where chronic bradykinin infusion in OZ rats improved insulin-mediated glucose uptake in incubated muscle [303]. The improvement in glucose uptake was largely attributed to bradykinin-NO-mediated activation of GLUT-4 translocation in muscle cells, thus increasing insulin-mediated glucose uptake. Bradykinin has also been reported to enhance NO release via activation of eNOS independently of the insulin signalling pathway, possibly resulting in a synergistic effect on NO production [304]. However, evidence that bradykinin increases insulin sensitivity *in vivo* is not overwhelming.

A number of investigators have reported that insulin stimulates NO production in endothelial cells [104] and thus NO mediates insulin's ability to induce dilation in skeletal muscle vasculature [59, 100, 103]. For this reason, increased NO bioavailability mediated by enhanced bradykinin activity, has also been proposed to improve insulin sensitivity by augmenting total blood flow to muscle *in vivo* [299]. However, increasing bulk blood flow through either insulin-sensitive [37] or insulin-resistant [305] muscle alone does not improve glucose uptake, indicating that muscle glucose uptake is not necessarily dependent of changes in total blood flow. In humans, direct intra-arterial infusion of bradykinin increased total muscle blood flow but failed to improve insulin sensitivity in obese, insulin resistant subjects [306]. Additionally, Mahajan and colleagues (2004) demonstrated that bradykinin infusion in rats increased femoral artery blood flow to a similar extent as methacholine, while only the latter enhanced insulin-mediated microvascular recruitment and muscle glucose uptake [307]. Therefore, it would be logical to assume that while bradykinin can affect total flow, it most likely does not result in increased nutritive perfusion through muscle (microvascular recruitment). However, both of these studies involved acute bradykinin infusions. Whether chronic increases in bradykinin activity, such as occurs during prolonged ACE inhibition, can affect insulin sensitivity *in vivo* is not known.

1.4.3.3. Angiotensin (1-7) and insulin resistance

More recent advances have culminated in the identification of a new sub-branch of the RAS involving the production of shorter angiotensin peptides termed Ang-(1-9) and Ang-(1-7) (reviewed by Santos and colleagues 2008 [269]). Briefly, metabolism of Ang-(1-9) by ACE or NEP (Fig. 1.5) results in the production of Ang-(1-7) and subsequent activation of the Mas receptor. This receptor is ubiquitous in distribution. In the vasculature activation of Mas by Ang-(1-7) has been reported to result in Akt/eNOS-mediated vasodilation [308]. In human endothelial cells activation of the Ang-(1-7)/Mas pathway directly opposes AT1R signalling, resulting in reduced NADPH oxidase-mediated oxidative stress [309]. This would imply that Ang-(1-7) is a counter-regulatory mechanism that may be important in attenuating/preventing AngII-mediated vasoconstriction. Interestingly, Walters and colleagues (2005) also demonstrated that blocking the AT2R reduces Ang-(1-7) mediated vasodilation [310]. These data suggest that as well as activating the Mas receptor, Ang-(1-7) can also stimulate the AT2R and thus induce NO-mediated vasodilation.

While the effects of increased Ang-(1-7) have thus far been poorly studied, one recent report has revealed that chronically infusing Ang-(1-7) can improve insulin resistance. In fructose fed rats, Giani and colleagues (2009) demonstrated that chronic infusion of Ang-(1-7) results in the amelioration of insulin resistance via improved insulin signalling in skeletal muscle, liver and adipose tissue [311]. However, in streptozotocin injection-induced diabetic rats (a model of type 1 diabetes), increasing Ang-(1-7) concentrations accelerated renal injury [312]. Thus, while the potential insulin sensitising effects of the Ang-(1-7)/Mas pathway are attractive, the research regarding this pathway is still in its infancy and further work is required to explain the roles this pathway may play in disease states.

1.4.4. AngII, insulin resistance and microvascular control

Interestingly, however, even though Lteif and colleagues (2012) detected no improvement in either whole body insulin sensitivity or insulin-mediated change in total

leg blood flow following ARB treatment, the authors did report an improvement in leg glucose uptake [283]. This finding has also been observed by Galleti and colleagues (1999) in the human forearm following 8 week ACE inhibition in hypertensive, insulin resistant subjects [313]. The authors reported that while insulin-mediated forearm glucose uptake was significantly improved by ACE inhibition, no such improvement in total forearm blood flow was detected [313]. For these reasons, both previous studies proposed that the improvement in limb glucose disposal was the results of enhanced myocyte insulin sensitivity and not haemodynamic improvement following inhibition of ACE or the AT1R. However, there is convincing evidence that increasing total limb blood flow alone does not necessarily improve limb glucose disposal [37, 38, 50] and that increasing microvascular blood flow in muscle (microvascular recruitment) appears to be more important [61, 62, 65]. Since neither of these previous studies assessed microvascular perfusion in muscle, and based their assessment of insulin's effects on total limb blood flow, whether the improved limb glucose disposal was the result of improvement in insulin-mediated microvascular recruitment in muscle cannot be dismissed. Furthermore, Jonk and colleagues (2011) recently reported that a single dose of irbesartan (ARB) can significantly improve insulin-mediated microvascular recruitment in hypertensive individuals [314]. Therefore, while improvement in myocyte insulin sensitivity cannot be excluded following inhibition of ACE or the AT1R in these previous studies, the improvement in limb glucose uptake may also be in part the result of improved insulin-mediated microvascular recruitment in muscle.

The vascular effects of AngII have been reported to regulate microvascular blood flow in muscle using systemic infusion of AT1R or AT2R antagonists [108]. Chai and colleagues (2009) demonstrated that by antagonising the AT2R, which is responsible for AngII-mediated vasodilation, basal microvascular perfusion in muscle could be significantly reduced [108]. Conversely, antagonising the AT1R, which is responsible for AngII-mediated constriction, increased basal microvascular perfusion of muscle [108]. Thus modulation of AngII action between these two receptors could influence muscle microvascular blood flow in insulin resistance. Subsequently, the same authors demonstrated that antagonism of the AT1R during a hyperinsulinaemic euglycaemic clamp resulted in enhanced insulin-mediated microvascular recruitment, whereas antagonism of the AT2R resulted in reduced insulin-stimulated microvascular

recruitment [109]. Interestingly co-infusion of both antagonists resulted in no net-change in insulin-mediated microvascular recruitment compared to insulin alone [109]. Together the studies by Chai and colleagues indicate that AngII may contribute to the control of microvascular perfusion in muscle at basal and during hyperinsulinaemia, however, this only appears to be important when the activity of one of the receptors is altered, allowing activity of the other receptor to dominate. Moreover, it has been reported that increased expression/activity of the AT1R is associated with the development of enhanced arterial constriction and insulin resistance [315]. This implies that impairment of microvascular perfusion by increased AT1R activity may contribute to the development of insulin resistance

1.5. Summary of aims

Skeletal muscle has been shown to be the main site of insulin mediated glucose uptake in the body and accounts for approximately 75-90% of glucose uptake following a meal [11]. In addition to the classical action of insulin to stimulate glucose uptake in muscle, insulin also enhances flow to the microvasculature to facilitate access of glucose and insulin to the myocyte. Indeed, insulin-mediated microvascular recruitment in muscle has been shown to be responsible for approximately 40-50% of insulin-mediated muscle glucose uptake *in vivo* [100, 167, 177]. Furthermore, in states of insulin resistance such as obesity, this microvascular action of insulin is significantly reduced [98, 219] and is associated with reduced insulin-mediated muscle glucose uptake.

There is mounting evidence that dysregulation of the RAS is associated with the development of insulin resistance and T2D. A number of studies have demonstrated that increased AngII activity can lead to skeletal muscle insulin resistance by directly inhibiting the insulin signalling pathway. Furthermore, it has recently been demonstrated that increased AngII activity, specifically via the AT1R directly impairs insulin-mediated microvascular recruitment in muscle [109]. Whether this effect of AngII to modulate insulin's microvascular actions in skeletal muscle can result in the development of muscle insulin resistance has not been previously investigated. Therefore, the main hypothesis of the current thesis is that dysregulation of the RAS, specifically in states

where there is increased AngII-mediated vasoconstriction via the AT1R, is detrimental to insulin-mediated microvascular recruitment in muscle and results in the development of muscle insulin resistance.

To test this hypothesis three primary aims were developed:

1. To determine whether dysregulation of the RAS attenuates insulin's vascular actions in muscle.

2. To determine whether increased AngII sensitivity/activity attenuates insulin's vascular actions in muscle.

3. To determine whether opposing AngII activity improves insulin's vascular actions in muscle during states of insulin resistance.

CHAPTER 2

MATERIALS AND METHODS

2.1 Animal care

All animals were cared for in accordance to the Australian code of practice for the care and use of animals for scientific purposes (2004, National Health and Medical Research Council). All experiments were approved by the University of Tasmania Animal Ethics Committee.

Two different rat strains were used in the present thesis. Male Sprague Dawley rats were obtained from the University of Tasmania central animal facility. Female obese Zucker rats were obtained from Monash Animal Services, Victoria. All animals were housed in temperature and light controlled conditions (22°C, 12 hr light/12 hr dark cycle) and provided free access to water and commercially manufactured diets. Details of the various dietary and pharmacological interventions are provided in each chapter accordingly.

2.2 Background

Following feeding protocols, rats were subjected to one of two experimental protocols. The first was the *in vivo* anaesthetised rat preparation. This procedure involves surgical cannulation of the carotid artery and both jugular veins during anaesthesia to facilitate blood sampling and intravenous infusions, respectively. The advantage of the procedure is that it allows for determination of whole body responses to a variety of stimuli *in vivo*, including assessment of whole body insulin sensitivity utilising the hyperinsulinaemic euglycaemic clamp procedure [27, 316]. Moreover, this technique has been adapted to allow for assessment of insulin's vascular and metabolic activity in skeletal muscle using various methods [37, 223].

The second protocol utilised was the constant flow pump-perfused rat hindleg preparation [317]. This technique involves surgical isolation of blood flow to one hindleg by ligation of a number of blood vessels in the abdominal cavity. Following blood flow restriction, the descending aorta and ascending vena cava are cannulated to facilitate

perfusion of the hindleg vasculature using a modified Krebs Heneseleit bicarbonate buffer gassed with carbogen. After attachment to the perfusion apparatus, the animals are sacrificed and constant flow through the hindleg is maintained using a peristaltic pump. The advantage of this preparation is that it allows for determination of vascular and metabolic responses to external stimuli, such as infusions of vasoconstrictors or insulin in an intact hindleg vascular system. Moreover, since total flow is maintained at a constant rate, this technique can be utilised to determine insulin's metabolic actions in skeletal muscle irrespective of insulin-mediated effect on flow.

2.3. Anaesthetised rat experiments

2.3.1. Surgical procedure

Following an overnight fast, rats were anaesthetized with an intraperitoneal injection of pentobarbital sodium (50mg/kg body wt.). A tracheostomy was performed to allow spontaneous respiration. Polyethylene cannulas (PE58; Microtube Extrusions, North Rocks, NSW, Australia) were inserted into both jugular veins to facilitate intravenous infusions. A further cannula was inserted into the carotid artery, which was subsequently connected to a pressure transducer (Transpac IV; Abbott Critical Systems, Morgan Hill, CA, USA) to allow for arterial blood sampling and for monitoring of mean arterial blood pressure. Using a 1.5cm incision, the right femoral artery was exposed and carefully separated from the femoral vein and saphenous nerve. Following ligation of the epigastric vessels, an ultrasonic flow probe (VB series 0.5mm; Transonic Systems, Ithaca, NY, USA) was positioned around the femoral artery to allow measurement of total femoral artery blood flow (FBF) (see Fig. 2.1). The flow probe was connected to a flow meter (Model T106, Transonic Systems) and along with the pressure transducer was connected to an IBM compatible PC that recorded blood pressure and FBF (at a frequency of 100Hz) using WINDAQ data acquisition software (DATAQ instruments, Akron, OH, USA). Rat anaesthesia was maintained by continuous infusion of aqueous pentobarbital sodium (0.6mg/min/kg body wt.) through one jugular vein. Body temperature of the rat was maintained at 37°C using a heated pad and heating lamp.

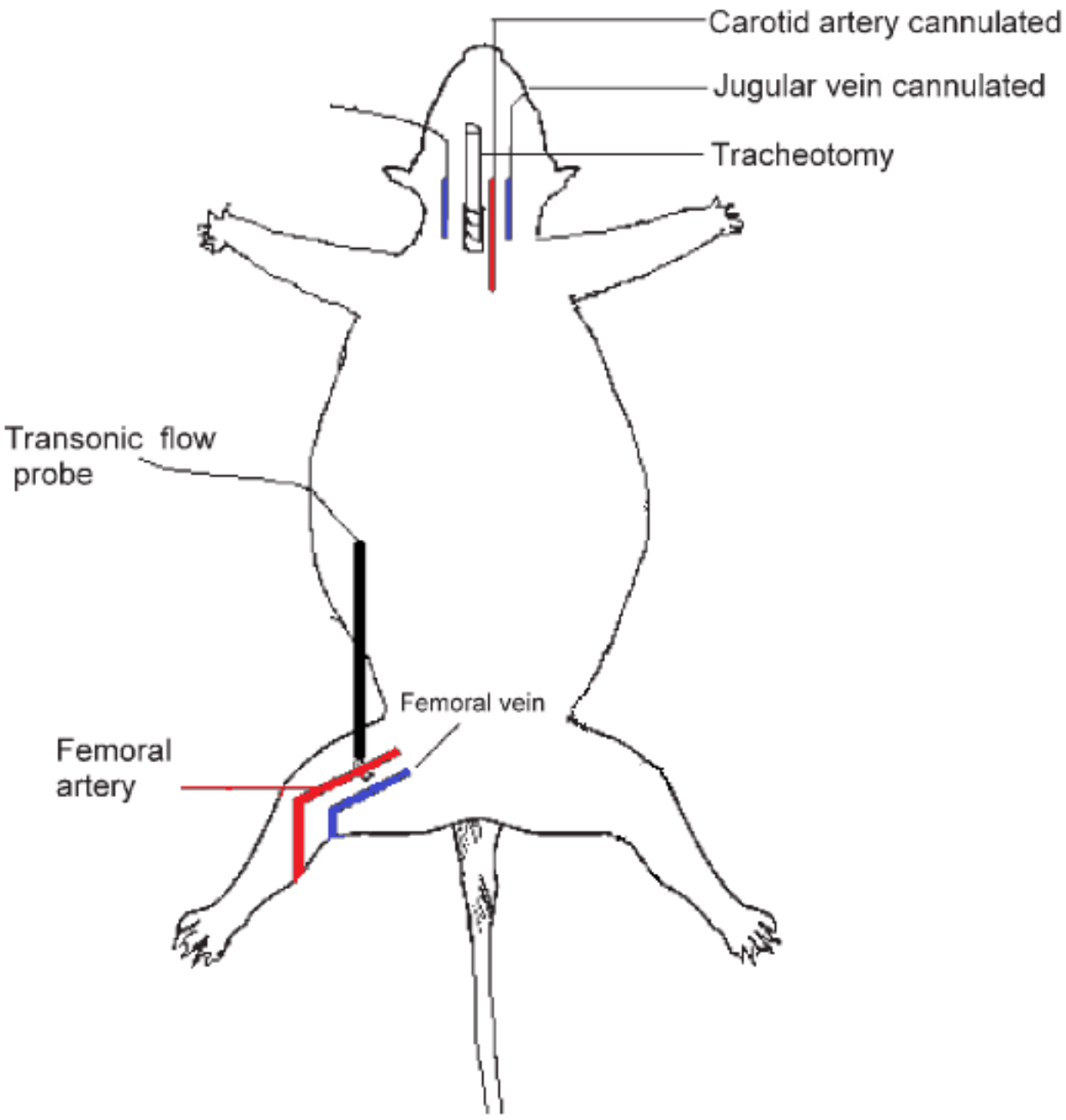

Figure 2.1. Schematic of surgical procedure performed for the anaesthetised rat preparation.

Following tracheotomy, cannulae were inserted into the carotid artery and both jugular veins. The carotid artery facilitated blood pressure and arterial blood/plasma glucose measurements while the jugular veins facilitate intravenous infusions of anaesthetic, insulin/saline and glucose. Surgery was also performed on one hindleg and the femoral artery carefully separated. A transonic flow probe was positioned around the isolated artery to facilitate measurement of FBF. Adapted from Mahajan and colleagues (2004) [307].

2.3.2. Plasma biochemistry

Blood and plasma glucose levels were determined using a glucose analyser (YSI 2300, Yellow Springs Instruments, OH, USA). Plasma insulin concentrations were determined by ELISA (Mercodia AB, Uppsala, Sweden). Fasting plasma FFA levels were determined using an enzymatic kit assay (NEFA C; Wako Pure Chemical Industries, Osaka, Japan). Haematocrit was calculated by dividing blood glucose concentration by the plasma glucose concentration.

2.3.3. Whole body glucose kinetics (Ra and Rd)

Whole body glucose turnover (rate of appearance and disappearance of glucose) was determined using a primed (2µCi), continuous infusion of 3-[^{3}H]-D-glucose (0.1µCi/min, specific activity of 16.6Ci/nmol, Perkin Elmer, IL, USA) along with the saline/insulin infusion. 15min prior to and at the conclusion of the experiment arterial plasma samples (25µL) were collected. The plasma samples were quick frozen in liquid nitrogen, evaporated to dryness to remove H_2O and re-suspended in distilled H_2O (100µL). Biodegradable counting scintillant (3mL, Amersham, Arlington Heights, IL, USA) was added to each sample and [^{3}H]-glucose radioactivity determined using a scintillation counter (Tri-Carb 2800TR, Perkin Elmer). The rate of glucose appearance (Ra) and rate of glucose disappearance (Rd) were calculated using the isotope dilution equation:

$$Ra + GIR = Rd = F/SA$$

Where

GIR = Glucose infusion rate

F = rate of tracer infusion

SA = Specific activity of glucose, calculated by dividing the plasma radioactivity by the plasma glucose concentration.

Hepatic glucose production (Ra) was calculated by subtracting the GIR from the Rd as previously described [318].

2.3.4. Muscle glucose uptake

Hindleg glucose uptake (HGU) was calculated as the end A-V glucose difference multiplied by FBF and expressed as µmol/min. Muscle specific glucose uptake was assessed by uptake of 2-deoxy-D-[1-^{14}C]glucose (2DG; 0.1mCi/mL; Perkin Elmer) as described previously [227, 319]. Briefly, 45 min prior to the conclusion of the experiment a 200µL bolus of 2DG was given (20µCi). Arterial plasma samples (25µL) were collected 5, 10, 15, 30 and 45 min after the 2DG bolus to assess plasma clearance of 2DG. At the conclusion of the experiment calf muscle was freeze clamped in liquid nitrogen, excised and kept at -80°C until required. The frozen muscle was powdered under liquid nitrogen and approximately 100mg of muscle powder was homogenised with 1.5mL of distilled water using a Heidolph silent crusher M at 26,000 RPM. The homogenate was centrifuged at 13,000 RPM at 4°C for 10 min and 1mL of supernatant was assessed for free and phosphorylated 2DG using an anion exchange column (AG-1X8; Bio-Rad Laboratories, CA, USA). Biodegradable counting scintillant (Amersham) was added to each radioactive sample and radioactivity was measured using a scintillation counter (Perkin Elmer). From this measurement and the specific activity of 2DG in plasma, the rate of glucose uptake (R`g), reflecting the glucose uptake by muscle, was calculated as previously described by others [319] and expressed as µg/g/min.

2.3.5. Skeletal muscle microvascular perfusion

Microvascular perfusion in muscle was assessed by metabolism of exogenously infused 1-methyl xanthine (1-MX) as previously published [37, 227]. Briefly, a bolus of allopurinol (10µmol/kg; Sigma Aldrich) was given 5min prior to 1-MX infusion to partially inhibit the activity of xanthine oxidase to ensure a constant saturating arterial level of 1-MX. Infusion of 1-MX (0.4mg/min/kg body wt.) was initiated 60min prior to the end of the experiment. At the end of the experiment 100µL of arterial plasma was added to 20µL of perchloric acid (2M) to precipitate the proteins. Hind-leg venous plasma, obtained from the femoral vein using a 29G needle, was collected and 100µL was mixed with 20µL of perchloric acid (2M). The samples were centrifuged at 13,000 RPM for 10 min and the supernatant assessed for 1-MX and oxypurinol concentrations

using reverse-phased high-performance liquid chromatography as previously described [77]. The metabolism of 1-MX in nmol/min was calculated using the following equation:

1-MX metabolism = ([1-MX]a – [1-MX]v) x plasma FBF

Where [1-MX]a and [1-MX]v are the plasma concentrations of 1-MX (µmol/L) obtained from arterial and venous blood samples respectively. Plasma FBF was used to calculate 1-MX metabolism to adjust for any differences in haematocrit between the groups. Plasma FBF was calculated as the total FBF multiplied by the haematocrit.

2.4. Isolated perfused hindleg experiments

2.4.1. Perfusion media

The media used for all perfusions was an erythrocyte free preparation with modified Krebs Heneseleit bicarbonate buffer.

Krebs Henseleit Buffer

118mM	NaCl
4.74mM	KCl
1.19mM	KH_2PO_4
1.18mM	$MgSO_4$
25mM	$NaHCO_3$
8.3mM	D-glucose

Along with the compounds above the Krebs buffer also contained 40g/L bovine serum albumin (BovoStar fraction V, Bovogen Biologicals, Victoria, Australia). Once all ingredients were dissolved the buffer was filtered under pressure through a 0.45µm pore size and kept at -20°C until required. When required the buffer was defrosted, gassed for >30min with carbogen (95% O_2: 5% CO_2) before $CaCl_2$ was added to a final concentration of 2.54mM.

2.4.2. Surgical preparation of perfused hindlimb experiments

Surgery was performed as outlined by Ruderman and colleagues (1971) [317] with additional modifications as detailed by Colquhoun and colleagues (1988) [320]. Following an overnight fast, rats were anaesthetized with an intraperitoneal injection of pentobarbital sodium (50mg/kg body wt.). Using strong twine, the base of the tail, closest to the anus, and the tarsus of the foot to be perfused were ligated tightly to restrict blood flow. A midline abdominal incision was made and skin reflected. The abdominal wall was incised from the pubic syphysis to the xiphoid process. The superior epigastric vessels and iliolumbar vessels were ligated on both the left and the right side of the rat. Ligatures were placed around the internal spermatic vessels, the seminal vessels and all other vessels supplying the testes, the neck of the bladder and the seminal vesicles. The testes and seminal vesicles were excised and the bladder drained of urine. Two ligatures were placed around the descending colon, proximal to the mesenteric arteries, with a small gap between them. An incision was made through the descending colon between the two ligatures and the intestine and connective tissue were separated to the level of the renal vessels. Another ligature was places around the duodenum, including the superior mesenteric vessels. Following this the entire gastrointestinal tract was removed from the visceral cavity by incising directly below the duodenal ligature. Flow was restricted to the contra-lateral leg by isolating and ligating the common iliac vessels supplying that leg. Two ligatures were placed around each of the iliolumbar vessels and tightly ligated to restrict flow. The aorta and vena cava were carefully separated using cotton tipped swabs. Once the vessels were isolated, two pairs of surgical grade silk ligatures were loosely placed around each vessel, the first just above the iliolumbar vessels and the second pair directly below the renal vessels. 0.1ml/100g of body wt. of Heparin (1000IU/ml, porcine mucous; Hospira Australia, Mulgrave, VIC, Australia) was injected

into the vena cava at the junction of the renal vein and allowed to circulate through the rats vasculature. The superior ligature around the vena cava was tied off and the vena cava was cannulated using an 18G catheter containing a 20G needle. Once the cannula was inside the needle was retracted and the cannula was inserted until it was positioned approximately at the aortic bifurcation, where it was secured in place using the lower silk ligature. Next, the superior ligature of the aorta was tied off and a small incision was made in the vessel wall. The aorta was then cannulated similarly to the vena cava using a needle threader and a 20G cannula filled with 0.9%NaCl and positioned at the same level as the vena cava cannula. 0.9% NaCl solution was pushed through the aortic cannula to ensure appropriate surgical preparation. See figure 2.2 for further details.

Once cannulation was complete the rat was moved to the perfusion cabinet and the aortic cannula was connected to the line supplying oxygenated perfusion buffer. The exit of buffer from the venous cannula was monitored to ensure that no blood clots formed within the exit line. Once fully connected to the perfusion apparatus the rat was euthanized with an intracardiac Pentobarbitone injection and a twine ligature was tied around the rat at the level of the L3 vertebra to prevent and blood flow to the rest of the body. The entire surgical procedure was completed within 20 min and at least 30 min was allowed for equilibration before any procedure was begun.

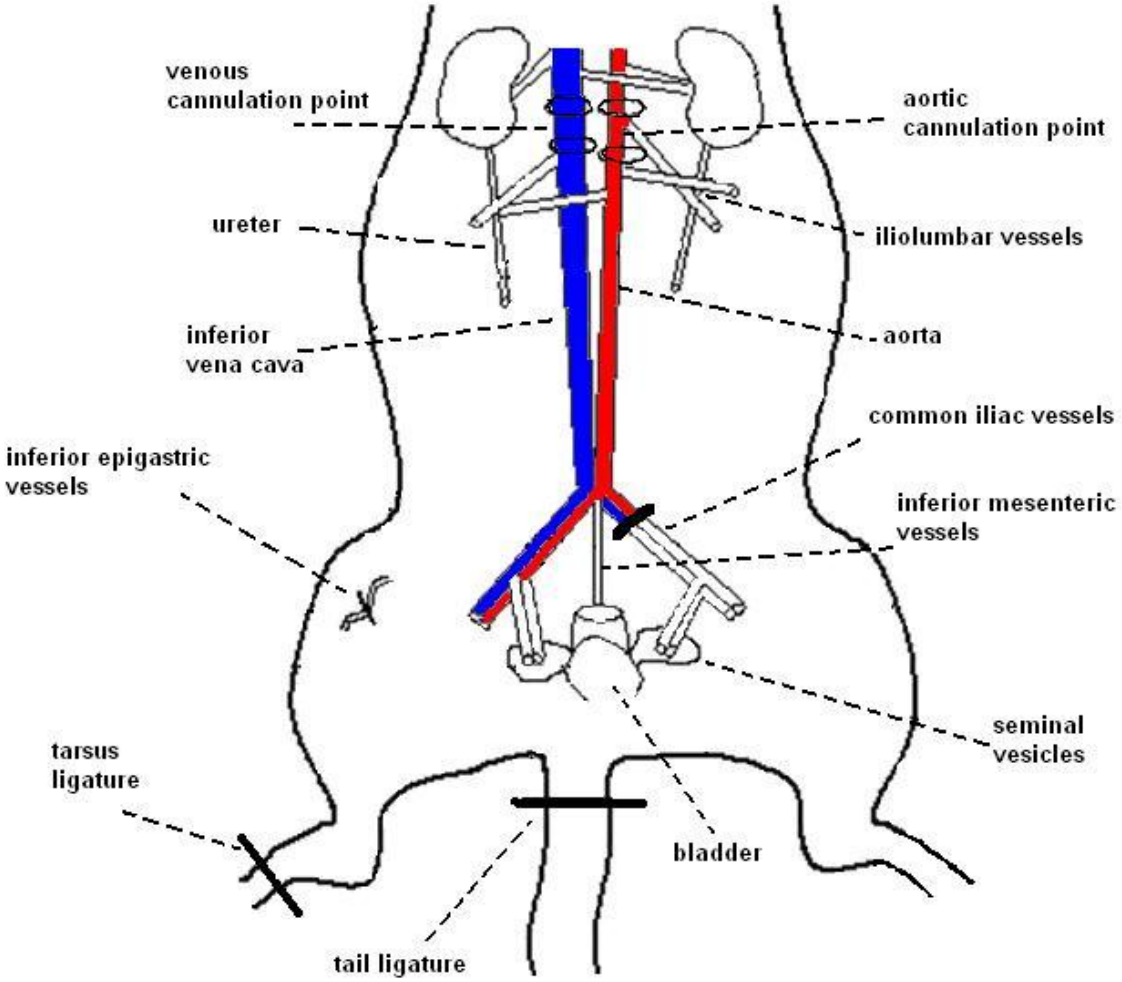

Figure 2.2. Schematic of the surgical isolation of blood flow to a single hindleg.

A schematic of the vessels, and organs, that are ligated during the surgical preparation of the isolated perfused rat hindleg (see text in section 2.4.2 for further details). Modified from Dora (1993) [321]. Following surgery flow was permitted to only go through the isolated hindleg, in this case the right hindleg.

2.4.3. Perfusion apparatus

A non-recirculating perfusion was performed using a peristaltic pump (Masterflex, Cole-Palmer, USA) at a constant flow rate of 8mL/min (~0.4 ml/min/g muscle wt.) at 32°C. The femoral artery flow rate *in vivo* in a healthy animal is approximately 1mL/min. As such, 8mL/min was chosen in this preparation to ensure adequate delivery of oxygen to the hindleg. The perfusion medium was continuously stirred, gassed with carbogen and the temperature maintained at 32°C using a glass, water-jacketed heat exchanger prior to entering a Silastic lung (also gassed with carbogen to maintain constant arterial pO_2). The arterial pressure was continuously monitored using a pressure transducer proximal to the aortic cannula, and any changes in perfusion pressure reflected changes in vascular resistance. The perfusion pressure was recorded throughout each perfusion using WINDAQ® data acquisition software (DATAQ Instruments Inc., USA).

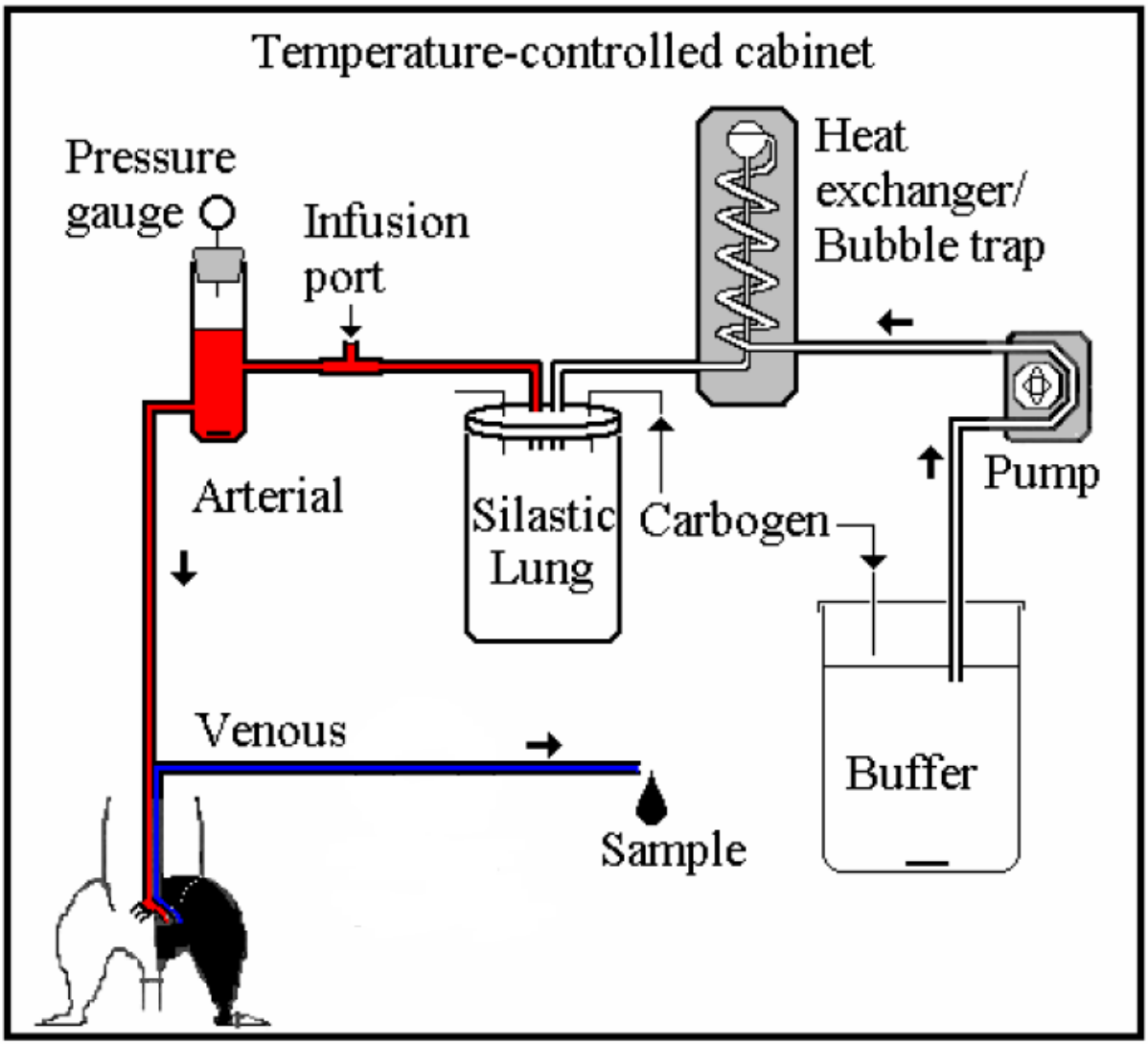

Figure 2.2. Perfusion apparatus for single hindleg perfusion experiments.

All perfusions were conducted at 32°C with erythrocyte free perfusion media (see text in section 2.4.3 for further details). Modified from Dora and colleagues (1993) [321].

CHAPTER 3

HIGH SALT DIET INDUCES MICROVASCULAR INSULIN RESISTANCE IN MUSCLE *IN VIVO*

3.1. Introduction

As described in Chapter 1, the RAS is an enzymatic pathway that regulates the production of AngII, an important regulator of blood pressure and cardiovascular homeostasis. AngII can bind to both type I (AT1R) and type II (AT2R) receptor subtypes. It is generally accepted that the major physiological responses to AngII are mediated by AT1R receptors which cause vasoconstriction, sodium retention, free radical production and cell proliferation [322]. However, AngII can also activate AT2R receptors which generally counteract the effects of AT1R receptors [322]. Investigations have uncovered the RAS pathway in almost every tissue including heart, blood vessels, skeletal muscle, adipose tissue, brain and the pancreas [322, 323], suggesting a broader role for the RAS than previously thought. Furthermore, dysregulation of RAS (usually increased activity) has been implicated in the development of a number of diseases including hypertension, insulin resistance and T2D [284, 323]. Specifically, overactivity of AngII via the AT1R has been shown to be detrimental to normal insulin action in adipose tissue, skeletal muscle and the vasculature [163, 203, 292-294]. In addition, ACE inhibition and ARB treatment in a number of animal models has provided more definitive evidence for involvement of AngII in the development of insulin resistance [163, 254, 324].

A number of animal models have been developed that display insulin resistance predominantly due to increased RAS activity. Most of these are the product of either increased systemic or local tissues RAS activity. These models include the TGR (mREN-2) rat, the chronically infused AngII rat and the high salt (HS) fed rat. The TGR (mREN-2) rat is genetic model of an overactive RAS developed by transfection of a second renin gene (mouse), thus increasing the renin expression and in turn resulting in an increase in other RAS components such as AngII. These rats exhibits severe hypertension associated with increased AngII concentrations and develop insulin resistance associated with reduced skeletal muscle insulin sensitivity [203]. Habibi and colleagues (2008) reported that these rats exhibit alterations in pancreatic function resulting from increased ROS production and therefore develop hyperinsulinaemia [325]. Similarly, the chronic AngII infused rat has also been reported to exhibit insulin resistance in skeletal muscle [290]. However, similar to the TGR (mREN-2) rats, these animals exhibit significant AngII-

mediated hypertension and vascular remodelling following as little as 4 weeks of AngII infusion [326]. In contrast, increasing dietary salt has been reported to result in either mild [327] or no [328, 329] effects on blood pressure and development of hypertension in this model appears to be largely dependent on the degree of salt sensitivity of the animals. However, 4-8 week HS fed rats have been reported to exhibit skeletal muscle insulin resistance associated with endothelial dysfunction and increased tissue RAS activity [327-330]. For these reasons the 4 week HS fed rats was chosen to investigate the effects of increase RAS activity on microvascular and skeletal muscle insulin sensitivity.

It has been reported that AngII is significantly more abundant in the arterial wall than circulating AngII, is independently regulated, and has an important role in pathophysiology of vascular disease [331-333]. However, early perspectives on the links between dietary HS and the RAS were dominated by the notion that HS intake reduces systemic RAS activity. This perspective, however, was solely based on circulating activity of RAS components such as renin and AngII, which indeed decrease following increased salt intake, but the contribution of local AngII generation and activity was largely ignored. However, emerging evidence indicates that HS intake up-regulates the AngII precursor, angiotensinogen, enhances angiotensin I conversion into AngII by ACE, and increases AngII signalling in cells, culminating in increased NADPH oxidase activity within the arterial wall [163, 334-336]. HS intake has also been reported to increases aortic AT1R mRNA, AT1R density, and AngII binding capacity *in vivo* [330, 337, 338]. Furthermore, incubation of vascular smooth muscle cells in an increased salt concentration results in time-dependent elevation of AT1R mRNA levels [330]. Nickenig and colleagues (1998) also noted that the HS-induced AT1R up-regulation led to an enhanced functional response of vascular smooth muscle cells upon stimulation with AngII, via an increase of intracellular Ca^{2+} in response to the HS concentration [330]. Thus, it would seem that increased salt can directly induce vascular AT1R up-regulation *in vitro* as well as *in vivo*.

A number of studies have demonstrated that HS feeding also results in the development of whole body and skeletal muscle insulin resistance [327, 339, 340]. The mechanisms

responsible for the development of insulin resistance following HS feeding remain unclear. However, reports have indicated that HS feeding also leads to endothelial dysfunction by reducing NO bioavailability [329, 341-343] and increasing vascular AngII activity [163, 330, 338, 344, 345]. Since insulin's effects on the vasculature are mediated at least in part by NO [59, 100, 103], HS intake, via increased AngII activity, has been reported to be detrimental to insulin-mediated vasodilation [163]. However, whether reduced insulin-mediated microvascular recruitment may contribute to the development of insulin resistance in the HS fed rat is not currently known. Therefore, the aim of the current study was to determine whether feeding rats a HS diet attenuates insulin-mediated microvascular recruitment in muscle and contributes to skeletal muscle insulin resistance.

3.2. Materials and Methods

3.2.1. Animals

Male Sprague Dawley rats (4 weeks of age) were obtained from the University of Tasmania Central Animal Facility. Animals were provided with semi-purified diets (AIN-93G, Specialty Feeds, Glen Forest, WA). On arrival, the rats were split into two groups and provided either a control (0.31% NaCl wt./wt.; CTRL,) or high salt (8.00% NaCl wt./wt.; HS) diet *ad libitum* for 3-4 weeks (Table 3.1). Water was freely available.

3.2.2. Anaesthetised rat experiments

3.2.2.1. Experimental procedure

Surgery was performed as outlined in chapter 2, section 2.3.1. Following surgical preparation, 1 hr was allowed for FBF and mean arterial blood pressure to stabilise, at which time a 2 hr infusion of either saline (10μL/min) or insulin (10mU/min/kg) was initiated. Since insulin stimulates glucose disposal, a 30% glucose solution (wt./vol.) was infused at a variable rate to maintain fasting blood glucose concentrations over the course of the experiment. For this reason, arterial blood glucose levels were assessed every 10 min in the first hour, and every 15 min in the second hour using a glucose

analyser (YSI 2300) and the glucose infusion rate (GIR) was adjusted accordingly. See Fig. 3.1. for a more detailed protocol. Plasma biochemistry, whole body glucose kinetics (Ra and Rd), muscle glucose uptake and microvascular recruitment were assessed as outlined in chapter 2, sections 2.3.2-5.

3.2.3. Xanthine oxidase activity

Homogenising Buffer

50mM	Na_2HPO_4
0.1mM	EDTA (K^+)
4.0mM	Dithiothretiol
0.5mg/mL	Trypsin Inhibitor

pH was adjusted to 7.4 using orthophosphoric acid.

Xanthine oxidase (XO) activity in skeletal muscle was assessed as previously described [346]. Briefly 400mg of muscle was homogenised in 2mL of homogenising buffer and samples were centrifuged at 50 000*g* at 4°C for 30 min (Sorvall WX Ultra 90, Thermo Electron Corporation, Ashville, NC, USA). Homogenate was crudely fractionated using a de-salting column (Sephadex G-25 medium; GE Healthcare, Rydalmere, NSW, Australia) and 1mL fractions were sequentially collected. Fractions 4 and 5 collected the proteins and were assayed for XO activity. 100µL from either fraction 4 or 5 were added to 900µL of assay buffer (homogenising buffer with 0.1mM xanthine). The mixed solutions were incubated for 1 hr at 37°C and the reaction stopped by adding 250µL of the assay mix to 50µL of 2M perchloric acid. Production of uric acid was measured using HPLC (Gemini 5µm C18, 255 x 4.6mm column) with 50 mM $NH_4H_2PO_4$ buffer at pH 3.5 (orthophosphoric acid) using a flow rate of 1.2 mL/min. The total protein content of each fraction was determined using the Bradford protein assay (Bio Rad, Hercules, CA, USA). Activity of XO was expressed as the amount of uric acid produced per mg of protein per min (pmol/mg of protein/min).

	CTRL	HS
Protein	19.4 %	19.4 %
Carbohydrate	68 %	60 %
Fat	7.0 %	7.0 %
Crude Fibre	5.1 %	3.2%
NaCl	0.31 %	8.00 %
Total Digestible Energy	16.1 MJ/Kg	15.5MJ/Kg

Table 3.1. Macronutrient composition of the control (CTRL) and 8% salt (HS) diets as wt./wt.

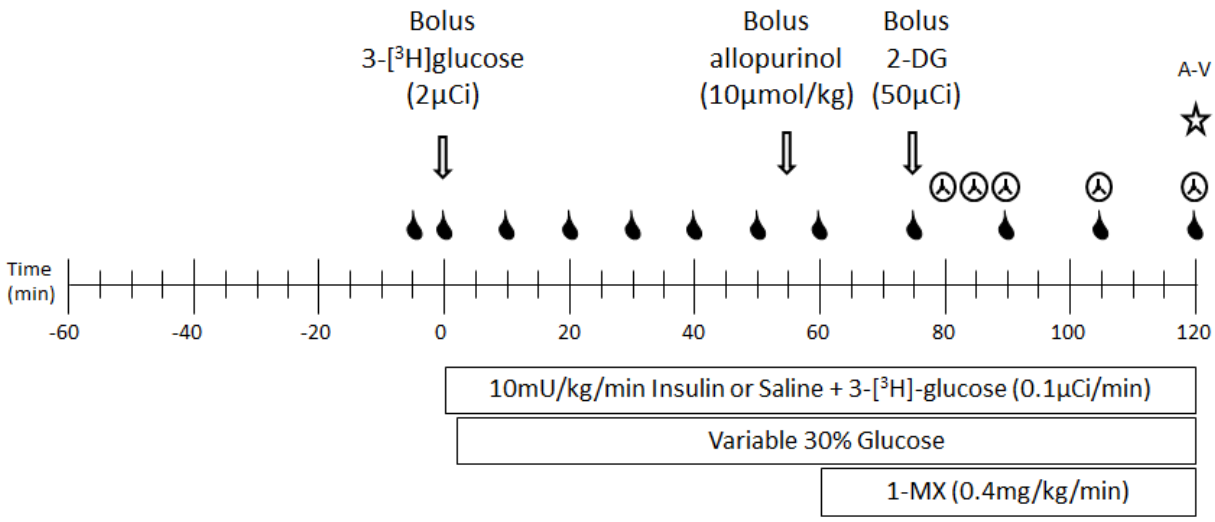

Figure 3.1. Experimental protocol for hyperinsulinaemic euglycaemic clamp.

After surgical preparation and 60 min of equilibration, a continuous infusion of saline (10µL/min) or insulin (10 mU/min/kg) was commenced and continued for 120 min. A 30% (wt./vol.) glucose infusion was initiated shortly after the commencement of the insulin infusion to maintain baseline glycaemia as assessed by arterial blood glucose sampling (). After 55 min of saline or insulin infusion, a bolus of allopurinol (10 µmol/kg) was administered. At 60 min, infusion of 1-methylxanthine (1-MX, 0.4 mg/kg/min) was initiated and maintained until the end of the experiment. At 75 min a bolus of 2-deoxy-D-[1-^{14}C]-glucose (2-DG; 20µCi) was administered. Radioactive plasma samples () were collected at 80, 85, 90, 105 and 120 min to determine the plasma 2-DG clearance. At the conclusion of the experiment arterial and femoral vein plasma samples ( ) were collected for the determination of hindleg glucose uptake and 1-MX metabolism. Immediately following sacrifice, calf muscle (gastrocnemius, plantaris and soleus group) was freeze clamped in liquid nitrogen and stored at -80°C.

3.2.3. Data and Statistics

Data are presented as the means ± SEM and statistics were performed using SigmaStat (Systat Software Inc, 2004). Comparisons between CTRL and HS fed rats were made using un-paired Student's t-test. Comparison between SAL and INS measurements in each group were performed by one-way ANOVA. Comparison of time-series measurements in each group was performed by two-way repeated measures ANOVA. When a significant difference of $p<0.05$ was found, pairwise comparisons by Student-Newman-Keuls test was used to assess treatment differences.

3.3. Results

3.3.1. Physical and biochemical characteristics of rats following 3-4 week feeding intervention

Following 3-4 weeks of dietary intervention there was no difference in body weight or epididymal fat mass between the CTRL and HS groups (Table 3.2). After an overnight fast there was no difference in plasma glucose, insulin, FFA or lactate between the CTRL and HS animals. During anaesthesia, no difference in mean arterial blood pressure was detected between the two groups. However, the HS fed animals exhibited a 46% lower basal FBF and a 95% higher FVR when compared to CTRL ($p<0.001$). The calculated haematocrit was also increased in HS fed rats compared to compared to CTRL ($p<0.001$).

	CTRL	HS	p value
Body weight (g)	227 ± 4	230 ± 6	0.721
Epididymal fat pad (g)	1.20 ± 0.07	1.28 ± 0.09	0.546
Mean arterial pressure (mmHg)	102 ± 2	101 ± 2	0.640
Basal FBF (mL/min)	0.98 ± 0.04	0.53 ± 0.02	<0.001
Basal FVR (mmHg.min/mL)	109 ± 6	195 ± 10	<0.001
Haematocrit (%)	43.6 ± 0.5	49.2 ± 0.9	<0.001
Fasting plasma glucose (mmol/L)	6.52 ± 0.13	6.24 ± 0.17	0.192
Fasting plasma insulin (pmol/L)	86 ± 8	97 ± 9	0.496
Fasting plasma lactate (mmol/L)	0.80 ± 0.03	0.79 ± 0.04	0.266
Fasting plasma FFA (mmol/L)	0.71 ± 0.03	0.67 ± 0.03	0.383

Table 3.2. Physical and biochemical characteristics of rats following 3-4 week feeding intervention.

Measures were collected immediately prior to the commencement of the saline infusion or insulin clamp. Epididymal fat pads were excised and weighed at the conclusion of the experiments. FBF; femoral artery blood blow, FVR; femoral artery vascular resistance, FFA; free fatty acids. Data are means ± SEM for n=10-20 rats in each group. Comparisons between groups were made using un-paired Students t-test.

3.3.2. Effects of HS diet on whole body insulin sensitivity

Feeding rats a HS diet appeared to reduce blood glucose measures and insulin sensitivity compared to CTRL fed rats. During the hyperinsulinaemic euglycaemic clamp procedure the blood glucose concentrations in the HS groups tended to be lower than the respective CTRL groups (Fig. 3.2.A, $p<0.05$). This was likely the result of the increased haematocrit following HS feeding since plasma glucose concentrations were not different between the two groups (Table 3.2). The glucose infusion rate (GIR) required to maintain euglycaemia during insulin infusion (a measure of whole body insulin sensitivity) was significantly lower in the HS-INS group compared to the CTRL-INS groups from 30 min until the conclusion of the insulin clamp. Following 2 hrs of insulin infusion the GIR in the HS-INS group was approximately 20% lower than in the CTRL-INS group (Fig. 3.2.B; 19.4 ± 0.4 vs. 16.1 ± 0.5 mg/kg/min; $p<0.001$).

Reduced whole body glucose disposal (Rd) was evident without development of liver insulin resistance (Ra) following HS intervention. Compared to saline, insulin infusion markedly increased Rd ($p<0.001$) in both CTRL (Fig. 3.3.B; 7.5 ± 0.5 vs. 23.5 ± 0.5 mg/kg/min) and HS (6.4 ± 0.5 vs. 18.3 ± 0.5 mg/kg/min) groups. While no difference in Rd was detected during saline infusion between CTRL and HS fed rats (7. 5 ± 0.5 vs. 6.4 ± 0.5 mg/kg/min, $p=0.158$), the insulin-mediated increase in Rd was higher in CTRL compared to HS fed rats ($p<0.001$).

No difference in Ra was detected between the CTRL and HS animals following 2 hr of saline infusion indicating that the basal liver glucose output was similar between the two groups (Fig. 3.3.A; 7.5 ± 0.5 vs. 6.4 ± 0.5 mg/kg/min; $p=0.076$). The rate of liver glucose output following 2 hrs of insulin infusion was significantly ($p<0.001$) supressed in the CTRL (7.5 ± 0.5 vs. 4.1 ±0.4 mg/kg/min) and HS (6.4 ± 0.5 vs. 2.1 ± 0.4 mg/kg/min) groups. Interestingly, compared to CTRL, insulin-mediated inhibition of liver glucose output was markedly greater in the HS group ($p=0.002$) indicating that the HS rat model has enhanced liver insulin sensitivity.

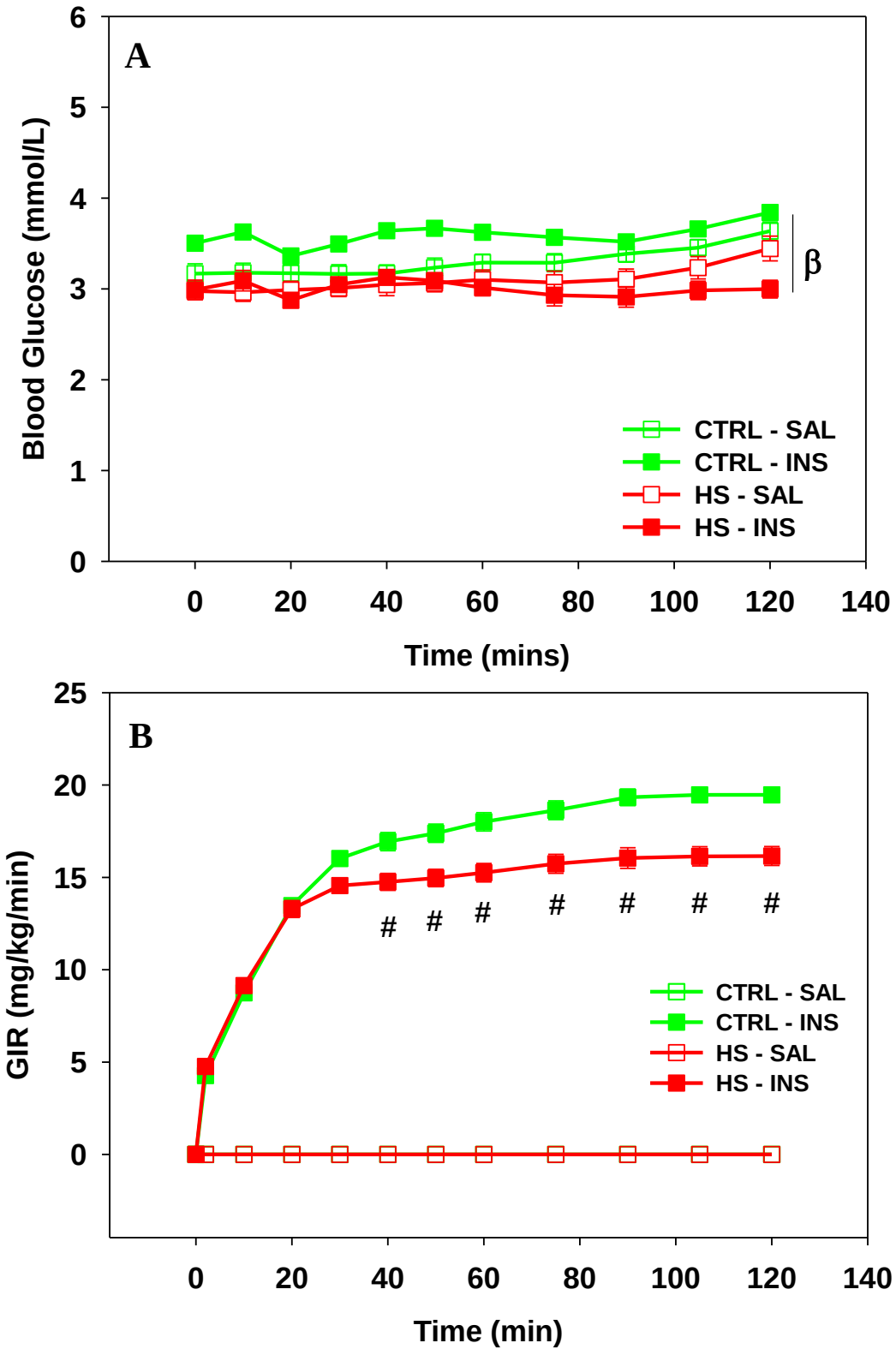

Figure 3.2. Dietary effects on whole body insulin sensitivity.

Blood glucose (A) and glucose infusion rate (GIR; B) during saline infusion or insulin clamp of CTRL and HS rats. Data are means ± SEM for n=10-12 rats in each group. β CTRL-INS significantly different ($p<0.05$) compared to HS-INS at every time point. # Significantly different ($p<0.05$) from CTRL-INS using two-way repeated measures ANOVA.

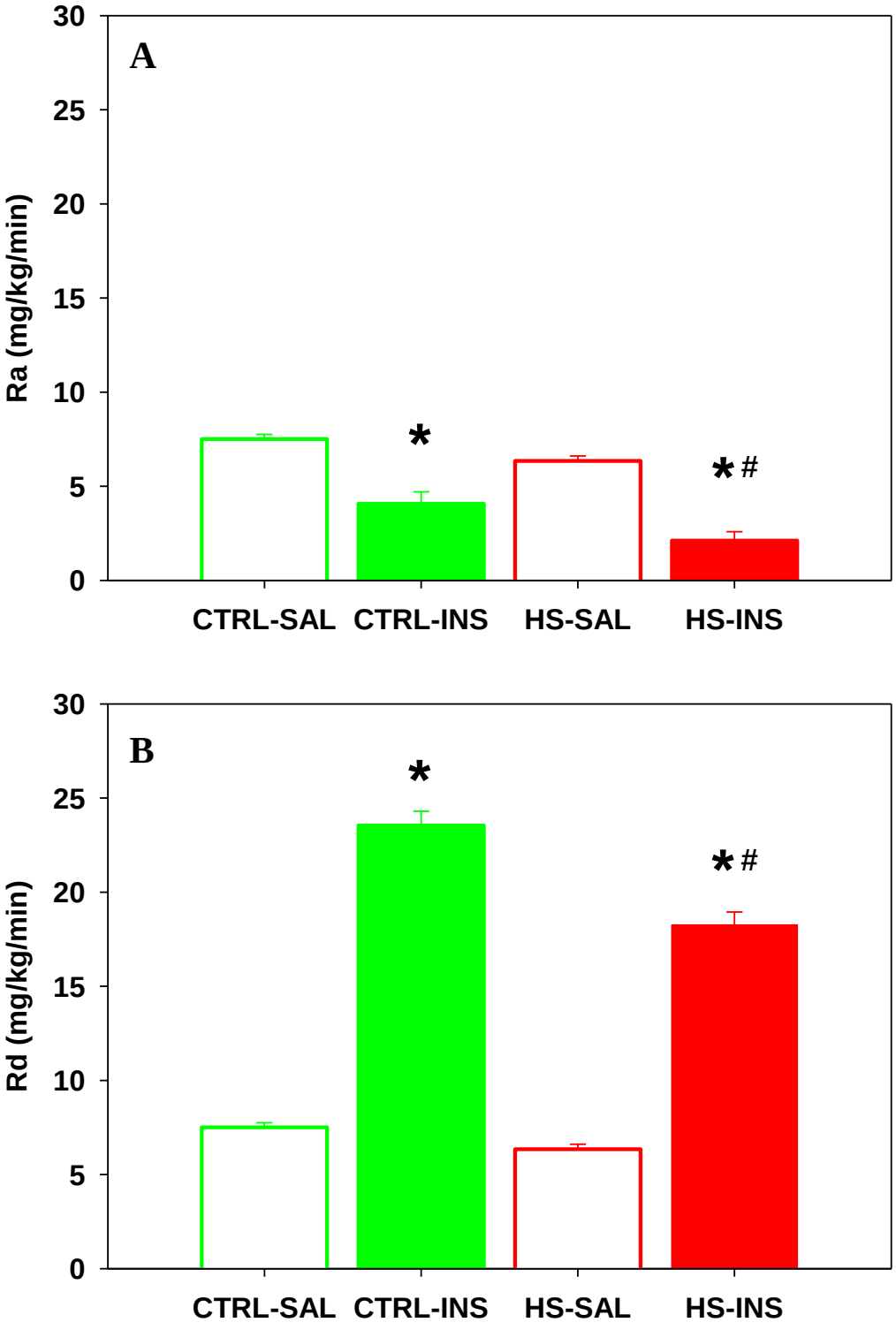

Figure 3.3. Dietary effects on whole body glucose metabolism *in vivo*.

Rate of glucose appearance (Ra; panel A) and disappearance (Rd; panel B) at the conclusion of saline infusion or insulin clamp in CTRL and HS rats. Data are means ± SEM for n=10-12 rats in each group. * Significantly different ($p<0.001$) from respective SAL. # Significantly different ($p<0.01$) from CTRL-INS using one-way ANOVA.

3.3.3. Effects of HS feeding on insulin-mediated hindleg and muscle specific glucose uptake

While basal hindleg glucose uptake appeared not to be different between the two groups, hyperinsulinaemia revealed moderate insulin resistance in hindlegs of HS fed rats compared to CTRL fed rats. During saline infusion hindleg glucose uptake was not different between CTRL and HS fed rats (Fig 3.4.A; 0.02 ± 0.04 vs. 0.07 ± 0.04 µmol/min, p=0.289). Compared to their respective saline groups, 2 hrs of insulin infusion significantly increased hindleg glucose uptake in both CTRL (0.02 ± 0.04 vs. 0.59 ± 0.03 µmol/min, p<0.001) and HS animals (0.07 ± 0.04 vs. 0.49 ± 0.04 µmol/min, p<0.001). However, insulin stimulated hindleg glucose uptake tended to be higher in CTRL rats compared to the HS fed rats (p=0.054).

Skeletal muscle glucose uptake appeared not to be affected by the HS diet at basal, however, insulin infusion revealed significant attenuation of insulin-mediated muscle glucose uptake following HS feeding. No difference in R`g was found following saline infusion between CTRL and HS animals (Fig. 3.4.B; 1.6 ± 0.8 vs. 1.3 ± 0.8 µg/g/min, p=0.799). However, insulin stimulated significant (p<0.001) increases in R`g in both CTRL (1.6 ± 0.8 vs. 24.0 ± 0.7 µg/g/min) and HS (1.3 ± 0.8 vs. 17.8 ± 0.7 µg/g/min) fed rats compared to their respective saline groups. Insulin-mediated R`g was significantly (p<0.001) reduced in HS fed rats compared to the CTRL fed rats.

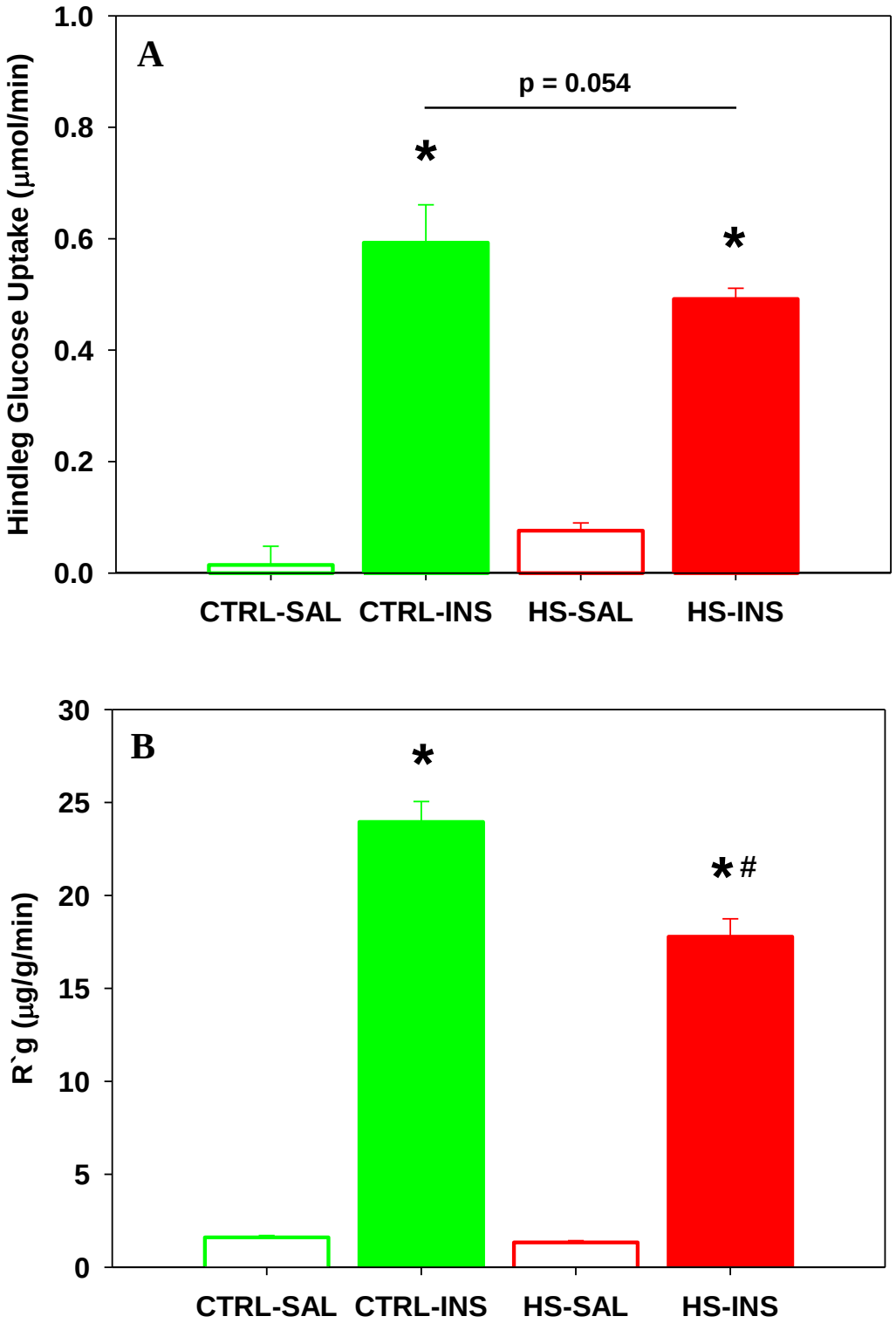

Figure 3.4. Hindleg and muscle specific glucose uptake *in vivo*.

Hindleg glucose uptake (A) and muscle specific glucose uptake (B) during saline infusion or insulin clamp in CTRL and HS rats. Data are means ± SEM for n=10-12 rats in each group. * Significantly different ($p<0.001$) from respective SAL. # Significantly different ($p<0.001$) from CTRL-INS using one-way ANOVA.

3.3.4. Effects of HS diet on basal and insulin-mediated FVR

HS feeding resulted in a significant increase in the basal femoral artery vascular resistance (FVR) and this was not affected by saline infusion in either HS or CTRL fed rats. However, insulin infusion significantly reduced FVR in both HS and CTRL groups. FVR was calculated from the FBF and mean arterial blood pressure. Compared to CTRL fed rats, HS fed rats were found to have elevated basal FVR (Table 3.2; $p<0.001$). Following 2 hrs of saline infusion no change in FVR was detected in either CRTL (Fig. 3.5; 109 ± 7 vs. 108 ± 7 mmHg.min/mL, $p=0.818$) or HS (188 ± 8 vs. 186 ± 11 mmHg.min/mL, $p=0.397$) animals. However, the FVR in HS fed rats remained significantly ($p<0.001$) elevated compared to CTRL following 2 hrs of saline infusion. Insulin infusion in CTRL animals reduced FVR compared to basal (109 ± 6 vs. 91 ± 8 mmHg.min/mL, $p<0.001$). Insulin infusion for 2 hrs also reduced FVR in HS animals compared to basal (204 ± 11 vs. 145 ± 4 mmHg.min/mL, $p<0.001$). However, the FVR in HS-INS rats remained significantly elevated above the CTRL-INS ($p<0.001$) following 2 hrs of insulin infusion.

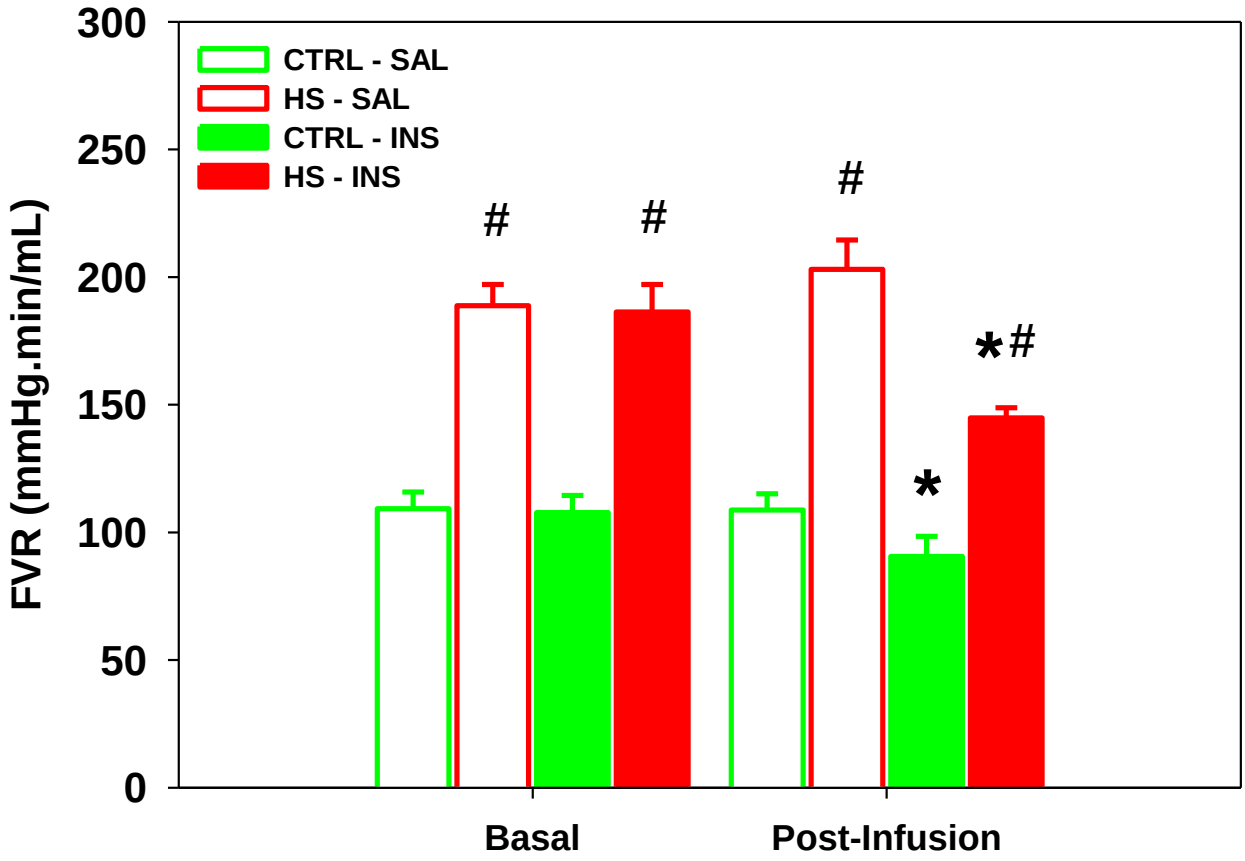

Figure 3.5. Femoral artery vascular resistance (FVR) at basal and following 120 min of saline or insulin infusion.

Basal and post-saline infusion or post-insulin clamp FVR in CTRL and HS rats. Data are means ± SEM for n=10-12 rats in each group. * Significantly different ($p<0.01$) from respective basal. # Significantly different ($p<0.001$) from respective CTRL using two-way ANOVA.

3.3.5. Effects of HS diet on basal and insulin-mediated FBF and 1-MX metabolism

Feeding rats a HS diet appeared to decrease basal FBF compared to CTRL fed rats, but insulin infusion induced comparable increases in FBF in both groups. HS fed rats were found to have significantly (Table 3.2; $p<0.001$) reduced basal FBF compared to CTRL fed rats. Saline infusion for 2 hrs did not affect FBF in either CTRL (0.97 ± 0.06 vs. 1.00 ± 0.05 mL/min, p=0.711) or HS (0.55 ± 0.03 vs. 0.53 ± 0.03 mL/min, p=0.951) fed rats. Insulin infusion for 2 hrs increased FBF in CTRL (1.00 ± 0.05 vs. 1.28 ± 0.09 mL/min, $p<0.001$) and HS (0.52 ± 0.04 vs. 0.69 ± 0.03 mL/min; $p<0.001$) fed rats. While the FBF in the CTRL animals remained higher following insulin infusion ($p<0.001$), the insulin-mediated change in FBF was not significantly different between the CTRL and HS groups (Fig. 3.6.A; 27.4 ± 6.0 vs. 39.1 ± 11.2 %, p=0.234).

Although HS feeding appeared not to affect insulin's effect on FBF, insulin's effects on microvascular recruitment were found to be attenuated following HS feeding. Metabolism of 1-MX was not different between the CTRL and HS animals following 2 hrs of saline infusion (Fig. 3.6.B; 3.99 ± 0.24 vs. 3.70 ± 0.35 nmol/min, p=0.523). Insulin infusion in CTRL fed rats stimulated metabolism of 1-MX compared to saline (3.99 ± 0.24 vs. 5.04 ± 0.30 nmol/min, p=0.021). However, insulin-mediated 1-MX metabolism did not increase in the HS animals when compared to saline infusion (3.70 ± 0.35 vs. 3.73 ± 0.29 nmol/min, p=0.956). Moreover insulin-mediated 1-MX metabolism in HS fed rats was significantly (p=0.003) reduced compared to CTRL fed rats. Although the arterial concentration of 1-MX was significantly higher in the HS animals compared to CTRL (28.1 ± 1.3 vs. 35.5 ± 2.1 µmol/L; p=0.004), there was no difference in the circulating oxypurinol concentration (6.27 ± 0.32 vs. 5.71 ± 0.16 µmol/L; p=0.190) or muscle xanthine oxidase activity between CTRL and HS groups (53.8 ± 11.8 vs. 61.5 ± 6.9 pmol/mg/min; p=0.501).

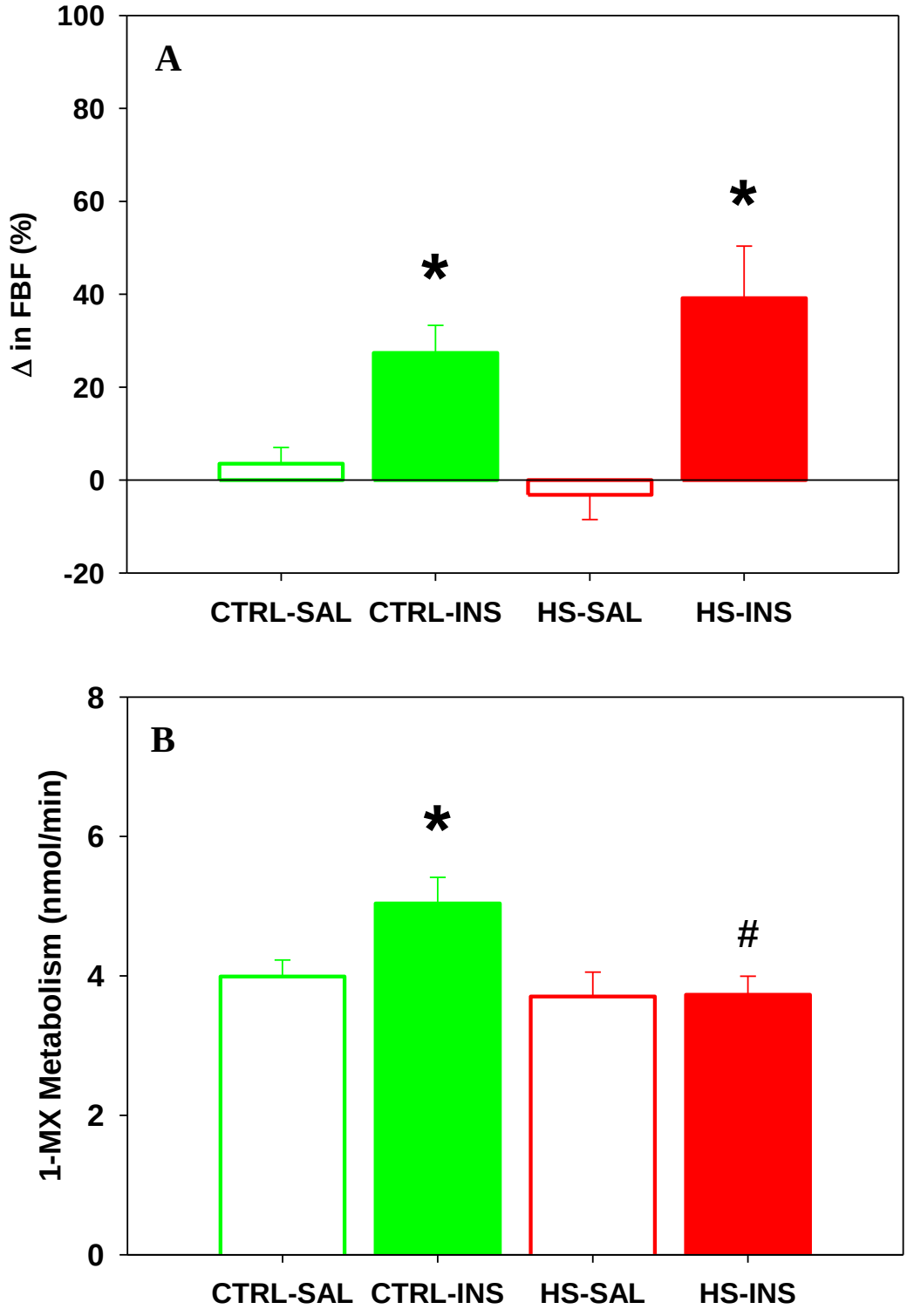

Figure 3.6. Vascular actions of saline and insulin *in vivo*.

Saline infusion or insulin clamp mediated change in FBF from basal (A) and 1-MX metabolism (B) in CTRL and HS rats. Data are means ± SEM for n=10-12 rats in each group. * Significantly different ($p<0.05$) from respective SAL. # Significantly different ($p<0.01$) from CTRL-INS using one-way ANOVA.

4.4. Discussion

The current study produced three main findings. Firstly, feeding animals a HS diet induced a state of whole body insulin resistance without reducing liver insulin sensitivity. Thus, whole body insulin resistance in the HS fed rats can be largely attributed to reduced muscle glucose uptake as confirmed by Rd. Secondly; the HS diet did not affect mean arterial blood pressure but markedly reduced basal FBF and increased FVR. Thirdly, the HS animals displayed normal FBF responses to insulin but exhibited significant attenuation in insulin-mediated microvascular recruitment. This is the first study to show that microvascular insulin resistance accompanies the development of whole body and skeletal muscle insulin resistance following HS feeding. This effect of the HS diet on insulin sensitivity was apparent despite an increase in liver insulin sensitivity. These data suggest that HS feeding results in an impairment of the microvascular response to insulin which in turn likely contributes to the development of skeletal muscle and whole body insulin resistance.

In the present study 3-4 weeks of HS feeding induced a state of insulin resistance as indicated by a marked decrease in GIR, muscle specific glucose uptake and a decrease hindleg glucose uptake during hyperinsulinaemia. Indeed, this is not the first study to show that HS feeding induces a state of whole body and muscle insulin resistance. Donovan and colleagues (1993) found that giving otherwise healthy men a HS diet for 5 days significantly reduced GIR during hyperinsulinaemic clamp conditions [340]. Similarly, Ogihara and colleagues (2001) reported reduced GIR and attenuation in skeletal muscle glucose uptake following HS feeding of rats [327]. Furthermore, in a subsequent study Ogihara and colleagues (2002) showed that during AngII infusion whole body and muscle insulin resistance also develops to a similar extent as occurs in HS feeding [327]. Moreover, when HS animals were infused with AngII that was a further attenuation of insulin sensitivity [290], suggesting that insulin sensitivity in HS fed animals can be further attenuated by increasing the circulating AngII concentrations. Indeed AngII, acting through the AT1R has been reported to directly inhibit insulin-stimulated muscle glucose uptake [200, 291, 294, 296]. In addition, other reports have demonstrated that HS feeding increases both AT1R and ACE expression [330, 345].

Therefore a plausible mediating factor in HS-induced muscle insulin resistance in the present study may be increased activity of AngII via the AT1R.

While skeletal muscle insulin resistance was apparent following HS feeding, the HS diet did not reduce liver insulin sensitivity. Interestingly, the liver was found to be slightly more sensitive to insulin following HS feeding. While it is well accepted that liver insulin resistance develops in a number of models of insulin resistance [142, 347, 348] the effects of HS feeding have been poorly studied and the findings are inconsistent. Ogihara and colleagues (2001) reported that HS feeding induced mild liver insulin resistance in Sprague Dawley rats [327]. When the same authors assessed liver insulin sensitivity in Dahl-salt sensitive and salt resistant rats they reported that HS feeding did not alter liver glucose output during insulin infusion in either group [339]. Interestingly in both previous studies feeding rats a HS diet was associated with significant increases in the liver insulin signalling [327]. This apparent increase in insulin signalling in hepatocytes may account for the slight increase in liver insulin sensitivity in the current study.

Whilst no evidence of hepatic insulin resistance was detected in the current study, a recent report has identified a possible role for the RAS in the development of liver insulin resistance. Takeshita and colleagues (2008) reported that hepatocyte insulin resistance may be mediated by TNFα and the AngII effects on plasminogen activator inhibitor 1 (PAI-1) activity [349]. In their study the authors reported that TNFα could also up-regulate angiotensinogen, ACE and AT1R mRNA in human hepatocytes [349]. Moreover, the authors reported that selective AT1R inhibition dose-dependently decreased the TNFα-induced PAI-1 production [349]. Thus it would appear that TNFα and RAS can co-ordinately stimulate PAI-1 production, suggesting a cross-talk between both systems, and a possible mechanism by which TNFα and AngII may induce insulin resistance in the liver. However, while the RAS may contribute to hepatic insulin resistance via this mechanism in human hepatocytes, the data from the current study would suggest that this is not the case during HS feeding. The effects of increased salt intake on hepatic insulin action have thus far been poorly studied and further investigation is required.

Similarly, the effects of HS intake on mean arterial blood pressure are not consistent throughout the literature [327, 329, 340]. Severity of HS-mediated blood pressure increases appears to depend on a number of factors. The underlying sensitivity to sodium overload appears to be important in determining the effects of HS diet on blood pressure homeostasis. For example the Dahl-salt sensitive rat, which is highly sensitive to sodium overload and HS feeding is regularly used to investigate the development of hypertension [350]. In normal Sprague Dawley rats, however, the results on blood pressure are not consistent with Ogihara and colleagues (2001) showing that 2 or 8 weeks of HS feeding significantly increased blood pressure [327], while Lenda and colleagues (2000) found no effect of 4-5 weeks of HS feeding on blood pressure [329]. Thus, similar to the report by Lenda and colleagues (2000), the lack of HS diet effects on blood pressure in the current study may possibly be attributed to the relative salt resistance of Sprague Dawley rats. Therefore, the use of HS fed rat in the current study appears to offer an opportunity to examine the involvement of RAS in the development of insulin resistance in the absence of hypertension, at least during anaesthesia.

However, while the effects of HS on blood pressure were negligible, basal FBF was reduced by approximately 45% and thus FVR was increased by approximately 80% in HS fed rats. These data are consistent with previous reports showing that HS feeding increases peripheral vascular tone in resistance vessels from kidney [338], mesenteric [337] and skeletal muscle [344]. In all three of these studies the increase in vascular tone/constriction following HS feeding was associated with significantly increased AT1R expression and activity. Thus, the increased FVR and reduced FBF in the current study may be partly attributable to increased AngII mediated constriction via the AT1R in the skeletal muscle resistance vessels. These data add further support to the notion that dysregulation of AngII activity, following HS feeding reduces the metabolic and haemodynamic actions of insulin *in vivo* and contributes to the development of insulin resistance in this model.

Interestingly, while the HS diet did not affect the insulin-stimulated increase in FBF compared to CTRL fed rats, during hyperinsulinaemia the HS fed rats exhibited significant attenuation of insulin-stimulated microvascular recruitment in muscle. A

number of studies have previously reported that in human and animal models of insulin resistance, reduced insulin-mediated muscle glucose uptake is associated with an attenuation in insulin-stimulated microvascular recruitment [167, 195, 196, 227, 242]. Thus, the data in the present study would support these previous findings since impairment of insulin-mediated microvascular recruitment was associated with attenuation of insulin-mediated muscle glucose uptake following HS diet. However, Ogihara and colleagues (2001) previously reported that HS diet also directly impairs insulin-mediated muscle glucose uptake [327]. Therefore, whether the reduced insulin-mediated muscle glucose uptake *in vivo* in the present study is a result of direct myocyte insulin resistance or impairment in insulin-mediated microvascular recruitment, or a combination of these factors, cannot be distinguished. However, since previous reports demonstrate that blocking insulin-mediated microvascular recruitment also reduces muscle glucose uptake [100, 167], it is reasonable to conclude that in the present study impairment in insulin-mediated microvascular recruitment following HS feeding may at least partly contribute to myocyte insulin resistance *in vivo*.

While the cause of microvascular insulin resistance was not determined in the current study, other reports have indicated a possible role for increased AngII activity. Chai and colleagues (2009) demonstrated that AngII receptors in the vasculature contribute to the regulation of microvascular perfusion at rest [108]. In a subsequent study the same authors also demonstrated that modulating AngII activity to favour AT1R activation is detrimental to insulin-mediated microvascular recruitment in normal, healthy rats [109]. In light of this evidence and previous reports showing that skeletal muscle vasculature exhibits increased AT1R's following HS feeding [344], the impairment in insulin-stimulated microvascular recruitment in the current study may in part be the result of enhanced vascular AT1R activity.

Lenda and Colleagues have previously shown that HS feeding increases the activity of XO in muscle microvasculature [329]. Since the measure of microvascular perfusion in the current study is dependent on 1-MX metabolism by XO, it was important to investigate if HS feeding increased XO activity in this study. XO activity was found to be similar between CTRL and HS groups. Further evidence for normal XO activity in

muscle of HS rats is that 1-MX metabolism in the saline infused animals was not different between the two groups. Furthermore, if indeed there was increased activity of XO one would expect to see a reduced arterial concentration of 1-MX in the HS fed rats. However, arterial concentration of 1-MX was actually found to be slightly increased in the current study and this may have been the result of the increase in haematocrit following HS feeding. Thus, the use of 1-MX metabolism as a measure of microvascular perfusion appears to be valid in this model since neither the metabolism of 1-MX during saline infusion or XO activity were affected by the HS diet.

4.4.4. Conclusions

In summary, the findings of the current study are in agreement with previous reports where HS feeding results in the development of whole body and skeletal muscle insulin resistance. However, this is the first study to demonstrate that HS feeding also attenuates insulin-mediated microvascular recruitment in muscle. Since insulin-mediated microvascular recruitment contributes to insulin-stimulated muscle glucose uptake the findings of the current study add further support to the concept that defects in insulin's ability to increase microvascular perfusion in muscle contribute to the development of muscle insulin resistance. Furthermore, since HS feeding has also been shown to up-regulate AngII activity, via increased vascular AT1R activation, the mechanism responsible for the attenuation of insulin-mediated microvascular recruitment following HS feeding may be related to enhanced AngII activity.

CHAPTER 4

EFFECTS OF LOCAL ANGIOTENSIN II INFUSION ON MUSCLE INSULIN SENSITIVITY *IN VIVO*

4.1. Introduction

In chapter 3 of the current thesis feeding rat a HS diet resulted in significant attenuation of insulin-mediated microvascular recruitment in muscle. The HS-induced microvascular defect was also associated with significant attenuation of whole body and skeletal muscle insulin sensitivity. There is convincing evidence in the literature that the HS-induced insulin resistance may be attributable to up-regulation of AngII activity. Specifically, through increased AT1R sensitivity/activity and decreased AT2R sensitivity/activity in the vasculature [163, 330, 338, 344]. Taken together, these findings raise the question as to whether increased vascular AngII activity interferes with insulin-mediated microvascular recruitment and therefore contributes to reduced insulin-mediated glucose uptake in muscle.

Inconsistent reports exist regarding the effect of AngII on insulin's vascular and metabolic actions *in vivo*. A number of previous studies have demonstrated that acute, systemic infusions of AngII combined with insulin, results in increased insulin-mediated glucose disposal in normal healthy subjects [287, 288, 351]. Buchanan and colleagues (1993) used systemic infusion of insulin and/or AngII to show that co-infusion of both increased whole body glucose disposal in healthy subjects to a greater extent than insulin alone [351]. Similarly, Fliser and colleagues (1997) reported that systemic AngII infusion in healthy subjects also increased insulin-mediated whole body glucose disposal [289]. Both of these previous studies attributed the increase in insulin-mediated whole body glucose disposal during AngII infusion to an augmentation of total leg blood flow [289, 351], thus implying an increase in leg muscle glucose uptake. However, given that others have shown that increasing total blood flow alone does not increase insulin-mediated muscle glucose uptake [37, 61], a reasonable conclusion may have been that AngII increased microvascular perfusion in these subjects to facilitate increased insulin-mediated muscle glucose uptake. However, this does not appear to be the case. Jonk and colleagues (2010) found that although systemic AngII infusion increased whole body insulin sensitivity in healthy individuals, this was associated with attenuation of insulin-mediated microvascular recruitment [287]. Therefore, these data suggest that increased microvascular recruitment is most likely not involved in the AngII-mediated increase in insulin sensitivity and if anything, that AngII infusion may be detrimental to insulin-

mediated microvascular recruitment. Indeed, this notion seems more likely since previous investigations have revealed that AngII directly inhibits the insulin signalling pathway in both vascular smooth muscle and vascular endothelial cells [202, 295].

Critically, insulin-mediated muscle glucose uptake was not directly assessed in these previous studies where AngII was reported to increase whole body insulin sensitivity [287, 289, 351]. Thus, whether the improvement in insulin-mediated whole body glucose disposal following AngII infusion is the result of an increase in insulin-mediated muscle glucose uptake cannot be directly concluded from these previous studies. Indeed, whether acute AngII has any direct effects on insulin-mediated glucose uptake by skeletal muscle cells is debatable. Patiag and colleagues (2000) reported that AngII does not affect insulin-mediated muscle glucose uptake *in vitro* in L6 myotubes [352]. However, other reports have found that AngII impairs insulin signalling in skeletal muscle cells *in vitro* [200] and also reduced insulin-mediated muscle glucose uptake [294, 296]. Given these reports demonstrating either negligible or inhibitory effects of AngII on insulin activity in skeletal muscle, it is reasonable to assume that the increased insulin-mediated whole body glucose disposal following AngII infusion is probably not related to an increase in muscle glucose uptake.

The inconsistencies in the previous studies where AngII has been found to enhance insulin-mediated whole body glucose disposal may be the product of the methodology utilised. In all of the *in vivo* studies AngII was infused systemically. For this reason, the effects of AngII on insulin's vascular and metabolic actions in muscle cannot be distinguished from the systemic effects of AngII on other organs such as the liver, heart and brain, which may in turn influence insulin sensitivity in their own right (reviewed by Bader (2010)) [323]. However, the relatively short half-life of AngII (~15 sec) suggests that it could be infused locally without systemic spill-over [259]. Indeed, one previous study has demonstrated that local AngII infusion *in vivo* can be achieved without systemic effects [353].

Jamerson and colleagues (1996) investigated whether local infusion of both AngII and insulin could alter forearm insulin sensitivity in healthy human subjects [353]. The authors found that AngII reduced total flow to muscle but increased A-V glucose extraction during insulin infusion, resulting in no net change in insulin-mediated glucose utilisation [353]. However, the authors also noted that local AngII infusion also resulted in a marked (~70%) increase in insulin concentration in the forearm compared to insulin infusion alone [353]. This increase in insulin concentration was most likely due to the protocol utilised. Since AngII decreased total flow to the forearm muscle and the insulin infusion was unchanged, the combined effect would be an increase in the forearm insulin concentration. For this reason, it cannot be discerned whether the increase in A-V glucose extraction during co-infusion of AngII and insulin was the result of the increase in the insulin concentration or possibly due to a haemodynamic and/or myocyte effect of AngII. Therefore, the primary aim of the current study was to determine whether local AngII infusion *in vivo* during systemic hyperinsulinaemic euglycaemic clamp effects insulin's vascular and metabolic actions in skeletal muscle. This aim was approached in two ways.

Since the HS model in chapter 3 of this thesis exhibited attenuation of vascular and metabolic actions of insulin and has been reported to display enhanced vascular activity and sensitivity to AngII [163, 330, 338, 344], the first aspect of the current study was to determine whether the HS model in the present thesis also displays enhanced vascular sensitivity/ responsiveness to AngII. To determine this AngII-mediated constriction was assessed in hindleg vasculature of CTRL and HS fed rats using the perfused rat hindleg preparation.

The second aspect of the current study was to determine the effects of local AngII infusion *in vivo*, into a single hindleg, on insulin's vascular and metabolic actions in skeletal muscle. To this end AngII was infused locally into one hindleg 15 min prior to and for the duration of the hyperinsulinaemic euglycaemic clamp procedure using the anaesthetised rat preparation. Since the HS fed rat has previously been reported to exhibit increased vascular sensitivity to AngII [163, 330, 338, 344], the effect of local AngII was assessed in both CTRL and HS fed rats. The novelty of these approaches is

that AngII is infused locally into the leg without causing systemic effects and thus allows for determination of AngII effects on insulin's vascular and metabolic action in skeletal muscle.

4.2. Methods

4.2.1. Animals

Male Sprague-Dawley rats (4 weeks of age) were obtained from the University of Tasmania Central Animal Facility. On arrival, the rats were split into two groups and provided either a standard laboratory chow (CTRL, Ridley Agri Products, Melbourne, VIC, Australia) or a semi-purified high salt (8% NaCl wt./wt.; HS) diet *ad libitum* for 3-5 weeks (Table 4.1). Water was freely available. Following the feeding protocol vascular sensitivity to AngII was assessed using the perfused hindleg preparation and insulin sensitivity during local hindleg infusion of AngII was assessed using the anaesthetised rat preparation.

4.2.2. Perfused rat hindleg experiments

4.2.2.1. AngII dose curve

Surgical procedure and experimental setup was carried out as previously outlined in chapter 2, sections 2.4.2 and 2.4.3. Following surgery and attachment to the perfusion apparatus, total flow through one hindleg was adjusted to a constant flow rate of 8ml/min for a 220g rat (equivalent to 0.4ml/min/g muscle). Following a 30 min equilibration period (to allow washout of red blood cells from the hindleg) an AngII dose curve was commenced to assess the vascular responsiveness of the hindleg. AngII was infused into the perfused hindleg from 0.1nM to 10nM in 10 min stepwise increments and is shown in more detail in Fig. 4.1.

	CTRL	HS
Protein	22.0 %	19.4 %
Carbohydrate	65.8 %	60.0 %
Fat	9.0 %	7.0 %
Crude Fibre	3.2 %	3.2%
NaCl	0.31 %	8.0 %
Total Digestible Energy	13.2MJ/Kg	15.5MJ/Kg

Table 4.1. Macronutrient composition of the chow (CTRL) and 8.0% salt (HS) diets as wt./wt.

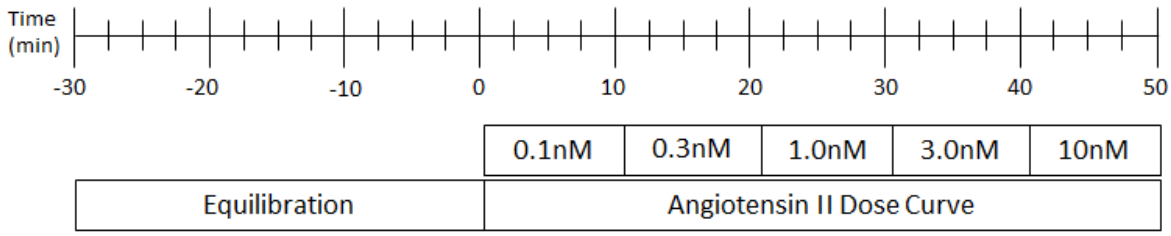

Figure 4.1. Experimental Protocol for Perfused Hindleg Preparation.

After surgical preparation and 30 min of equilibration, AngII infusion was initiated through the infusion port at a 1:100 of the total flow rate through the hindleg. An increasing AngII dose curve was used to assess the sensitivity of the vasculature to AngII mediated constriction and perfusion pressure was recorded continuously using Windaq® software.

4.2.3. Anaesthetised rat experiments

4.2.3.1. Surgical procedure

Surgery was performed as outlined in chapter 2, section 2.3.1 with the following additions. Following cannulation of the carotid artery and both jugular veins, both the left and right femoral arteries were isolated for FBF measurement. To facilitate the local infusion of AngII into one hindleg, the epigastric artery of one hindleg was cannulated using a 29g needle, see Fig. 4.2. Immediately following cannulation of the epigastric artery saline infusion at 1:100 of the FBF rate was initiated to maintain vessel compliance during the equilibration period.

4.2.3.2. Experimental procedure

Following surgical preparation, 1 hr was allowed for FBF and mean arterial blood pressure to stabilise, after which AngII was infused into one hindleg, to a final concentration of 5nM, via the epigastric artery at 1:100 of the FBF rate. Following 15 min of AngII infusion a 2 hr infusion of insulin (10 mU/min/kg) was initiated and a 30% glucose solution (wt./vol.) was infused at a variable rate to maintain fasting blood glucose concentrations over the course of the experiment. For this reason, arterial blood glucose levels were assessed every 10 min in the first hour, and every 15 min in the second hour using a glucose analyser (YSI 2300) and the glucose infusion rate (GIR) was adjusted accordingly. See Fig. 4.3. for a more detailed protocol. Plasma biochemistry, whole body glucose kinetics (Ra and Rd), muscle glucose uptake and skeletal muscle microvascular perfusion were performed as previously outlined in chapter 2, sections 2.3.2-5.

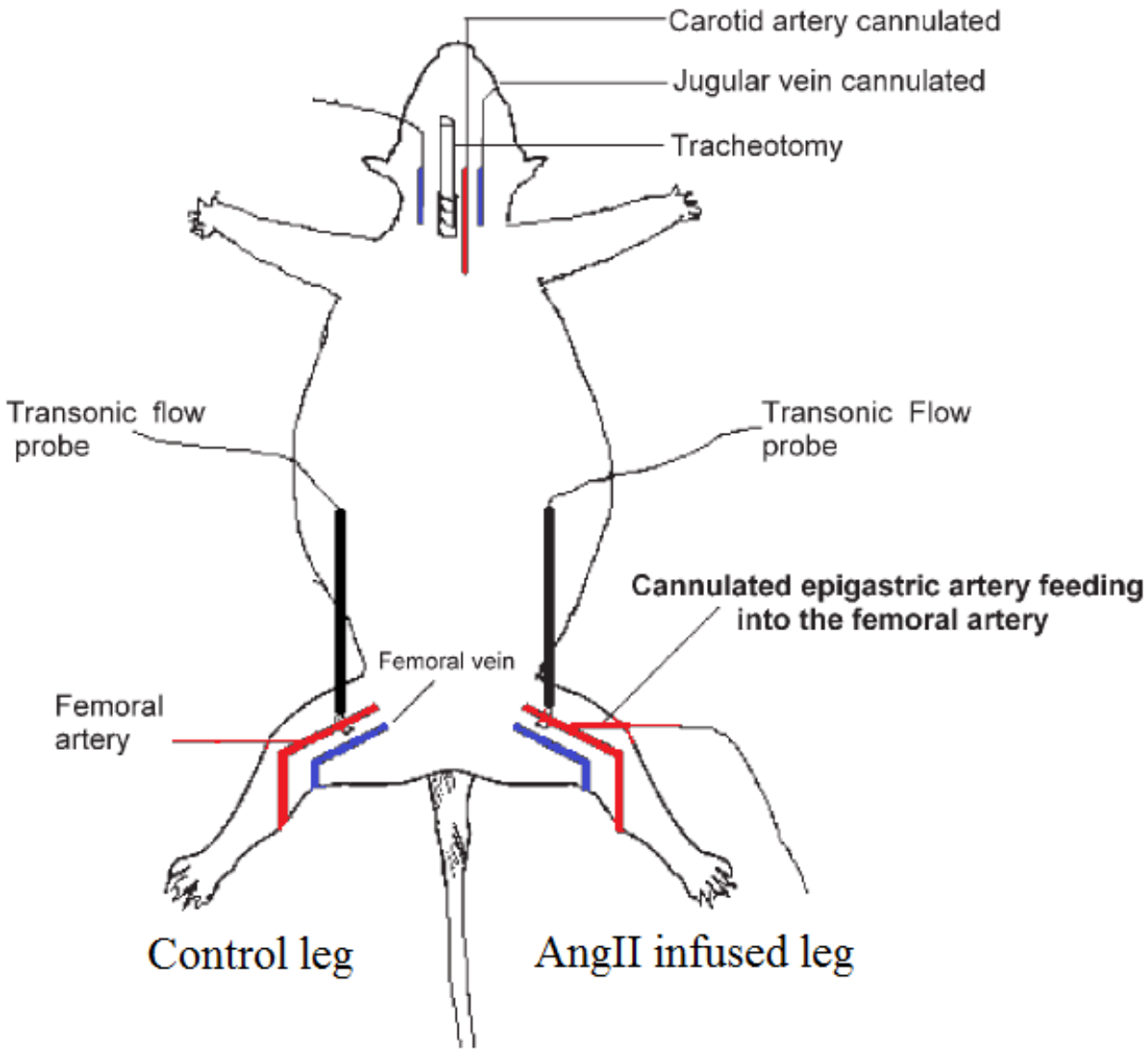

Figure 4.2. Schematic of surgical procedure performed for the anaesthetised rat preparation with epigastric artery cannulation.

Following tracheotomy, cannulae were inserted into the carotid artery and both jugular veins. The carotid artery facilitated blood pressure and arterial blood/plasma glucose measurements while the jugular veins facilitate intravenous infusions of anaesthetic, insulin/saline and glucose. Surgery was also performed on one hindleg and the epigastric artery was cannulated to facilitate local AngII infusion. Surgery was also performed on both hindlegs and the femoral artery of each was carefully separated. Transonic flow probes were positioned around each isolated femoral artery to facilitate simultaneous measurement of FBF. Adapted from Mahajan and colleagues (2004) [307].

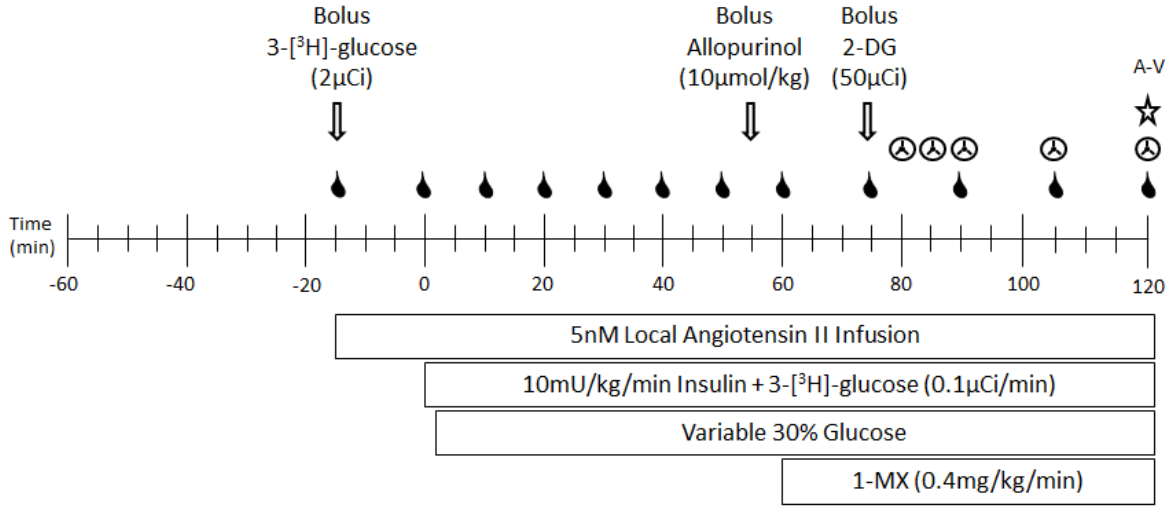

Figure 4.3. Experimental protocol for anaesthetised rat experiments.

After surgical preparation and 60 min of equilibration, AngII was infused into one hindleg to a final concentration of 5nM via the epigastric artery at 1:100 of the FBF rate. Following 15 min of AngII infusion a continuous infusion of insulin (10mU/min/kg) was commenced and continued for 120 min. A 30% (wt./vol.) glucose infusion was initiated shortly after the commencement of the insulin infusion to maintain baseline glycaemia as assessed by arterial blood glucose sampling (). After 55 min of insulin infusion, a bolus of allopurinol (10µmol/kg) was administered. At 60 min, infusion of 1-methylxanthine (1-MX, 0.4mg/kg/min) was initiated and maintained until the end of the experiment. At 75 min a bolus of 2-deoxy-D-[1-14C]-glucose (2-DG; 20µCi) was administered. Radioactive plasma samples () were collected at 80, 85, 90, 105 and 120 min to determine the plasma 2-DG clearance. At the conclusion of the experiment arterial and femoral vein plasma samples ( ) from both hindlegs were collected for the determination of hindlimb glucose uptake and 1-MX metabolism. Immediately following sacrifice, calf muscle (gastrocnemius, plantaris and soleus group) from each hindleg was freeze clamped in liquid nitrogen and stored at -80°C.

4.2.4. Data and Statistics

Data are presented as the means ± SEM and statistics were performed using SigmaStat (Systat Software Inc, 2004). Comparisons between CTRL and HS fed rats for the perfused hindleg preparation were made using un-paired Student's t-test. Comparisons between Control and AngII hindlegs in both CTRL and HS fed rats were made using paired Student's t-test. Comparison of time-series measurements in CTRL and HS fed rats were performed by two-way repeated measures ANOVA. When a significant difference of $p<0.05$ was detected, pairwise comparisons by Student-Newman-Keuls test was used to assess treatment differences.

4.3. Results

4.3.1. Physical and biochemical characteristics of CTRL and HS rats following 3-5 week feeding intervention

Following an overnight fast both groups of rats displayed similar characteristics (Table 4.2) compared to the respective groups from chapter 3, Table 3.2. While total body weight and fat mass were larger in the current study compared to the results obtained in chapter 3 of this thesis, the slightly longer feeding protocol used would explain this slight increase. As with the previous study there were no differences in fasting plasma glucose, lactate or insulin following 4-5 weeks feeding between the CTRL and HS groups. FBF was slightly reduced in the HS group compared to CTRL group and thus the HS animals displayed slightly elevated FVR. However, no difference in mean arterial blood pressure was detected. Basal FBF and FVR between control and AngII legs was not significantly different in CTRL rats ($p=0.466$) and $p=0.421$ respectively). HS fed rats exhibited slightly increased basal FBF in AngII legs compared to control legs but this difference did not reach significance ($p=0.174$). Similarly basal FVR was also slightly reduced in the AngII compared to control legs in HS rats but did not reach significance ($p=0.236$). Compared to rats in chapter 3, GIR (CTRL; 18.5 ± 1.1; HS, 17.1 ± 1.2 mg/kg/min), Ra (CTRL; 2.10 ± 1.42, HS; 2.02 ± 0.86 mg/kg/min) and Rd (CTRL; 21.5 ± 1.5, HS; 19.2 ± 0.9 mg/kg/min) during the hyperinsulinaemic euglycaemic clamp were not significantly affected by local AngII infusion.

	CTRL	HS	p value
Body weight (g)	270 ± 17	260 ± 12	0.421
Epididymal fat pad (g)	1.52 ± 0.34	1.42 ± 0.27	0.690
Fasting plasma glucose (mmol/L)	6.47 ± 0.31	6.58 ± 0.41	0.815
Fasting plasma insulin (pmol/L)	112 ± 33	114 ± 24	0.941
Fasting plasma lactate (mmol/L)	0.66 ± 0.06	0.76 ± 0.03	0.124
Mean arterial pressure (mmHg)	103 ± 5	108 ± 4	0.180
Control Leg			
Basal FBF (mL/min)	0.95 ± 0.18	0.72 ± 0.07	0.083
Basal FVR (mmHg.min/mL)	112 ± 25	147 ± 13	0.177
AngII Leg			
Basal FBF (mL/min)	1.09 ± 0.16	0.92 ± 0.06	0.259
Basal FVR (mmHg.min/mL)	99 ± 13	122 ± 8	0.332

Table 4.2. Physical and biochemical characteristics of CTRL and HS rats following 3-5 weeks feeing intervention.

Measures were collected at the completion of the 3-5 weeks of dietary intervention following an overnight fast. Basal mean arterial pressure, FBF and FVR were determined immediately prior to the local AngII infusion in the anaesthetised rat preparation. Epididymal fat pads were excised and weighed at the conclusion of the experiments. FBF; femoral artery blood blow, FVR; femoral artery vascular resistance. Data are means ± SEM for n=5 rats in each group.

4.3.2. Effects of AngII-mediated vasoconstriction in the perfused hindleg preparation

Rats fed a HS diet exhibited significantly increased AngII-mediated perfusion pressure changes compared to the CTRL fed rats. The basal perfusion pressures prior to AngII infusion were not different between CTRL and HS fed rats (32.8 ± 1.2 vs. 34.9 ± 0.6 mmHg, p=0.183). AngII increased perfusion pressure dose-dependently in the hindlegs of both CTRL and HS fed rats (Fig. 4.4). However, the HS group exhibited approximately 2-fold greater increase in perfusion pressure following each dose of AngII compared to CTRL. Perfusion pressure in both groups peaked approximately 4 min following the initiation of each dose of AngII. For this reason the perfusion pressure developed at 4 min for each dose of AngII was averaged and is shown in Fig. 4.5.

Infusion of AngII increased perfusion pressure in a dose-dependent fashion in both CTRL and HS groups. A significant increase in perfusion pressure from basal was detected following infusion of 3nM AngII (14.5 ± 1.9 mmHg, p<0.001) in the CTRL group and further increased following 10nM infusion (25.9 ± 2.4 mmHg, p<0.001). However, the HS group exhibited greater sensitivity to AngII as indicated by a leftward shift in the AngII dose response curve. Thus, a dose of 0.3nM was sufficient to increase perfusion pressure significantly compared to basal (6.4 ± 0.7 mmHg, p=0.046). Each subsequent dose of AngII in HS fed rats produced further increases in perfusion pressure compared to basal (p<0.001). Moreover compared to CTRL, AngII infusion in HS rats produced approximately 100% greater increase in perfusion pressure at 1.0nM (5.4 ± 1.3 vs. 13.4 ± 2.2 mmHg, p=0.014), 3.0nM (14.5 ± 1.9 vs. 32.5 ± 3.5 mmHg, p<0.001) and 10nM (25.9 ± 2.4 vs. 46.6 ± 3.4 mmHg, p<0.001).

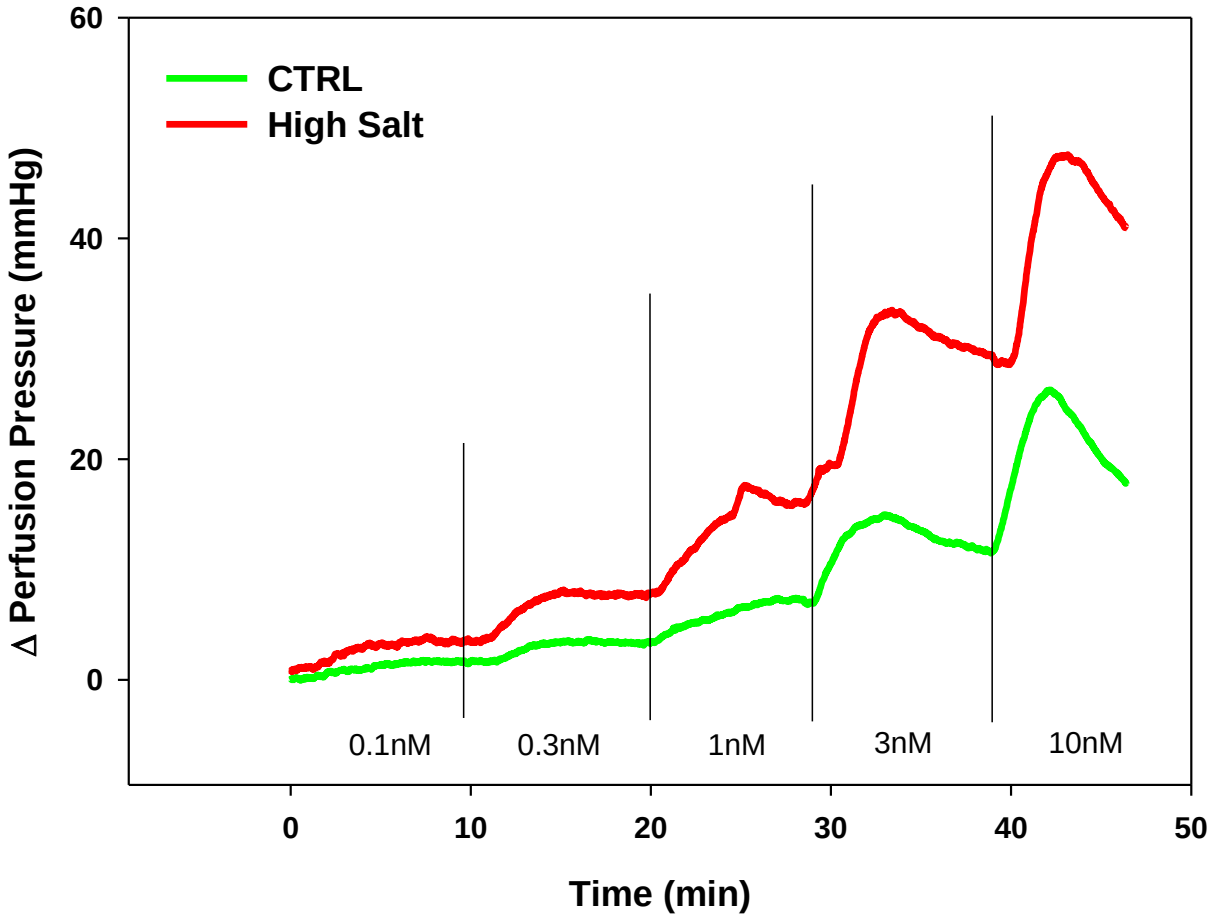

Figure 4.4. Time course of changes in perfusion pressure in response to AngII dose curve in perfused hindlegs of rats fed chow or HS diets.

Time course of AngII mediated changes in perfusion pressure with increasing dose of AngII. AngII concentration increased in 10 min step-wise increments as indicated. Data are means for n=5 rats in each group. SE bars were omitted for clarity.

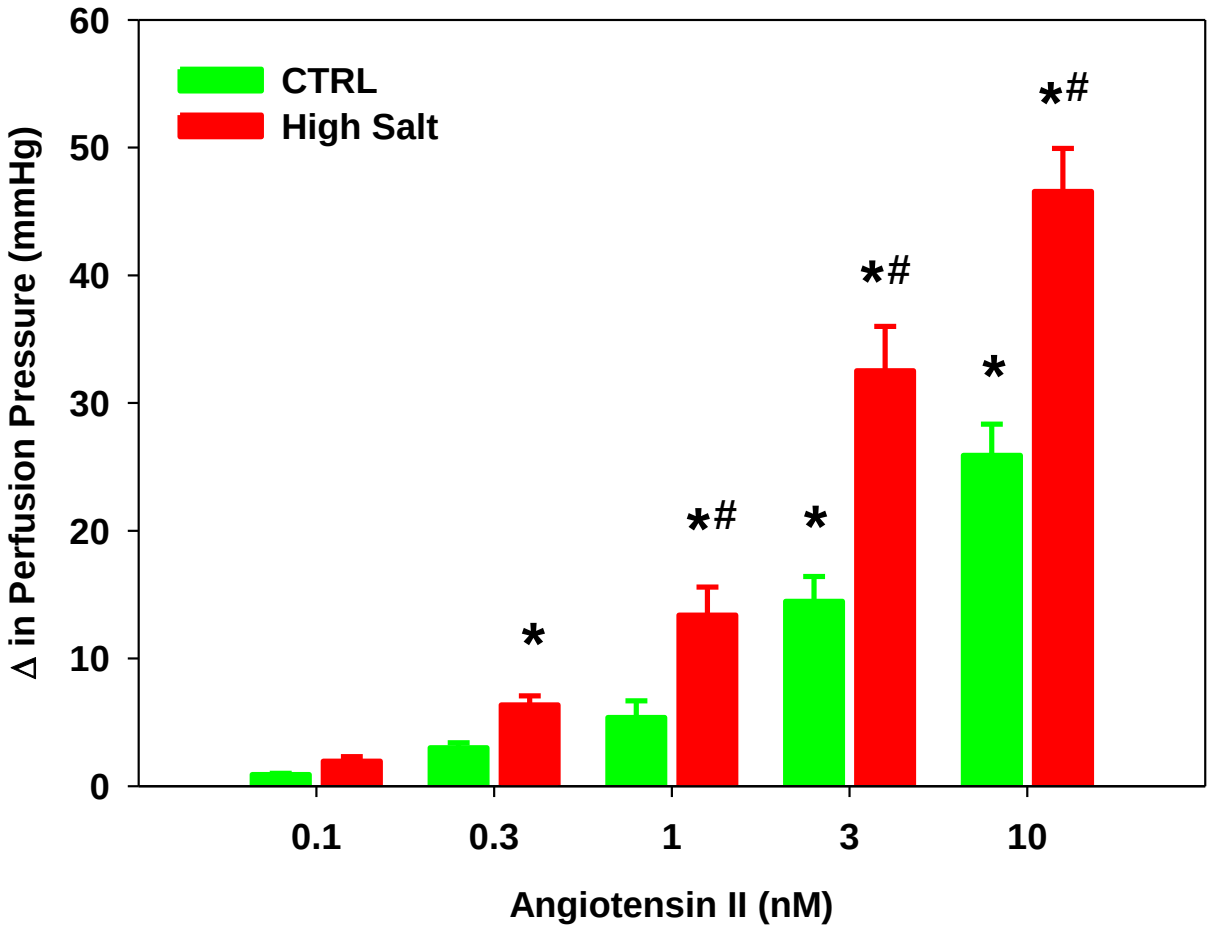

Figure 4.5. Maximum perfusion pressure changes in response to AngII dose curve in perfused hindlegs of rats fed CTRL or HS diets.

The maximum perfusion pressure obtained for each dose of AngII in perfused hindlegs of CTRL and HS fed rats. Data are means ± SEM for n=5 rats. * Significantly different ($p<0.05$) from the respective basal within the same dietary group using two-way repeated measures ANOVA. # Significantly different ($p<0.05$) from CTRL at the same AngII dose using two-way repeated measures ANOVA.

4.3.3. *In vivo* effects of 15 min of local AngII infusion on FBF

Local infusion of AngII for 15 min in CTRL fed rats decreased FBF in the AngII leg by approximately 20% but did not affect mean arterial blood pressure or control leg FBF. In CTRL fed rats, local infusion of AngII for 15 min reduced the FBF in the AngII leg by approximately 20% (Fig. 4.6; $p<0.05$). No significant change in FBF was detected in the control leg during AngII infusion into the AngII leg. FBF was significantly ($p<0.05$) reduced in the AngII leg following 10 min of AngII infusion and following 15 min was approximately 20% lower ($p<0.05$) compared to the control leg.

Local infusion of AngII for 15 min in either HS fed rats decreased FBF in the AngII leg by approximately 30% but did not affect mean arterial blood pressure or control leg blood flow. In HS fed rats, infusion of AngII for 15 min reduced FBF by approximately 30% in the AngII leg (Fig. 4.7; $p<0.001$). FBF was significantly decreased following 6 min of AngII infusion in the AngII leg and following 15 min, FBF was approximately 25% lower compared to control leg ($p<0.05$).

Local infusion of AngII for 15 min in legs of HS fed rats tended to reduce FBF more rapidly (within 6 min compared to 10 min) and to a greater extent than in legs of CTRL fed rats, however, this difference did not reach statistical significance (CTRL; -19.9 ± 6.1 vs. HS; 31.0 ± 2.5 %, $p=0.156$).

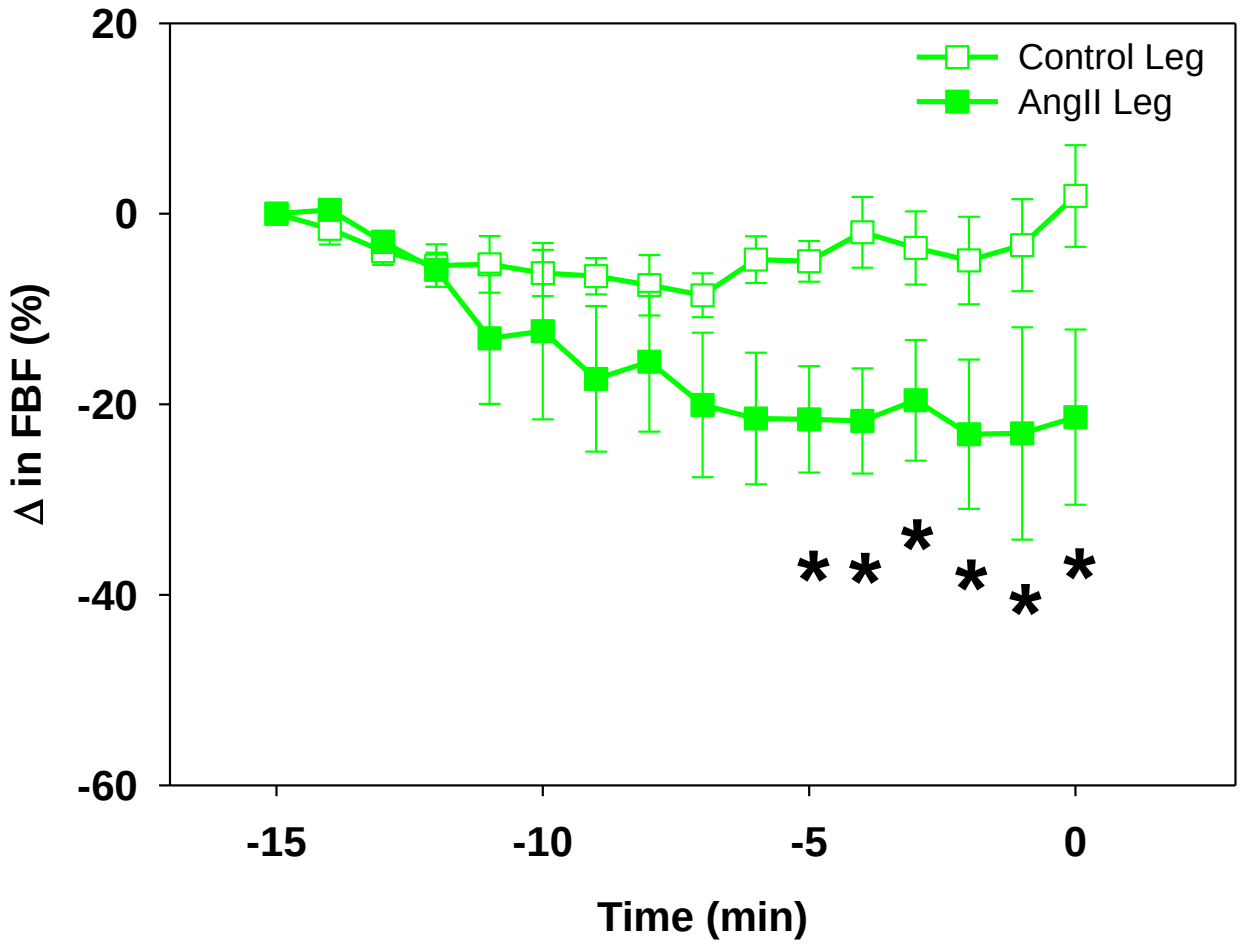

Figure 4.6. *In vivo* effects of 15 min AngII infusion on FBF in CTRL fed rats.

Time course of changes in FBF in control and AngII legs during 15 min of local AngII infusion. AngII was infused into the AngII leg at 1:100 of the FBF rate to a final concentration of 5nM. Data are means ± SEM for n=5 rats. * Significantly different ($p<0.05$) from control leg using two-way repeated measures ANOVA.

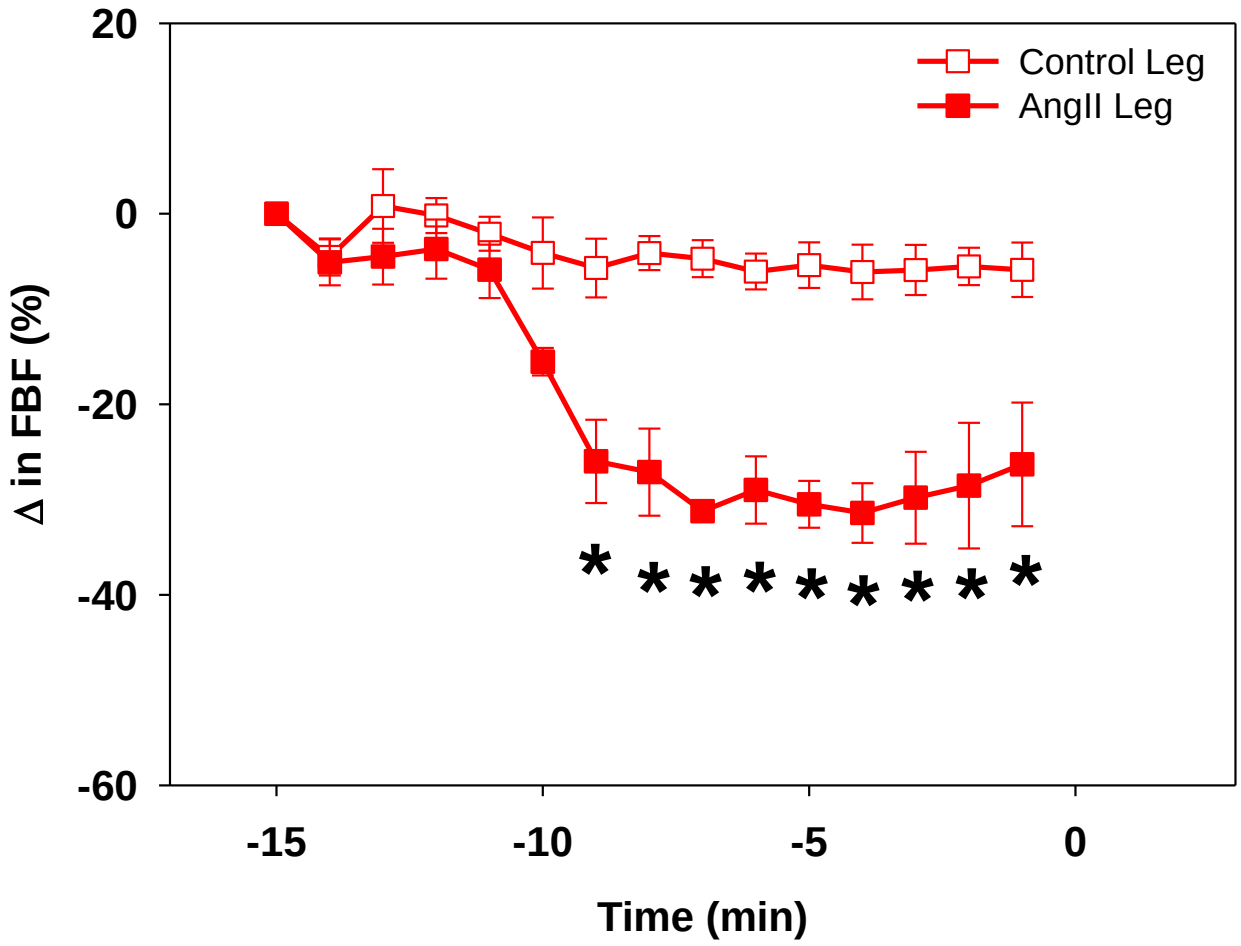

Figure 4.7. *In vivo* effects of 15 min AngII infusion on FBF in HS fed rats.

Time course of changes in FBF in control and AngII legs during 15 min of local AngII infusion. AngII was infused into the AngII leg at 1:100 of the FBF rate to a final concentration of 5nM. Data are means ± SEM for n=5 rats. * Significantly different ($p<0.05$) from control leg using two-way repeated measures ANOVA.

4.3.4. *In vivo* effects of local AngII infusion on basal and insulin-mediated change in FBF

While AngII infusion decreased basal FBF following 15 min in the AngII leg, 120min of insulin infusion increased FBF to a similar extent in both legs of CTRL fed rats. Representative time course of AngII and insulin mediated changes in FBF in CTRL fed rats is shown in Fig. 4.8. FBF at -15 min tended to be slightly increased in the AngII legs compared to the control legs of CTRL fed rats (Fig. 4.9; 0.95 ± 0.18 vs. 1.09 ± 0.16 mL/min, p=0.466). Following 15 min of AngII infusion in CTRL fed rats FBF decreased in the AngII leg (1.09 ± 0.16 vs. 0.86 ± 0.14 mL/min, p=0.043) but did not change in the control leg (0.95 ± 0.18 vs. 0.93 ± 0.18 mL/min, p=0.603). No difference in FBF at t=0 min was detected between the AngII and control legs (p=0.111) in CTRL fed rats. Infusion of insulin for 120 min increased FBF in control (0.93 ± 0.18 vs. 1.35 ± 0.11 mL/min, p=0.049) and AngII (0.86 ± 0.14 vs. 1.22 ± 0.23 mL/min, p=0.029) legs of CTRL fed rats. However, the insulin-mediated change in FBF was similar between the two legs in CTRL fed rats (p=0.217). Since no change in mean arterial blood pressure were detected during the course of AngII and insulin infusion (data not shown), FVR followed similar trends to that of the FBF response in both legs of CTRL rats.

Similar to CTRL fed rats, AngII infusion for 15 min reduced basal FBF in the AngII leg of HS fed rats, but insulin infusion for 120 min increased FBF to a similar extent in both legs. Representative time course of AngII and insulin mediated changes in FBF in HS fed rats is shown in Fig. 4.10. FBF at -15 min tended to be slightly increased in the AngII infused leg compared to the control leg of HS fed rats (Fig. 4.11; 0.77 ± 0.06 vs. 0.91 ± 0.05 mL/min, p=0.174). Following 15 min of AngII infusion in HS fed rats FBF decreased in the AngII leg (0.92 ± 0.06 vs. 0.66 ± 0.04 mL/min, p=0.004) but was not different in the control leg (0.71 ± 0.07 vs. 0.72 ± 0.10 mL/min, p=0.622). FBF at t = 0min was not different between AngII and control legs (p=0.384). 120 min insulin infusion in HS fed rats increased FBF in control (0.72 ± 0.10 vs. 0.91 ± 0.12 mL/min, p=0.037) and AngII legs (0.66 ± 0.04 vs. 0.80 ± 0.10 mL/min, p=0.033). No difference in the insulin-stimulated change in FBF was detected between the control and AngII legs of HS fed rats (p=0.269). Since no change in mean arterial blood pressure were detected during the course of AngII and insulin infusion (data not shown), FVR followed similar trends to that of the FBF response in both legs of HS fed rats.

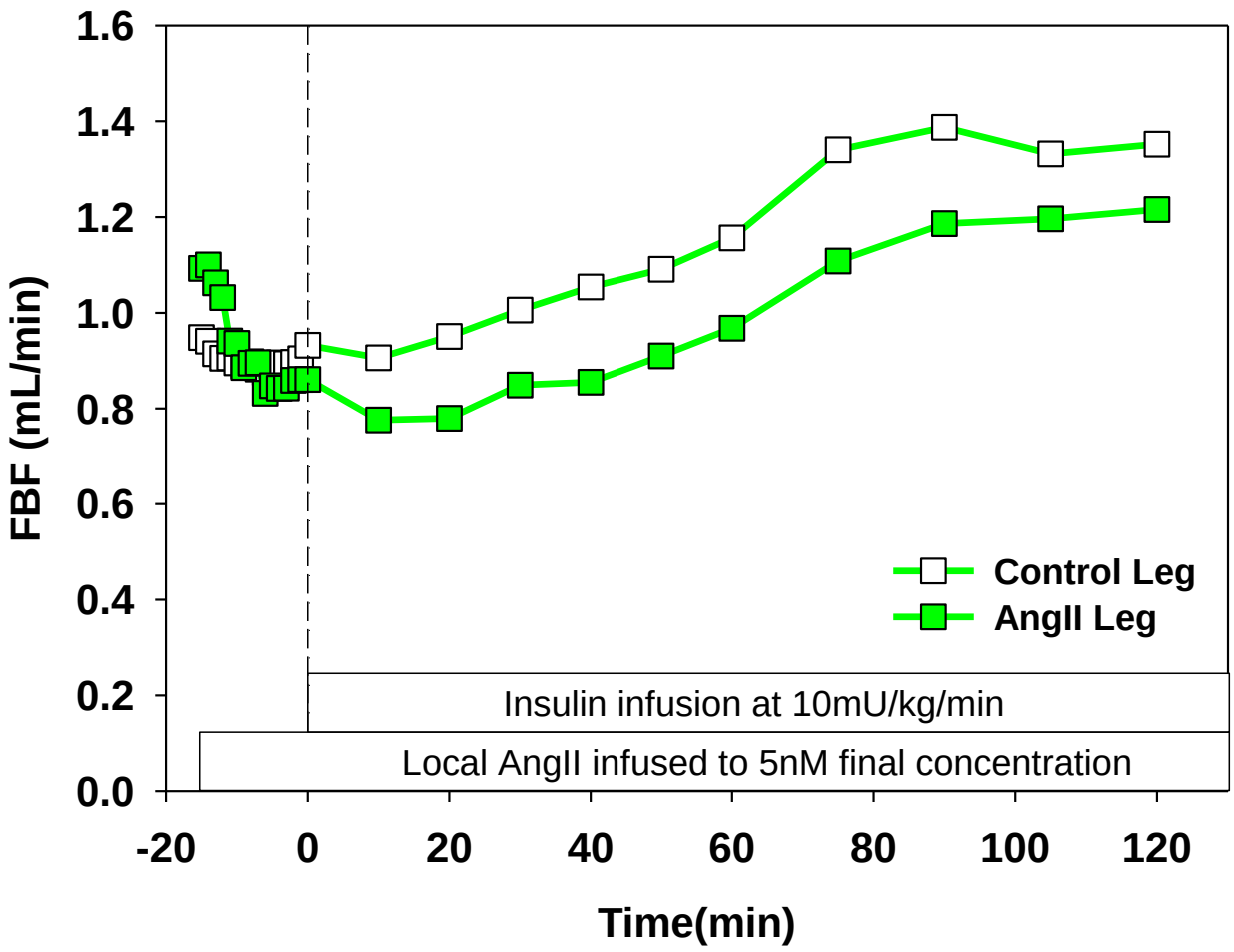

Figure 4.8. Representative traces showing the effects of AngII and insulin infusion on FBF in control and AngII legs of CTRL fed rats.

The time course of AngII and insulin infusion is shown above. AngII was infused locally only into the AngII Leg to a final concentration of 5nM via the epigastric artery. Insulin was infused systemically at a rate of 10mU/kg/min. Data are means for n=5, SE bars were omitted for clarity.

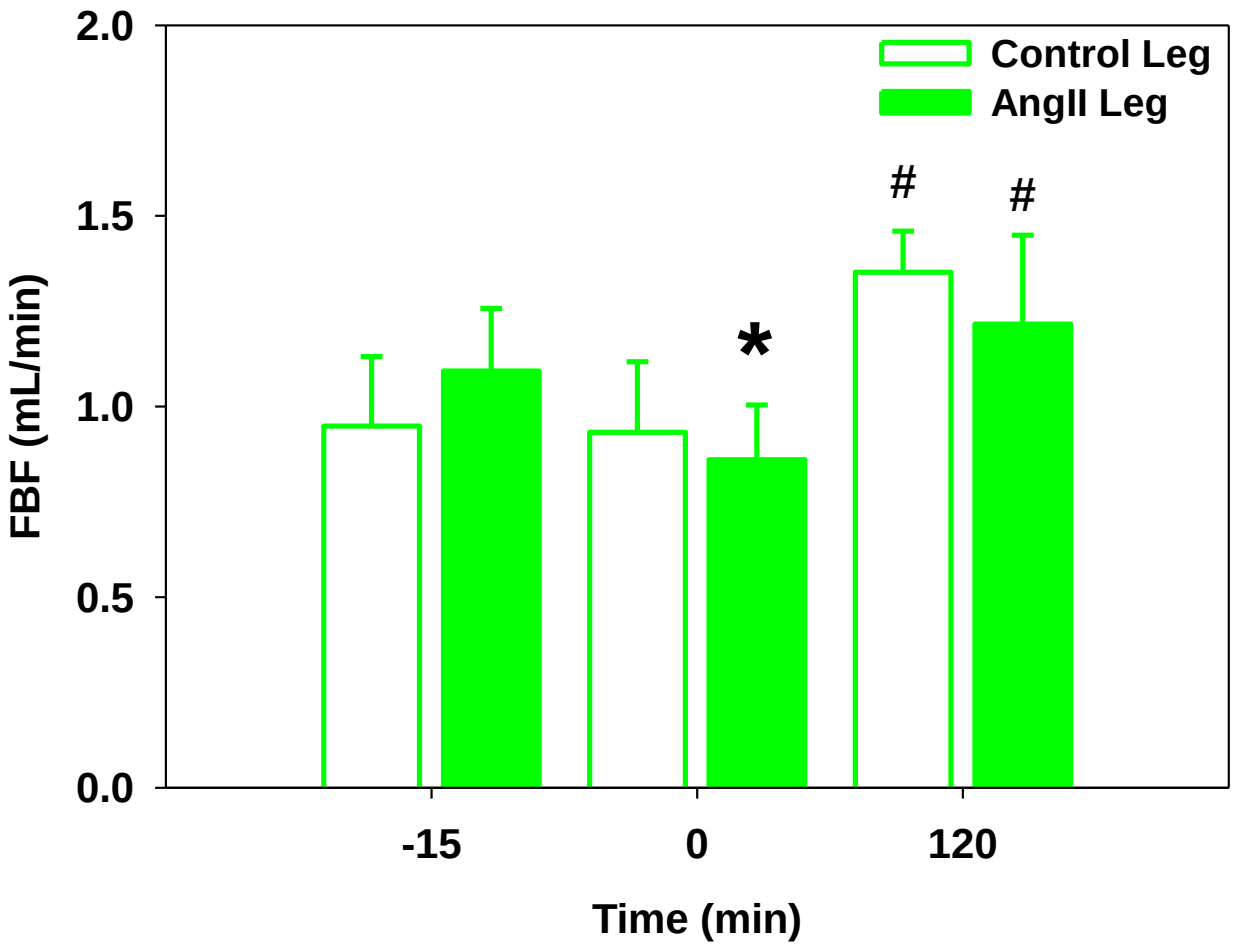

Figure 4.9. Effects of AngII and insulin infusion on FBF in control and AngII legs of CTRL fed rats.

Local AngII was infused to a final concentration of 5nM for 15 min in the AngII Leg. Systemic insulin infusion was initiated at 0 min and continued for 120 min. Data are means ± SE for n=5 rats. * Significantly different ($p<0.05$) compared to respective -15 min FBF. # Significantly different ($p<0.05$) compared to respective 0 min FBF using two-way repeated measures ANOVA.

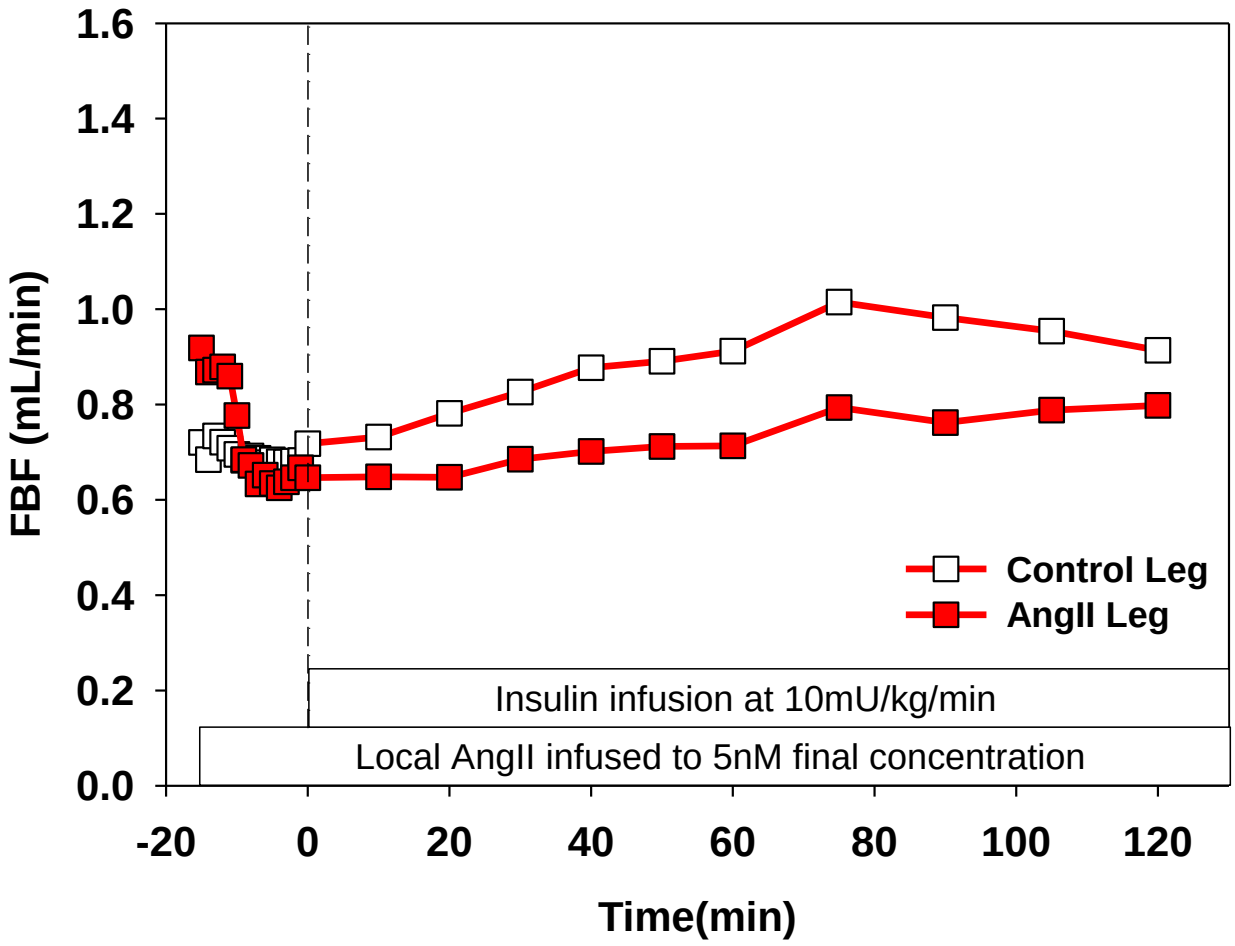

Figure 4.10. Representative traces demonstrating the effects of AngII and insulin infusion on FBF in control and AngII legs of HS fed rats.

The time courses of AngII and insulin infusion are shown above. AngII was infused locally only into the AngII Leg to a final concentration of 5nM via the epigastric artery. Insulin was infused systemically at a rate of 10mU/kg/min. Data are means for n=5, SE bars were omitted for clarity.

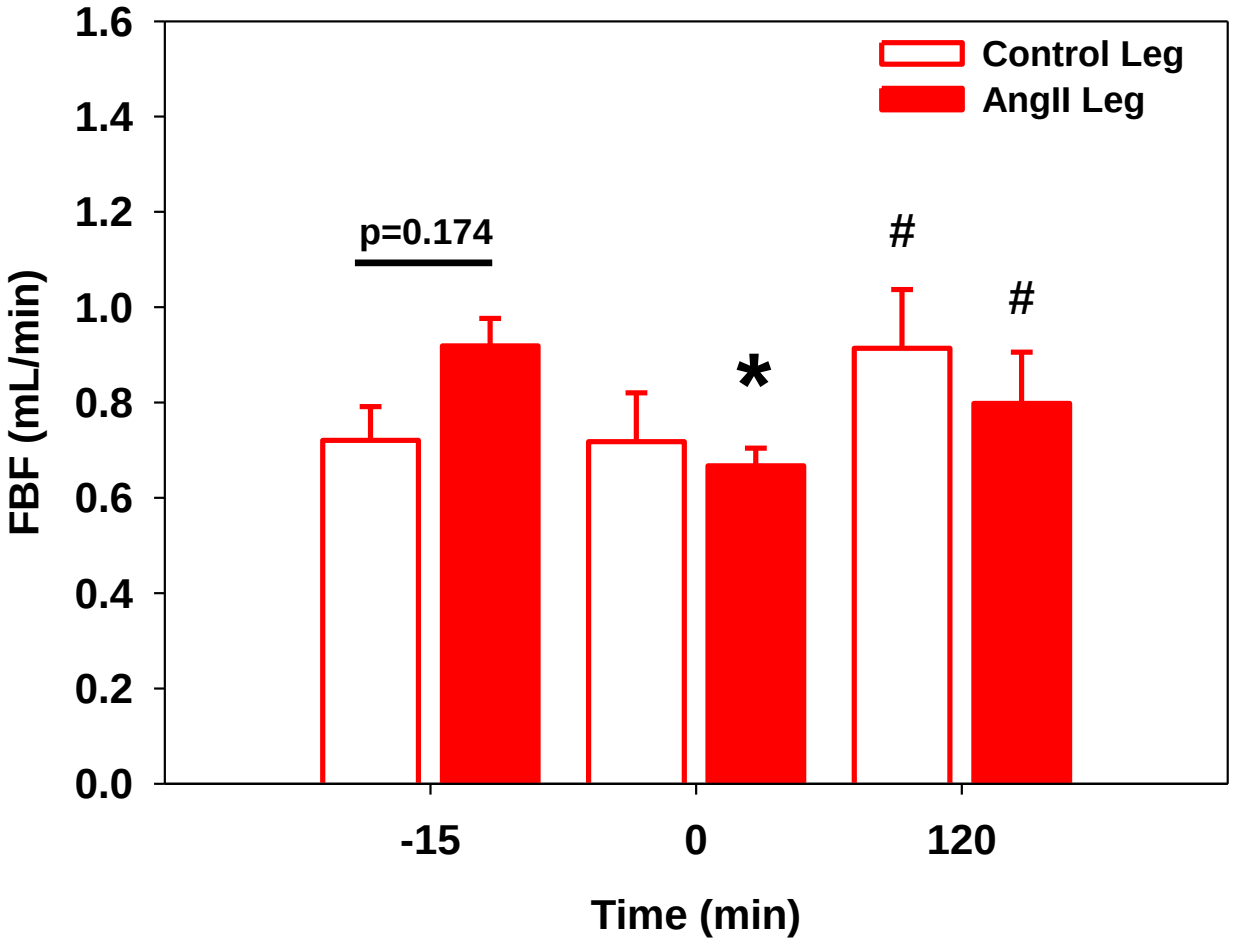

Figure 4.11. Effects of AngII and insulin infusion on FBF in control and AngII legs of HS fed rats.

Local AngII was infused to a final concentration of 5nM for 15 min in the AngII Leg. Systemic insulin infusion was initiated at 0 min and continued for 120 min. Data are means ± SE for n=5 rats. * Significantly different ($p<0.05$) compared to respective -15 min FBF. # Significantly different ($p<0.05$) compared to respective 0 min FBF using two-way repeated measures ANOVA.

4.3.5. Effects of local AngII infusion on insulin-mediated 1-MX metabolism

Local infusion of AngII did not alter metabolism of 1-MX between control and AngII legs in CTRL fed rats. However, infusion of AngII reduced 1-MX metabolism in the AngII leg when compared to the control leg of HS fed rats. Following 120 min of insulin infusion no difference in 1-MX metabolism was detected between control and AngII infused legs (Fig. 4.12; 5.40 ± 1.19 vs. 4.65 ± 0.86 nmol/min; p=0.171) in CTRL fed rats. However, metabolism of 1-MX was approximately 30% lower in AngII infused leg compared to the control leg (4.92 ± 0.95 vs. 3.66 ± 0.91 nmol/min; p=0.022) in HS fed rats. Metabolism of 1-MX was not different between CTRL and HS fed rats in either the control (p=0.740) or AngII (p=0.470) legs.

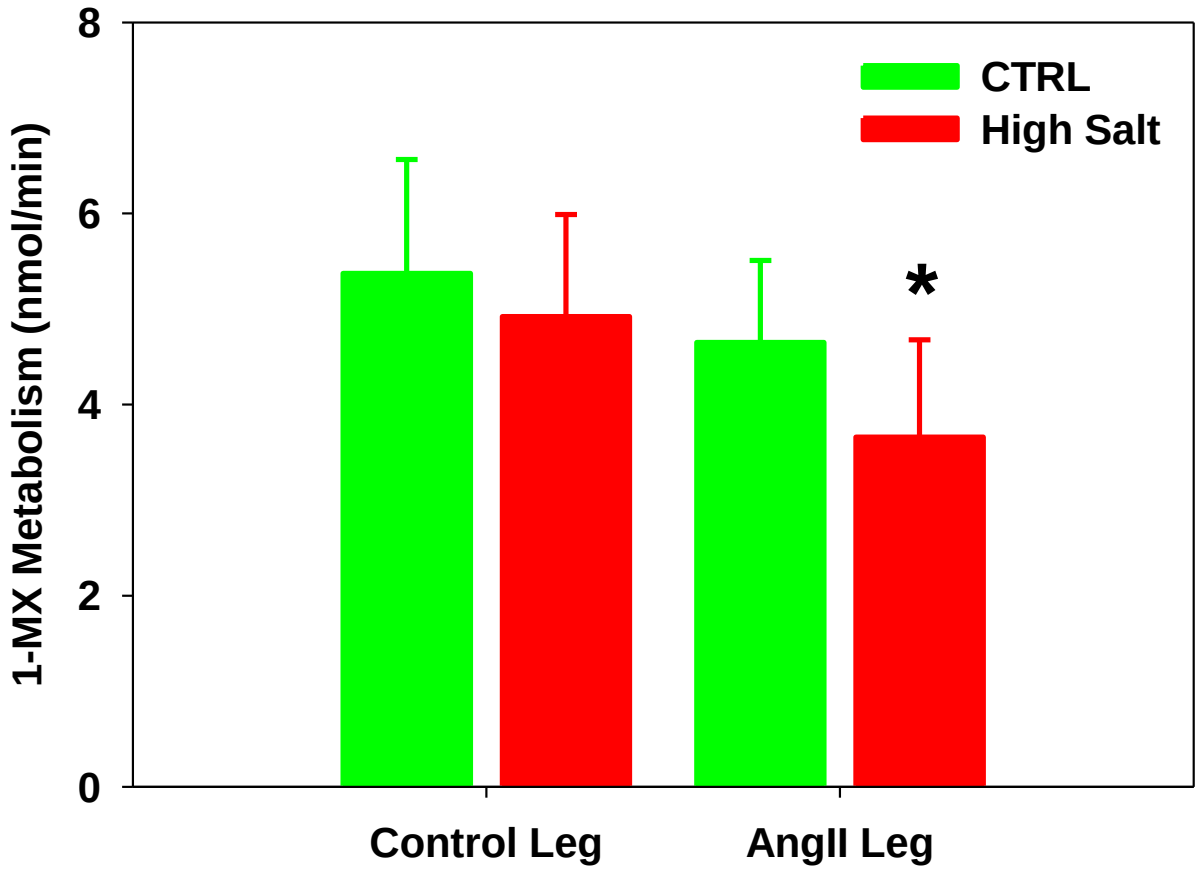

Figure 4.12. Effects of AngII and insulin infusion on 1-MX metabolism.

Effects of systemic insulin infusion on 1-MX metabolism in CTRL and HS fed rats during local AngII infusion into the AngII leg. Data are means ± SEM for n=5 rats. * Significantly different ($p<0.05$) compared to HS control leg.

4.3.6. Effects of local AngII infusion on insulin-mediated hindleg and muscle glucose uptake

After 120 min of infusion insulin-mediated hindleg glucose uptake was reduced in the AngII infused leg compared to the control leg in HS fed rats but no effect was found in the CTRL fed rats. AngII infusion did not affect hindleg glucose uptake between control and AngII legs (Fig. 4.13.A; 0.71 ± 0.09 vs. 0.67 ± 0.07 µmol/min; p=0.407) of CTRL fed rats. However, in HS fed rats AngII infusion caused a marked decrease in hindleg glucose uptake following hyperinsulinaemia (0.57 ± 0.10 vs. 0.37 ± 0.06 µmol/min; p=0.010). Hindleg glucose uptake was significantly (p=0.020) decreased in the AngII infused leg of HS fed rats compared to the AngII infused leg of CTRL fed rats. While hindleg glucose uptake tended to be lower in the control leg of HS fed rats compared to CTRL fed rats, no significant difference was detected between the two (p=0.215).

Following 120 min of infusion insulin-mediated R`g was reduced in the AngII infused leg compared to the control leg in HS fed rats but no effect was found in the CTRL fed rats. AngII infusion did not affect R`g between control and AngII infused legs (Fig. 4.13.B; 20.9 ± 3.2 vs. 20.1 ± 3.8 µg/g/min; p=0.323) of CTRL fed rats. However, in HS fed rats AngII infusion slightly, but significantly reduced R`g during hyperinsulinaemia (16.5 ± 0.6 vs. 15.0 ± 0.6 µg/g/min; p=0.014). Whilst R`g was slightly lower in both control (p=0.097) and AngII (p=0.176) infused legs of HS fed rats compared to the CTRL fed rats, this difference did not reach statistical significance.

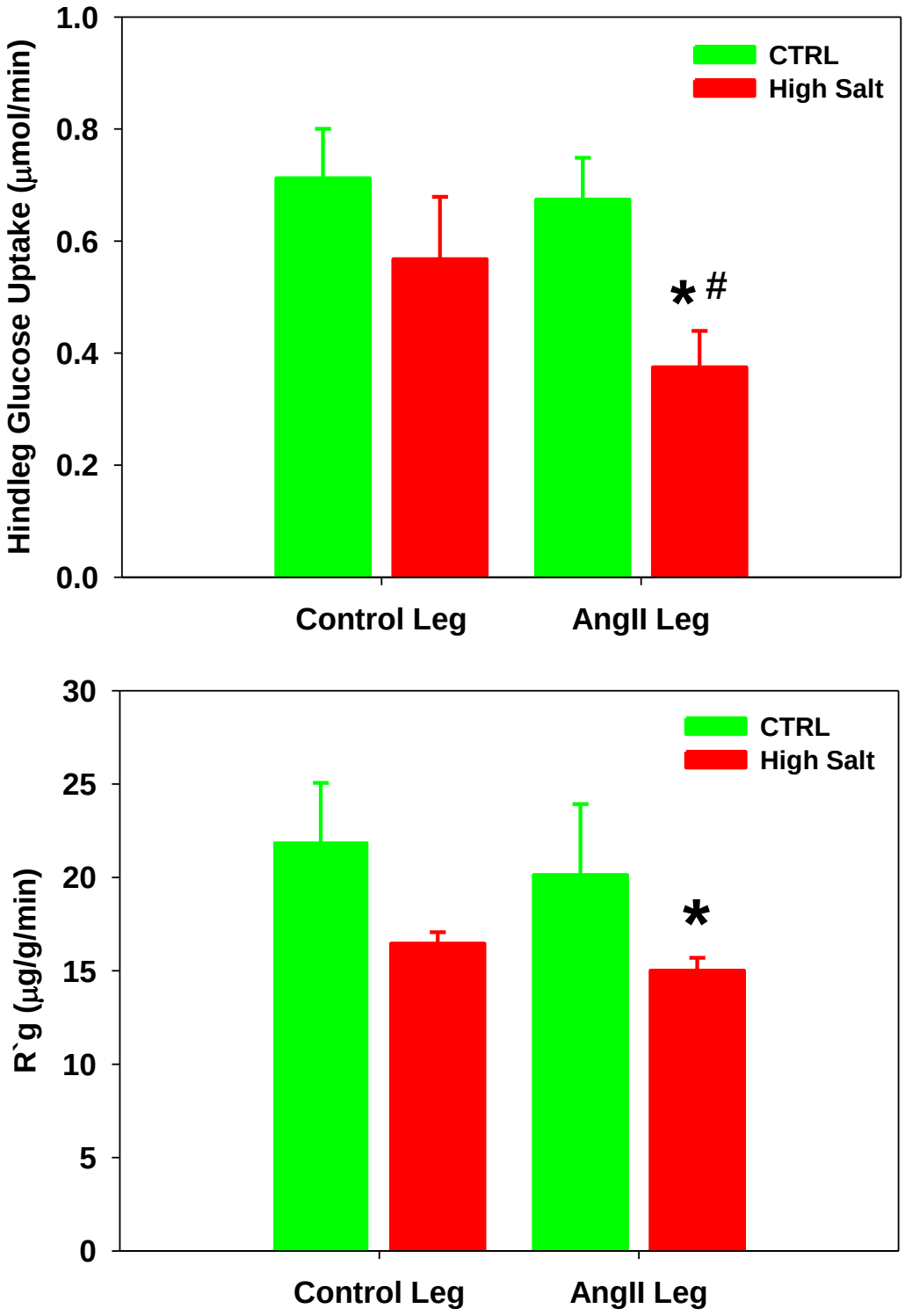

Figure 4.13. Insulin-mediated hindleg and muscle glucose uptake in CTRL and HS rats.

Hindleg glucose uptake (A) and muscle glucose uptake (B) in CTRL and HS fed rats following 2 hrs of insulin systemic infusion and local AngII infusion into the AngII leg. Data are means ± SEM for n=5 rats. * Significantly different ($p<0.05$) compared to respective control leg. # Significantly different ($p<0.05$) from CTRL-AngII leg.

4.4. Discussion

This study produced three main findings. Firstly, following 3-5 weeks of dietary intervention, HS fed rats exhibited markedly increased vascular sensitivity to AngII-mediated constriction compared to CTRL fed rats. Secondly, infusion of AngII locally into one hindleg attenuated microvascular recruitment and muscle glucose uptake in the presence of insulin compared to the control leg of HS fed rats. Thirdly, local AngII infusion markedly reduced basal FBF but did not attenuate insulin-mediated microvascular recruitment or muscle glucose uptake in CTRL fed rats. Together these data confirm that AngII infusion increases vasoconstriction *in vivo* and can interfere with insulin-mediated microvascular recruitment and muscle glucose uptake. Notably, however, the inhibitory effects of AngII were only present in the HS fed rats, while local AngII infusion had little effect on insulin action in hindlegs of CTRL fed animals, suggesting that AngII can impair insulin action in hindleg vasculature where there has been an up-regulation of AT1R sensitivity.

In the present study AngII infusion into the constant-flow perfused hindlimb preparation increased perfusion pressure in a dose dependent manner in the vasculature of both CTRL and HS fed rats. However, the HS rats exhibited a leftward shift in AngII sensitivity compared to the CTRL fed rats. It is also possible that the HS diet may have increased the vascular responsiveness to AngII, but this could not be determined in the present study as it was not possible to reach maximal AngII doses without excessive perfusion pressure. Similar to the perfused hindleg experiments, local 5nM AngII infusion in the anaesthetised rat preparation significantly reduced FBF compared to basal FBF in both CTRL and HS fed rats groups. However, as with the perfused hindleg experiments, this AngII effect appeared to be more pronounced in the HS compared to CTRL diet fed rats. Notably, however, FBF was slightly elevated in AngII infused legs of both CTRL and HS fed rats compared to the control leg. The increased basal FBF was most likely an artefact resulting from the extra surgery required to cannulate the epigastric artery of the AngII infused legs. Importantly, when the percentage change in FBF from basal was assessed following 15 min AngII infusion, the AngII infused legs of both CTRL (~20%) and HS (~30%) fed rats exhibited a marked drop in FBF compared to basal. Together these data suggest that the vascular sensitivity to AngII is significantly

enhanced in the HS fed rats compared to CTRL fed rats. These results are consistent with previous reports in the literature where HS feeding has been shown to increase peripheral constriction in a number of vascular beds including skeletal muscle resistance arterioles via increased AT1R activity [337, 338, 344].

Weber and colleagues (1999) reported that HS intake leads to a potentiation of the response of skeletal muscle resistance arterioles to AngII-mediated constriction [344]. In addition, this response appeared to be selective for AngII, since norepinephrine-mediated vasoconstriction was not altered following HS feeding [344]. This increase in AngII-mediated vasoconstriction following HS feeding has in part been attributed to an increase in the expression and/or sensitivity of the AT1R in the vasculature [337, 354, 355]. Indeed, Wang and colleagues (1998) reported that the vasculature of HS fed rats exhibited a 171% increase in AT1R expression compared to control diet fed rats [337]. Therefore, it would be reasonable to conclude that in the present study the increased AngII-mediated vascular response in HS fed rats both *in vivo* and *in vitro* can be partly attributed to an increase in AT1R-mediated vasoconstriction. However, others have also uncovered potential roles in the down-regulation of other RAS components that contribute to vasodilation following HS feeding. Gonzalez and colleagues (2005) reported that HS intake in rats significantly decreased the expression of AT2R in mesenteric resistance arteries [356]. Additionally, increasing salt intake has also been reported to reduce the concentration of Ang-(1-7) [357], which generally opposes AngII-mediated constriction. Thus, by increasing either AT1R expression, or decreasing AT2R expression and Ang-(1-7) concentration, or a combination of these factors, increased salt intake in the present study may shift the balance to favour AngII-AT1R mediated vasoconstriction.

It has previously been reported that acute, systemic infusions of AngII increases insulin-mediated whole body glucose disposal in healthy subjects [287, 288, 351]. However, the mechanisms responsible for this improvement in insulin sensitivity remain unclear. Buchanan and colleagues (1993) reported that AngII increased insulin-mediated whole body glucose disposal by increasing total leg blood flow in healthy subjects [351]. However, previous reports have identified that increases in total blood flow alone are not

sufficient to increase muscle glucose uptake but also requires an increase in the microvascular perfusion of muscle [37, 61]. Furthermore, Jonk and colleagues (2010) reported that systemic infusion of AngII actually decreased insulin-mediated microvascular recruitment [287]. Therefore, these data suggesting that the increased whole body glucose disposal following AngII infusion is most likely not the result of increased insulin-mediated haemodynamic effects in muscle. However, a direct effect on insulin-mediated muscle glucose uptake cannot be excluded, since no direct measure of muscle glucose uptake was obtained in theses previous studies. Although, this too appears unlikely since AngII has been previously shown to not only inhibit the insulin signalling pathway in myocytes [200] but also inhibit insulin-mediated muscle glucose uptake [294, 296]. Therefore, the effects of systemic AngII are difficult to interpret and the increased whole body glucose disposal may be the result of a central effect of AngII (possibly liver, brain or heart) rather than direct effects on insulin-mediated muscle blood flow and glucose uptake. Indeed, preliminary data from the present study found that systemic AngII infusion in CTRL fed rats was associated with a significant rise in blood glucose concentrations (~1.5mmol/L) at both pressor and sub-pressor doses (data not shown), suggesting a possible acute AngII effect on liver glucose output. For these reasons, local AngII infusion was used to determine the effects of AngII on insulin sensitivity in skeletal muscle in the present study.

Jamerson and colleagues (1996) previously found that local infusion of AngII did not affect insulin-mediated glucose extraction across the forearm compared to insulin alone in healthy subjects [353]. However, the results were confounded by a significant increase in hyperinsulinaemia during the local forearm infusions. In the present study insulin was infused systemically following 15 min of AngII infusion and therefore it would be reasonable to assume that the same insulin concentration reached both the control and AngII infused hindlegs. The data from the present study suggests that in CTRL diet fed rats, local AngII infusion has minimal effects on insulin sensitivity in muscle. Indeed, while local AngII infusion initially reduced FBF compared to basal, systemic insulin infusion for 120 min was able to overcome this vasoconstriction and moreover, insulin stimulated comparable increases in total FBF in both legs of CTRL diet fed rats. In addition, it was found that local AngII infusion did not significantly affect insulin-mediated microvascular recruitment, hindleg or muscle specific glucose uptake

compared to the control leg. Together, these data would suggest that acute 5nM AngII infusion has minimal effects on skeletal muscle insulin sensitivity in normal healthy animals. In light of these findings, it is unlikely that the enhanced insulin-mediated glucose disposal during systemic AngII infusion reported in the previous studies can be attributed to a direct effect of AngII on insulin's vascular and metabolic actions in skeletal muscle and is more likely the result of a central AngII effect.

In contrast to the CTRL fed rats local AngII infusion attenuated insulin-mediated microvascular recruitment, hindleg and muscle specific glucose uptake compared to the control leg of HS fed rats. This impairment of insulin sensitivity in muscle was associated with markedly increased AngII-mediated vasoconstriction as measured in the perfused hindleg preparation. Since previous studies have reported that increased salt intake is associated with an increase in AT1R expression [330, 338], it follows that increased AngII activity via the AT1R may be detrimental to insulin sensitivity in skeletal muscle. In two recent reports Chai and colleagues (2009 and 2011) demonstrated that both AT1 and AT2 receptors may be involved in the regulation of both basal [108] and insulin-stimulated microvascular perfusion of skeletal muscle [109]. The authors reported that at basal and during hyperinsulinaemia, systemic infusion of an AT1R receptor antagonist increased microvascular perfusion of muscle [109]. Conversely, systemic infusion of an AT2R antagonist reduced both basal and insulin-stimulated microvascular perfusion of skeletal muscle [109]. Thus, the authors proposed that modulating AngII activity to preferentially stimulate the AT1R may reduce microvascular perfusion of muscle and that AngII activation of the AT2R increases microvascular perfusion of muscle. It follows then that in the HS fed rat, where vascular AT1R expression has been reported to be increased and AT2R expression decreased [330, 338, 344, 356], local infusion of AngII likely results in the preferential activation of the AT1R. Therefore, the reduction in insulin-mediated microvascular recruitment during local AngII infusion in HS fed rats may be attributable to increased activation of the AT1R compared to the control leg.

As well as reducing insulin-mediated microvascular recruitment, local AngII infusion was also found to attenuate insulin-mediated hindleg and muscle glucose uptake

compared to the control leg of HS fed rats. The direct cause of the AngII-mediated attenuation of glucose uptake in muscle of HS fed rats was not directly investigated in the present study. However, previous studies have reported that AngII, via the AT1R, can directly inhibit the insulin signalling pathway in muscle and thus attenuate insulin-mediated glucose uptake in skeletal muscle [294, 296]. However, these previous studies assessed the AngII effect on insulin-mediated muscle glucose uptake in incubated muscles (*in vitro*), thus avoiding the contribution of insulin's vascular action. This is the first study to demonstrate that local AngII infusion *in vivo* significantly reduces insulin-mediated microvascular recruitment and is associated with a reduction insulin-mediated muscle glucose uptake in HS fed rats. However, others have shown that acute inhibition of insulin-mediated microvascular recruitment in muscle also reduces insulin-mediated muscle glucose uptake [100, 167, 177]. It follows then that AngII-mediated attenuation of insulin-mediated microvascular recruitment in muscle may be in part responsible for the decrease in insulin-mediated muscle glucose uptake. However, whether local AngII infusion *in vivo* also directly inhibits insulin-mediated muscle glucose uptake in HS fed rats cannot be excluded from the results of the current study. Thus, it is possible that the attenuation of muscle glucose uptake during local AngII infusion in HS fed rats may be the result of either reduced insulin-mediated microvascular recruitment, direct inhibition of insulin-mediated muscle glucose uptake or a combination of these factors.

Notably, although local AngII infusion significantly reduced the basal FBF in the AngII leg of both CTRL and HS fed rats, 120 min of systemic insulin infusion was able to overcome this and increased FBF to a similar extent as the control leg. Given that AngII attenuated insulin-mediated microvascular recruitment and muscle glucose uptake in the HS but not the CTRL fed rats, these data suggest that the effects of insulin to increase both microvascular perfusion and muscle glucose uptake are independent of insulin's effects on total FBF. Indeed, previous reports would tend to support this conclusion. Coggins and colleagues (2001) reported that insulin-mediated microvascular recruitment and muscle glucose uptake occurs without any changes in total limb blood flow in normal healthy subjects [61]. Moreover, Vincent and colleagues (2002 and 2004) reported that insulin-mediated microvascular recruitment in muscle occurs early, and precedes changes in total blood flow [38, 105]. Furthermore, blocking this early increase in insulin-mediated microvascular recruitment also partially inhibited insulin-mediated

muscle glucose uptake [105]. Therefore, taken together with these previous reports, the data from the current study also indicates that insulin-mediated muscle glucose uptake is not dependent on an increase in total FBF. Rather, it would appear to be partly dependent on the ability of insulin to increase microvascular perfusion of muscle.

4.4.3. Conclusions

The primary aims of this study were to examine hindleg sensitivity to AngII mediated constriction and to determine the effects of local AngII infusion on insulin-mediated microvascular recruitment and glucose uptake in muscle of CTRL and HS fed rats. The results indicate that in the HS fed rats, where AngII sensitivity/action is up-regulated, local AngII infusion was detrimental to insulin-stimulated microvascular recruitment and muscle glucose uptake. However, in the CTRL fed rats, where AngII activity is relatively normal, local AngII infusion did not affect insulin-stimulated microvascular perfusion or muscle glucose uptake. The discrepancy between the two models maybe due to differential sensitivity and/or expression of the AT1 and AT2 receptors. In the CTRL fed rats, the effects of AngII on insulin-mediated microvascular recruitment are likely mediated by balanced activation of AT1R mediated constriction and AT2R mediated dilation allowing for relatively normal insulin-stimulation of microvascular recruitment and thus muscle glucose uptake. Conversely in the HS model, the effects of AngII on skeletal muscle vasculature are likely mediated through increased activity of the AT1R, resulting in further increased FVR and thus further attenuation of insulin-stimulated microvascular perfusion and muscle glucose uptake. Thus, the data from the current study support the notion that HS feeding results in up-regulation of vascular AngII activity and further implicates increased AngII activity as detrimental to normal insulin-mediated microvascular recruitment and muscle glucose uptake *in vivo*.

CHAPTER 5

INHIBITION OF ANGIOTENSIN CONVERTING ENZYME IMPROVES HS DIET-INDUCED INSULIN RESISTANCE

5.1. Introduction

In chapter 3 of the current thesis rats fed a HS diet for 3-4 weeks exhibited attenuation of whole body insulin sensitivity compared to CTRL fed rats. Moreover, the HS fed rats exhibited marked attenuation of both insulin-mediated microvascular recruitment in muscle and insulin-mediated muscle glucose uptake compared to CTRL fed rats. Furthermore, the results in chapter 4 of the current thesis indicate that the development of insulin resistance following HS feeding may be due to an enhancement of vascular AngII sensitivity/activity. Indeed, local infusion of AngII was found to further inhibit both insulin-mediated microvascular recruitment in muscle and insulin-mediated muscle glucose uptake in the HS fed rats. Therefore, it would be reasonable to assume that inhibition of AngII activity would lead to an improvement in insulin sensitivity in the HS fed rat.

Reduction in concentration and activity of AngII can be achieved in two ways. Firstly AngII formation can be reduced by inhibiting the ACE, which synthesises AngII from angiotensin I. Secondly, AngII action can be attenuated by specifically blocking the AT1R using and ARB. Previous studies have reported that both ACE inhibition and ARB treatment can improve insulin sensitivity in a number of different models of insulin resistance [231, 255, 358, 359]. In obese Zucker rats, Henriksen and colleagues (1999) reported that ACE inhibition improves skeletal muscle insulin sensitivity by augmenting GLUT-4 translocation in muscle [255]. Similar results have been obtained by treating obese Zucker rats with an ARB [358]. Using the Dahl-salt sensitive, insulin resistant rat, Zhou and colleagues (2009) reported that ARB treatment improved whole body insulin sensitivity and improved insulin-mediated vasodilation in the aorta by reducing AngII-mediated ROS production [163]. Together, these data suggest that direct inhibition of AngII action by ACE inhibitors or ARBs improves insulin sensitivity in insulin resistant rats.

There is strong evidence, however, that inhibition of ACE may be of more benefit to insulin's vascular actions than ARBs. Feldman and Schmidt (2001) reported that long term treatment with the ACE inhibitor quinapril improved insulin-mediated vasodilation

in hypertensive individuals [360]. In fructose-fed hypertensive rats, Uchida and colleagues (2002) reported that phenylephrine-induced aortic constriction could be significantly attenuated by insulin-mediated dilation only following quinapril treatment [361]. Clerk and colleagues (2007) reported that quinapril treatment of Zucker Diabetic Fatty rats (ZDF; a model of late stage T2D) significantly improved whole body insulin sensitivity and increased insulin-mediated microvascular recruitment, although muscle glucose uptake was not measured [324]. While the exact mechanisms leading to improvement in vascular insulin sensitivity were not investigated in these previous studies, other reports have indicated that ACE inhibition goes further than just attenuation of AngII production. Indeed, inhibition of ACE has been reported to also reduce the expression of the AT1R in the vasculature, decrease bradykinin degradation and also reduce tissue ACE expression as well as activity [362-365]. Thus, ACE inhibitors may improve vascular and metabolic insulin sensitivity by inhibiting AngII action at various levels within the vasculature. Since the HS rats exhibit both enhanced vascular AngII sensitivity and also reduced microvascular insulin sensitivity, inhibition of AngII using an ACE inhibitor may be an appropriate target for improving insulin resistance in HS fed rats.

Therefore, the aim of the current study was to investigate whether inhibition of ACE in the HS fed rat could restore whole body and skeletal muscle insulin sensitivity. To this end, the effect of quinapril treatment on the vascular sensitivity to AngII was assessed using the perfused rat hindleg preparation. In addition, the effects of quinapril treatment on the haemodynamic and metabolic actions of insulin were determined *in vivo* using the anaesthetised rat preparation.

5.2. Methods

5.2.1. Animals

Male Sprague Dawley rats (4 weeks of age) were obtained from the University of Tasmania Central Animal Facility. On arrival, the rats were provided a high salt (8.00% NaCl wt./wt.; HS) AIN-93G semi-purified diet and received quinapril (HS+Q) in their drinking water at a dose of 1mg/kg/min for the duration of the feeding period (3-4 weeks). The results obtained following HS+Q treatment were compared to the HS results obtained in chapter 4 for the perfused hindleg experiments and chapter 3 for the anaesthetised rat experiments.

5.2.2. Perfused rat hindleg experiments

Surgical procedure and experimental setup were performed as previously outlined in chapter 2, sections 2.4.2 and 2.4.3. Following surgery and commencement of the perfusion, total flow through one hindleg was adjusted to a constant flow rate of 8ml/min for a 220g rat (equivalent to 0.4mL/min/g muscle). Following a 30 min equilibration period (to allow washout of red blood cells from the hindleg) an AngII dose curve was commenced to assess the vascular responsiveness of the hindleg. AngII was infused into the hindleg at 0.1, 0.3, 1.0, 3.0 and 10nM in 10 min stepwise increments and is shown in more detail in Fig. 5.1.

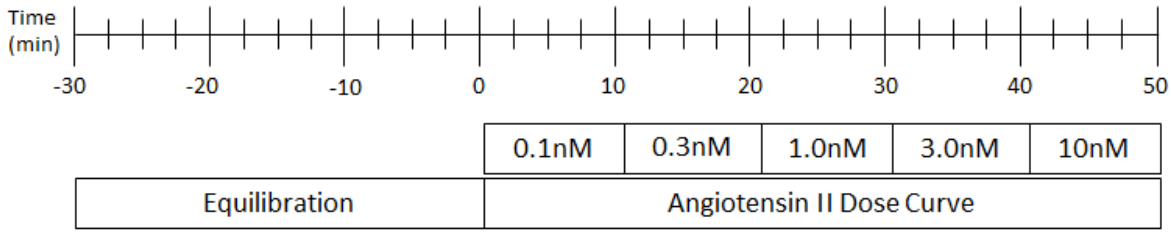

Figure 5.1. Experimental protocol for perfused rat hindleg preparation.

After surgical preparation and 30 min of equilibration, AngII infusion was initiated through the infusion port at 1:100 of the total flow rate through the hindleg to reach final concentration as indicated above. An increasing AngII dose curve was used to assess the sensitivity of the vasculature to AngII mediated constriction and perfusion pressure was recorded continuously using Windaq® software.

5.2.3. Anaesthetised rat experiments

Surgery was performed as previously outlined in chapter 2, section 2.3.1. Following surgical preparation, 1 hr was allowed for FBF and mean arterial blood pressure to stabilise, at which time a 2 hr infusion of insulin (10mU/min/kg) was initiated. Since insulin stimulates glucose disposal, a 30% glucose solution (wt./vol.) was infused at a variable rate to maintain fasting blood glucose concentrations over the course of the experiment. For this reason, arterial blood glucose levels were assessed every 10 min in the first hour, and every 15 min in the second hour using a glucose analyser (YSI 2300) and the glucose infusion rate (GIR) was adjusted accordingly. See Fig. 5.2 for a more detailed protocol. Plasma biochemistry, whole body glucose kinetics (Ra and Rd), muscle glucose uptake and microvascular recruitment in muscle were assessed as previously outlined in chapter 2, sections 2.3.2-2.3.5.

5.2.4. Data and Statistics

Data are presented as the means ± SEM and statistics were performed using Sigma-Stat (Systat Software Inc, 2004). Comparisons between CTRL, HS and HS+Q rats for the perfused hindleg preparation data were made using repeated measures two-way ANOVA. Comparisons between CTRL, HS and HS+Q groups were performed by one-way ANOVA. Comparison of time-series measurements in each group was performed by two-way repeated measures ANOVA. When a significant difference of $p<0.05$ was found, pairwise comparisons by Student-Newman-Keuls test was used to assess treatment differences.

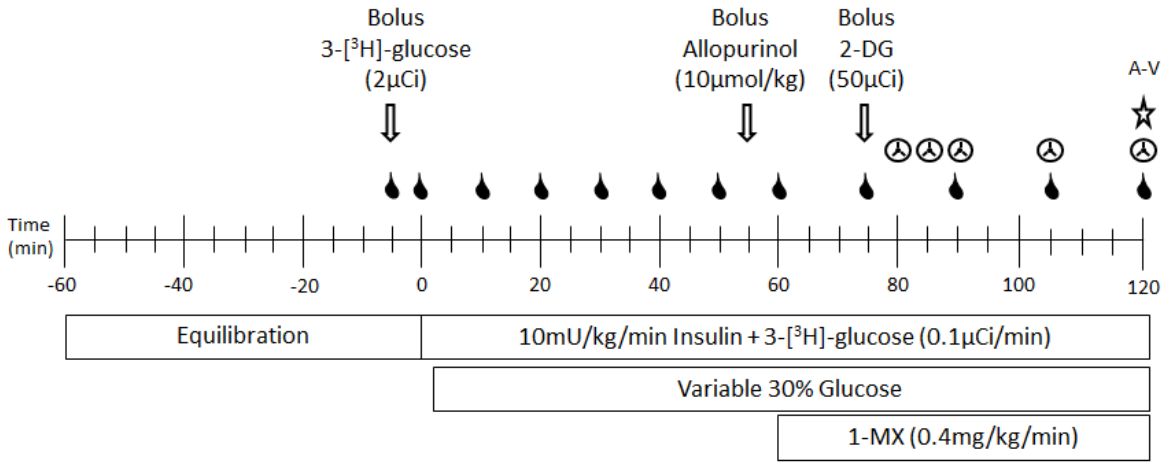

Figure 5.2. Experimental protocol for anaesthetised rat experiments.

After surgical preparation and 60 min of equilibration a continuous infusion of insulin (10mU/min/kg) was commenced and continued for 120 min. A 30% (wt./vol.) glucose infusion was initiated shortly after the commencement of the insulin infusion to maintain baseline glycaemia as assessed by arterial blood glucose sampling (). After 55 min of insulin infusion, a bolus of allopurinol (10µmol/kg) was administered. At 60 min, infusion of 1-methylxanthine (1-MX, 0.4mg/kg/min) was initiated and maintained until the end of the experiment. At 75 min a bolus of 2-deoxy-D-[1-^{14}C]-glucose (2-DG; 20µCi) was administered. Radioactive plasma samples () were collected at 80, 85, 90, 105 and 120 min to determine the plasma 2-DG clearance. At the conclusion of the experiment arterial and femoral vein plasma samples ( ) were collected for the determination of hindleg glucose uptake and 1-MX metabolism. Immediately following sacrifice, calf muscle (gastrocnemius, plantaris and soleus group) was freeze clamped in liquid nitrogen and stored at -80°C.

5.3. Results

5.3.1. Physical and biochemical characteristics of rats following 3-4 week feeding intervention

Following 3-4 week HS+Q animals were found to have similar body weight ($p=0.190$) and epididymal fat mass ($p=0.714$) compared to both CTRL and HS groups. No difference was detected in fasting plasma glucose ($p=0.421$), insulin ($p=0.185$), lactate ($p=0.449$) or FFA (0.463) concentrations between the three groups. The haematocrit was significantly reduced in the HS+Q group compared the HS ($p<0.001$) group and remained slightly elevated compared to the CTRL ($p=0.060$) group. While no change in mean arterial blood pressure was detected between the three groups ($p=0.862$) the HS+Q animals were found to have reduced basal FBF compared to CTRL fed rats ($p<0.001$) and elevated basal FBF compared to HS fed rats ($p=0.028$). Consequently the FVR was found to be elevated in the HS+Q group compared to CTRL ($p=0.003$) and reduced compared to HS ($p=0.013$) groups.

	CTRL	HS	HS+Q
Body weight (g)	227 ± 4	230 ± 6	214 ± 5
Epididymal fat pad (g)	1.20 ± 0.07	1.28 ± 0.09	1.17 ± 0.05
Mean arterial pressure (mmHg)	102 ± 2	101 ± 2	103 ± 3
Basal FBF (mL/min)	0.98 ± 0.04	0.53 ± 0.02 *	0.70 ± 0.09 *#
Basal FVR (mmHg.min/mL)	109 ± 6	195 ± 10 *	160 ± 19 *#
Haematocrit (%)	43.6 ± 0.5	49.2 ± 0.9 *	46.4 ± 1.4 #
Fasting plasma glucose (mmol/L)	6.52 ± 0.13	6.24 ± 0.17	6.43 ± 0.29
Fasting plasma insulin (pmol/L)	86 ± 8	97 ± 9	130 ± 23
Fasting plasma lactate (mmol/L)	0.80 ± 0.03	0.79 ± 0.04	0.76 ± 0.03
Fasting plasma FFA (mmol/L)	0.71 ± 0.03	0.67 ± 0.03	0.72 ± 0.02

Table 5.2. Physical and biochemical characteristics of rats following 3-4 week feeding intervention.

Measures were collected immediately prior to the commencement of the saline infusion or insulin clamp. Epididymal fat pads were excised and weighed at the conclusion of the experiments. FBF; femoral artery blood blow, FVR; femoral artery vascular resistance, FFA; free fatty acids. Data are means ± SEM for n=8-20 rats in each group.
* Significantly different ($p<0.05$) from CTRL using on-way ANOVA. # Significantly different ($p<0.05$) from HS using one-way ANOVA.

5.3.2. Effects of quinapril on vascular AngII sensitivity in the perfused rat hindleg preparation

The time course for changes in perfusion pressure in response to the AngII dose curve is shown in Fig. 5.3. Each increase in AngII concentration stimulated a greater increase in perfusion pressure in the hindlegs of all three groups. However, while the HS+Q group exhibited greater increases in perfusion pressure following each dose of AngII compared to CTRL, the AngII response was reduced compared to HS. Perfusion pressure in all three groups peaked approximately 4 min following the initiation of each dose of AngII. For this reason the perfusion pressure developed at 4 min for each dose of AngII was averaged for each of the treatment groups and is shown in Fig. 5.4.

Quinapril treatment appeared to reduce the vascular sensitivity of HS fed rats to AngII-mediated constriction, but remained elevated compared to CTRL fed rats. Compared to CTRL rats, HS and HS+Q groups were significantly more sensitive to AngII from 1.0nM onward. At a dose of 1.0nM AngII, the HS and HS+Q groups exhibited significant ($p<0.001$ for both) increases in perfusion pressure compared to their respective basal (HS; +13.3 ± 2.2 and HS+Q; +12.7 ± 0.4 mmHg) but were not different from each other ($p=0.964$).

Increasing AngII to 3.0nM significantly ($p<0.001$) increased perfusion pressure in the HS+Q (+24.9 ± 1.8 mmHg) and HS (+32.5 ± 5.1 mmHg) groups compared to their respective basal. However, the AngII-mediated perfusion pressure increase at 3.0nM was significantly ($p=0.010$) reduced in the HS+Q compared to the HS group, but remained significantly ($p<0.001$) elevated above the CTRL (+14.5 ± 1.9 mmHg) group.

Similarly, at a concentration of 10nM AngII, the HS and HS+Q groups displayed significantly ($p<0.001$) increased perfusion pressure compared to their respective basal (HS; + 46.6 ± 3.4 and HS+Q; +34.8 ± 2.6 mmHg). However, while the perfusion pressure change in the HS+Q group was significantly ($p<0.001$) reduced compared to the HS group it remained significantly ($p<0.001$) higher than the CTRL (25.9 ± 2.4) group.

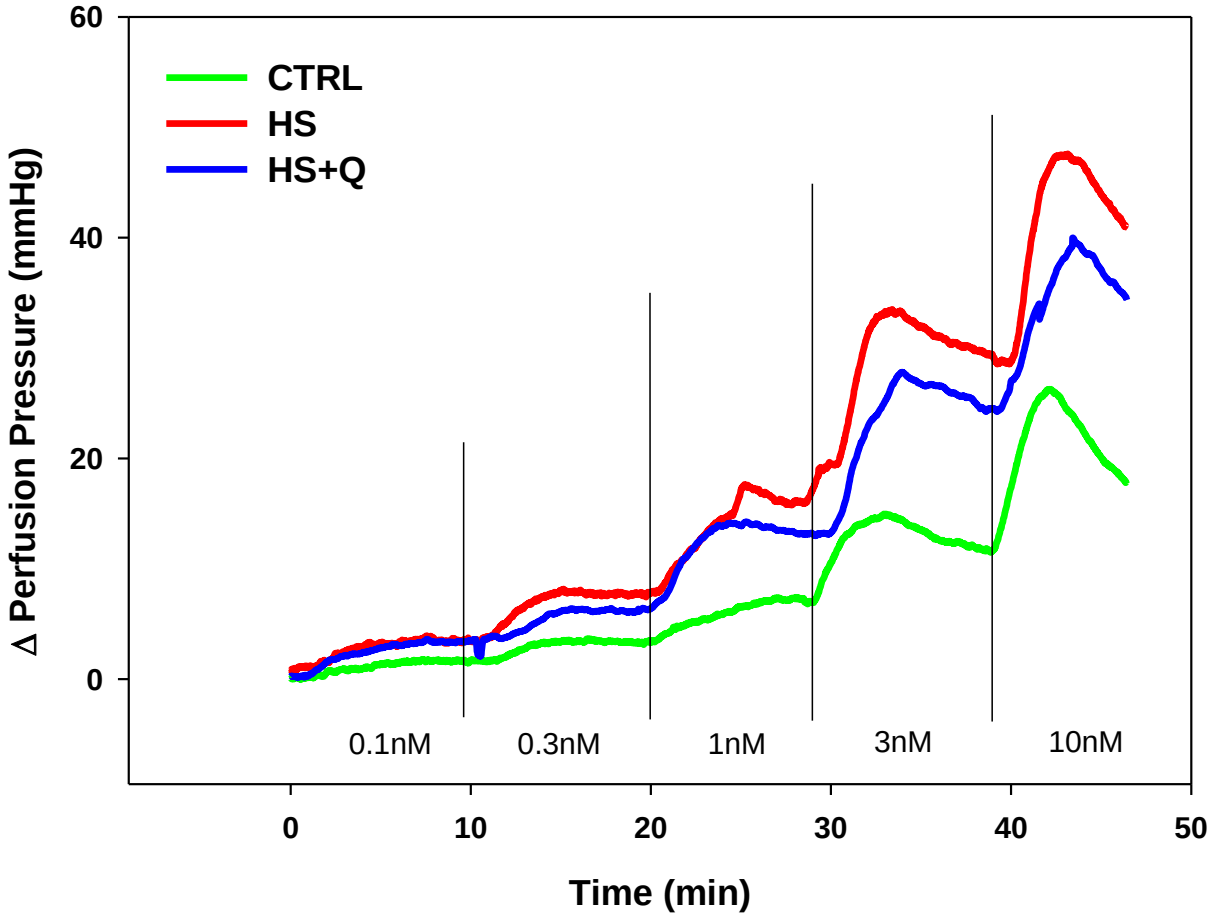

Figure 5.3. Time course of changes in perfusion pressure in response to AngII dose curve in perfused hindlegs of rats fed CTRL, HS or HS+Q diets.

Time course of AngII mediated changes in perfusion pressure at increasing doses of AngII. AngII infusion began at a concentration of 0.1nM and every 10 min the dose was increased as indicated. Data are means for n=5 rats in each group. SE bars were omitted for clarity.

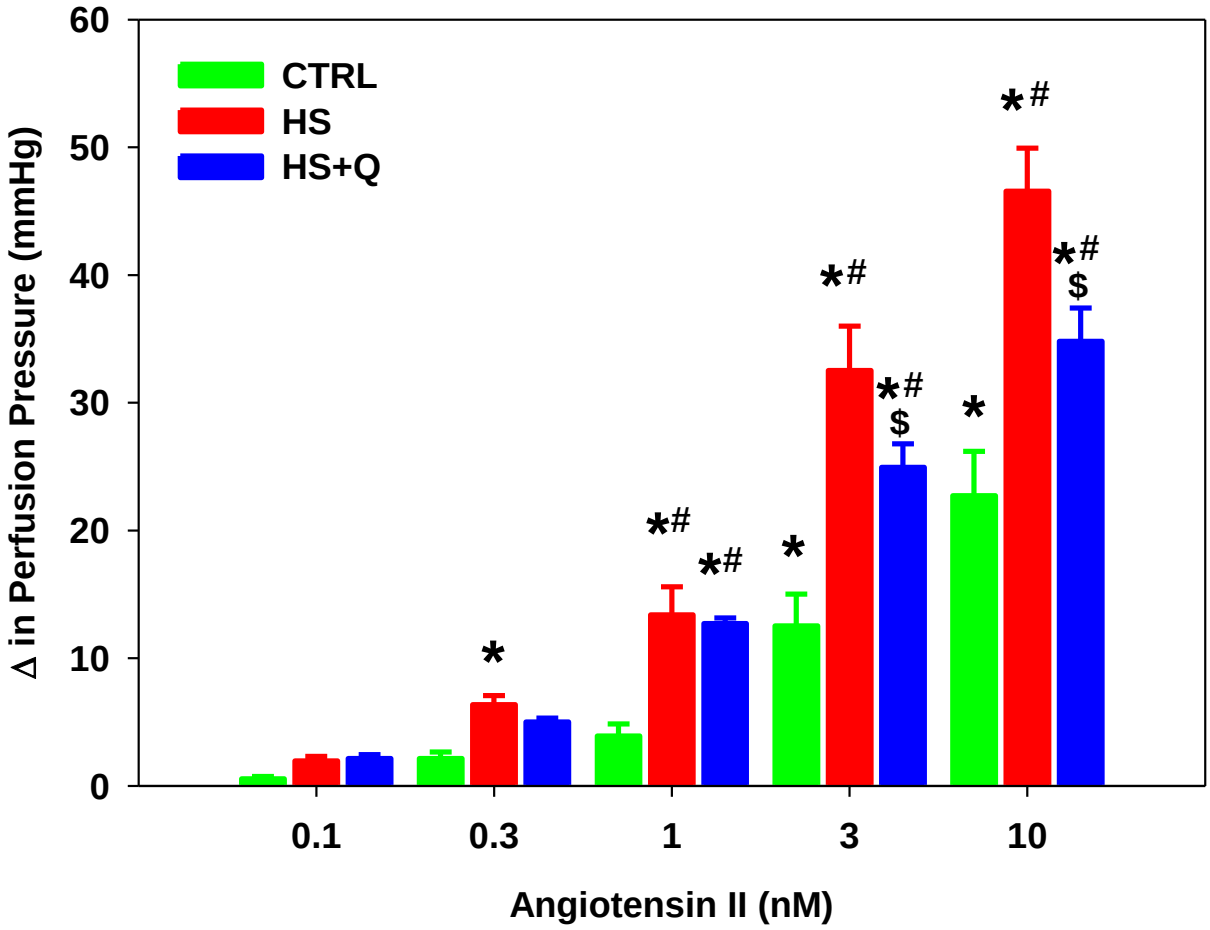

Figure 5.4. Perfusion pressure changes in response to AngII dose curve in perfused hindlegs of rats fed CTRL, HS or HS+Q diets.

The maximum perfusion pressure obtained for each dose of AngII in the three dietary groups. Data are means ± SEM for n=5 rats in each group. * Significantly different ($p<0.05$) from basal within the same dietary group. # Significantly different ($p<0.05$) from CTRL at the same dose. $ Significantly different ($p<0.05$) from HS at the same dose using two-way repeated measures ANOVA.

5.3.3. *In vivo* effects of quinapril on whole body glucose metabolism

Quinapril appears to have increased the blood glucose measures and improved insulin sensitivity in HS fed rats compared to untreated HS rats. Blood glucose was reduced at all time-points in HS+Q rats when compared to CTRL rats (Fig. 5.5.A, $p<0.001$). However, compared to the HS group the HS+Q group was found to have elevated blood glucose measures at every time points ($p<0.05$) except 20, 30 and 40 min. The glucose infusion rate (GIR) required to maintain glycaemia during 10mU/min/kg insulin infusion was significantly improved in HS+Q animals compared to untreated HS animals from 50 min until the conclusion of the experiment (Fig. 5.5.B, $p<0.01$). Following 2 hrs of insulin infusion the GIR was 23% higher in the HS+Q group compared to the HS group (20.1 ± 0.6 vs. 16.3 ± 0.1 mg/kg/min, $p<0.001$). Moreover, the HS+Q group exhibited significantly increased GIR at 105 and 120 min compared to CTRL animals ($p<0.05$).

The effects of quinapril on whole body insulin sensitivity appear not to be mediated by increased liver insulin sensitivity. No difference in the insulin mediated suppression of liver glucose output was detected between HS and HS+Q groups (Fig. 5.6.A; 2.2 ± 0.5 vs. 0.7 ± 0.9 mg/kg/min, $p=0.116$). Compared to CTRL the HS+Q group displayed increased suppression of liver glucose output during hyperinsulinaemia (4.1 ± 0.6 vs. 0.7 ± 0.9 mg/kg/min, $p=0.002$). Whole body glucose disposal (Rd) was found to be increased in the HS+Q group compared to the HS group (Fig. 5.6.B; 18.3 ± 0.7 vs. 21.8 ± 0.7 mg/kg/min, $p=0.002$), while no difference was detected compared to the CTRL group (23.5 ± 0.7 vs. 21.8 ± 0.7 mg/kg/min, $p=0.104$).

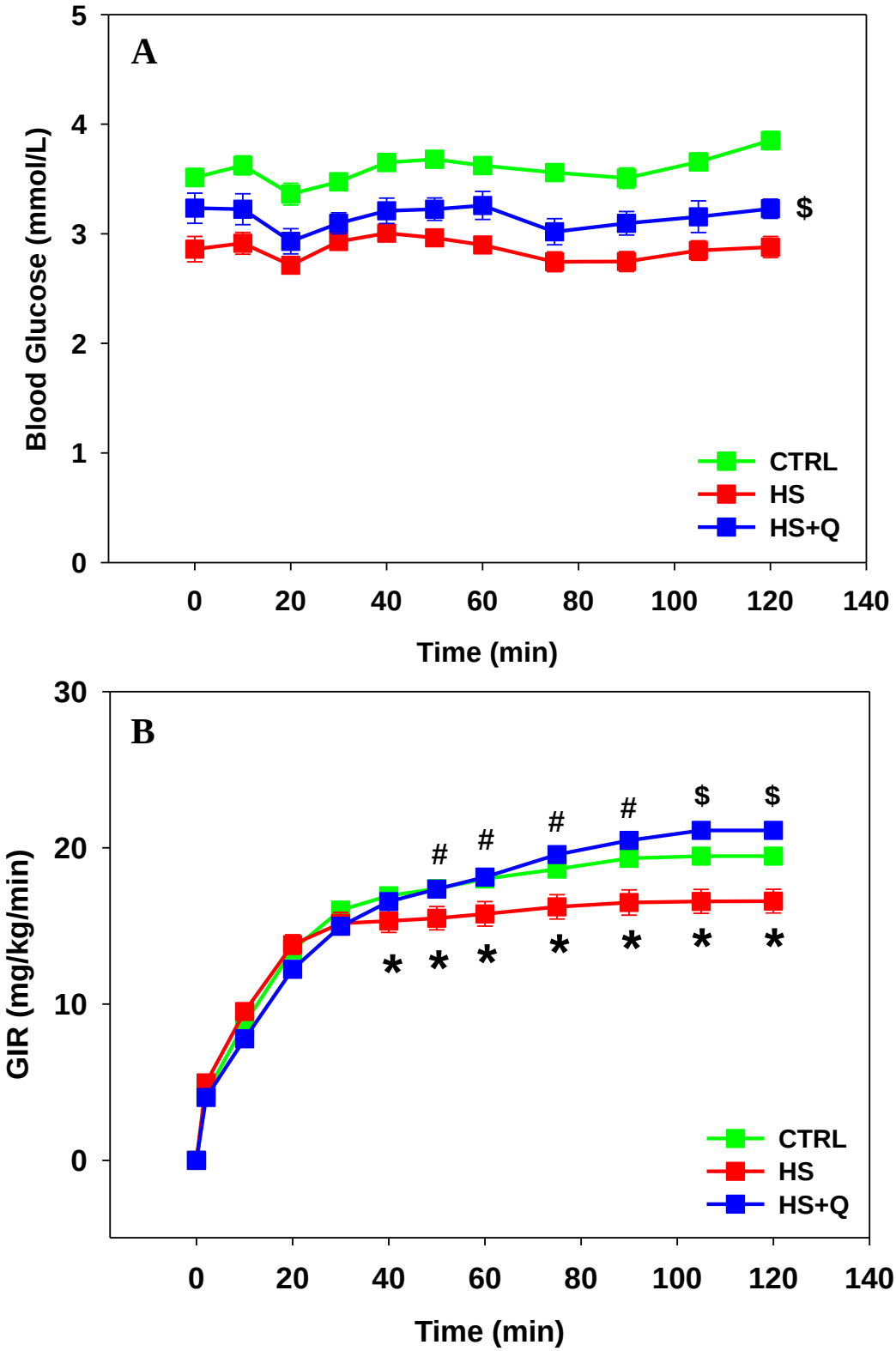

Figure 5.5. Quinapril effects on whole body insulin sensitivity.

Blood glucose (A) and glucose infusion rate (GIR; B) during insulin clamp of CTRL, HS and HS+Q rats. Data are means ± SEM for n=8-12 rats in each group. * Significantly different ($p<0.05$) compared to CTRL. # Significantly different ($p<0.05$) compared to HS. $ Significantly different compared to both CTRL and HS groups using two-way repeated measures ANOVA.

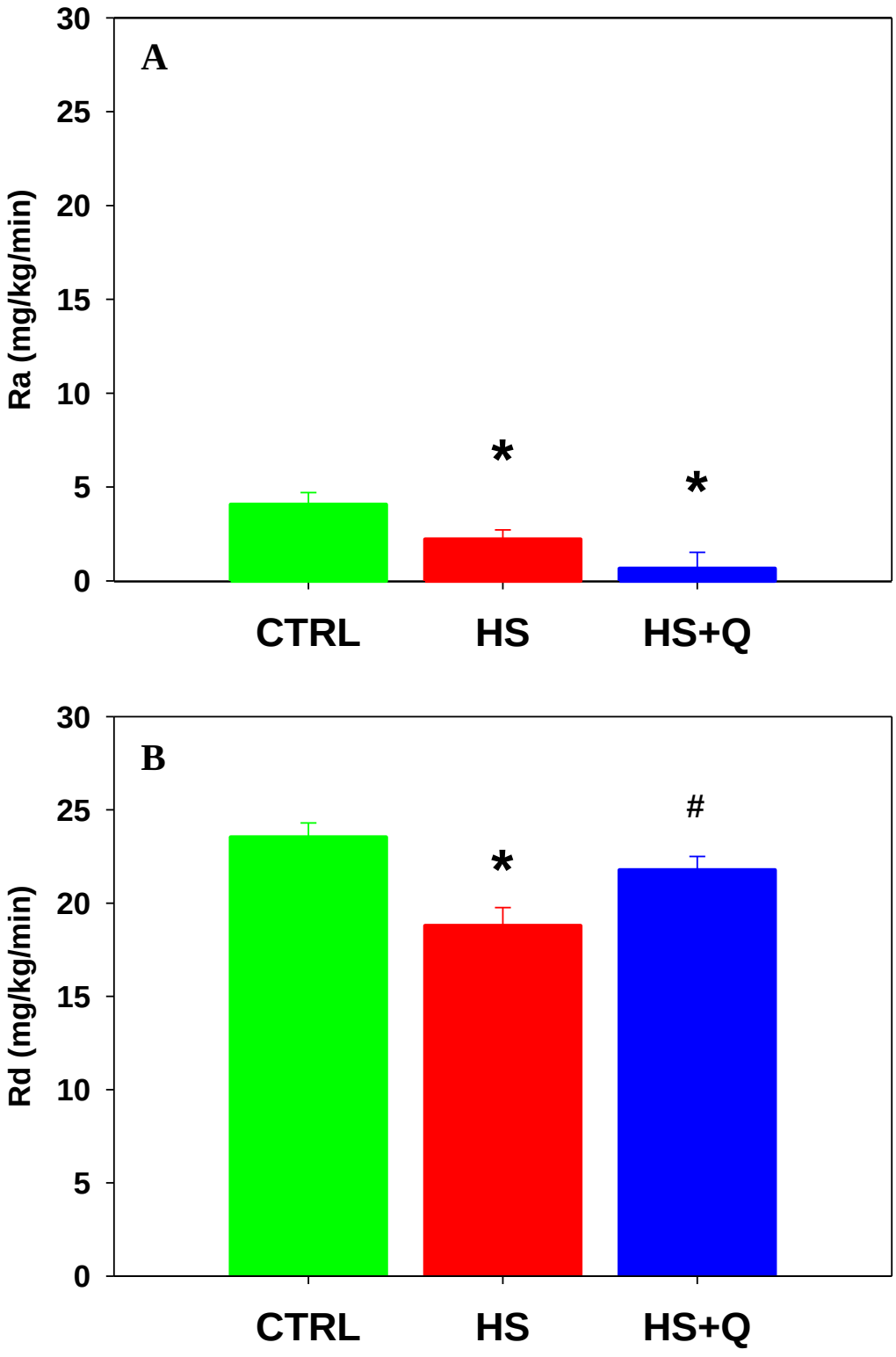

Figure 5.6. Quinapril effects on whole body glucose kinetics *in vivo*.

Rates of glucose appearance (Ra; panel A) and disappearance (Rd; panel B) at the conclusion of insulin clamp in CTRL, HS and HS+Q rats. Data are means ± SEM for n=8-12 rats in each group. * Significantly different ($p<0.05$) from CTRL. # Significantly different ($p<0.01$) from HS using one-way ANOVA.

5.3.4. Effects of quinapril on hindleg and muscle glucose uptake *in vivo*

The improvements in GIR following quinapril treatment were reflected in similar improvement in muscle insulin sensitivity. Following 3-4 weeks of HS+Q treatment hindleg glucose uptake during hyperinsulinaemia was significantly increased compared to HS group (Fig. 6.7.A; 0.62 ± 0.06 vs. 0.47 ± 0.02 µmoles/min, p=0.045). No difference in hindleg glucose uptake was detected when comparing HS+Q animals to the CTRL animals (0.62 ± 0.06 vs. 0.65 ± 0.06 µmoles/min, p=0.726). Similarly R`g during hyperinsulinaemia was improved in the HS+Q group compared to the HS group (Fig. 6.7.B; 21.4 ± 1.0 vs. 17.5 ± 1.0 µg/kg/min, p=0.018). While R`g tended to be reduced in the HS+Q group, compared to CTRL group no significant difference was detected (21.4 ± 1.0 vs. 24.0 ± 1.0 µg/kg/min, p=0.105).

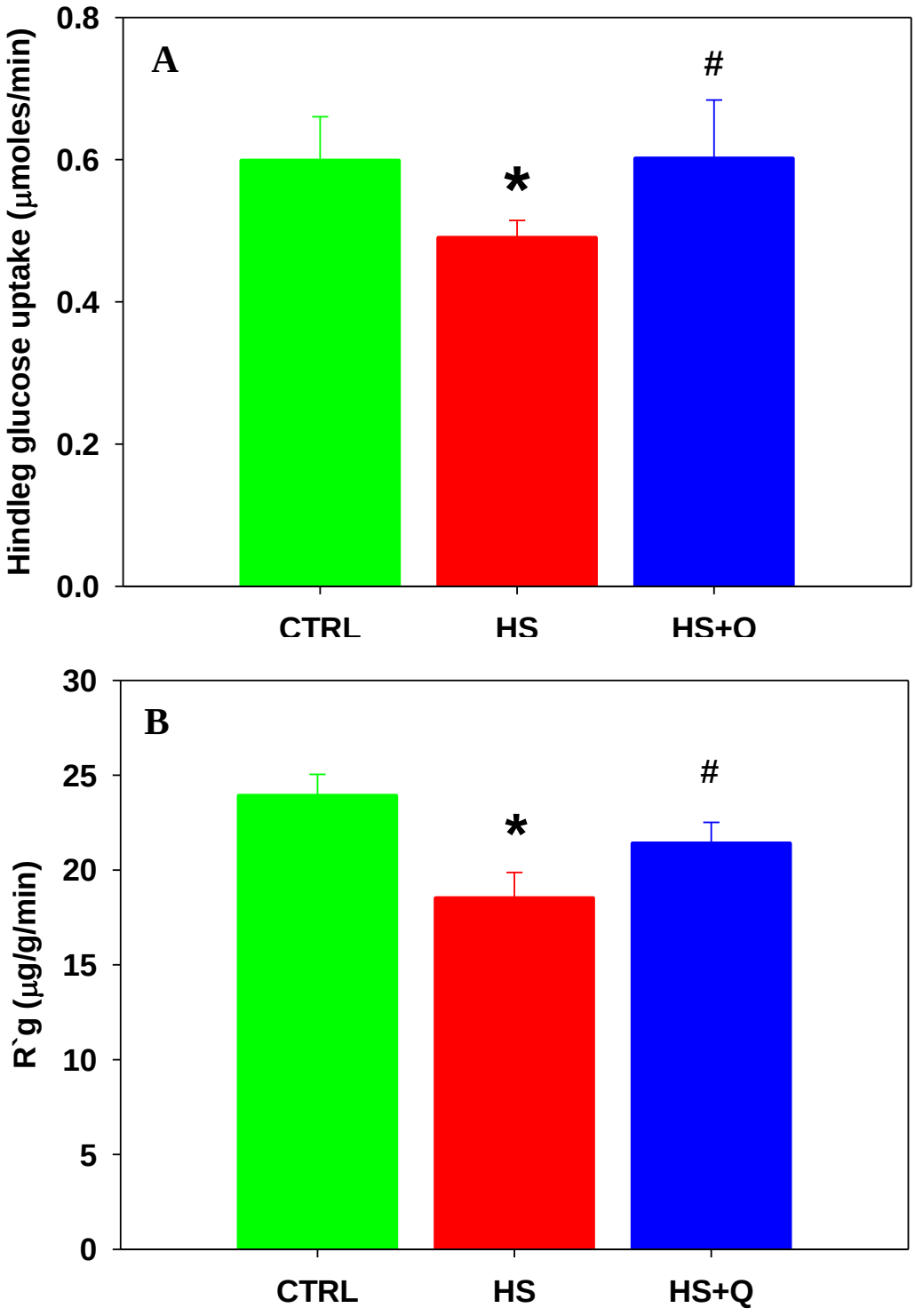

Figure 5.7. Hindleg and muscle specific glucose uptake *in vivo*.

Hindleg glucose uptake (A) and muscle specific glucose uptake (B; R`g) during insulin clamp in CTRL, HS and HS+Q rats. Data are means ± SEM for n=8-12 rats in each group. * Significantly different ($p<0.05$) from CTRL. # Significantly different ($p<0.05$) from HS using one-way ANOVA.

5.3.5. Effects of quinapril on insulin's vascular actions *in vivo*

Treating HS fed rats with quinapril significantly reduced basal FVR compared to the untreated HS fed rats (Table 5.1, p=0.013). However the basal FVR in the HS+Q group remained elevated compared to CTRL rats (p=0.003). Following 120 min of insulin infusion, FVR was reduced in all three groups compared to their respective basal (p<0.05). However, while post-insulin FVR in the HS+Q group remained elevated compared to the CTRL group (133 ± 13 vs. 91 ± 10 mmHg.min/mL, p=0.013) no difference was detected between HS and HS+Q groups (144 ± 10 vs. 133 ± 13 mmHg.min/mL, p=0.486).

Quinapril treatment slightly improved basal FBF in HS fed rats but did not affect the insulin-mediated increase in FBF. Basal FBF was increased in HS+Q rats compared to untreated HS rats (Table 5.1, p=0.028) but remained lower compared to CTRL rats (p<0.001). Following 2 hrs of insulin infusion FBF increased in CTRL (1.00 ± 0.05 vs. 1.28 ± 0.09 mL/min, p<0.001), HS (0.52 ± 0.04 vs. 0.69 ± 0.03 mL/min; p<0.001), and HS+Q (0.70 ± 0.08 vs. 0.84 ± 0.11, p=0.030) groups. No difference in the insulin-stimulated change in FBF was detected between the three groups (Fig. 5.9.A, CTRL; 27.4 ± 6.0 vs. HS; 39.1 ± 11.2 vs. HQ+Q; 21.4 ± 6.7 %, p=0.723).

Although quinapril did not affect insulin's action on total flow, it did improved insulin-mediated microvascular recruitment in HS fed rats. Insulin-stimulated 1-MX metabolism was enhanced in HS+Q rats compared to untreated HS rats (Fig. 5.9.B, 4.64 ± 0.37 vs. 3.58 ± 0.23 nmol/min, p=0.037). While metabolism of 1-MX tended to be slightly lower in the HS+Q group compare to CTRL no significant difference was detected (4.54 ± 0.37 vs. 5.04 ± 0.36 nmol/min, p=0.405).

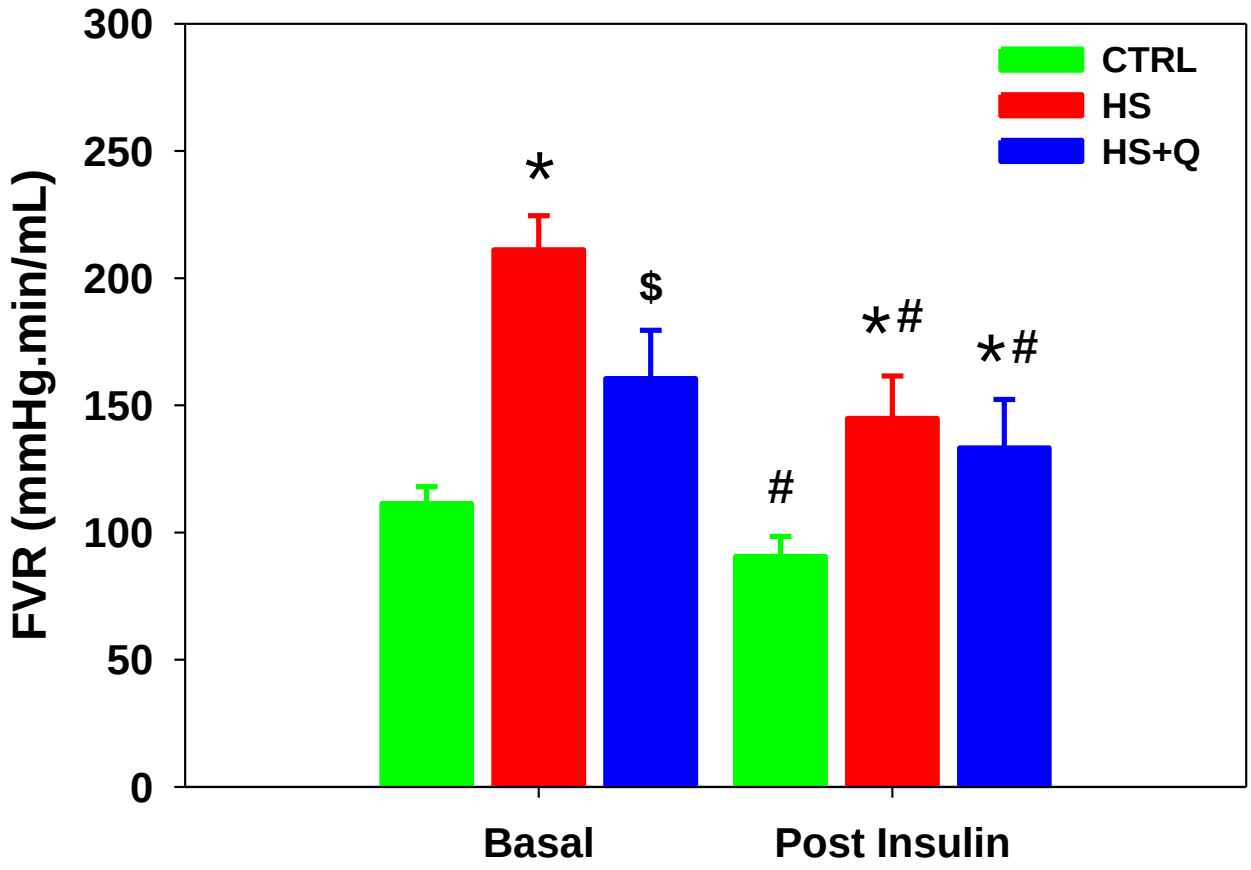

Figure 5.8. FVR before and after 120 min of insulin infusion.

Basal and post-insulin clamp FVR in CTRL, HS and HS+Q rats. Data are means ± SEM for n=8-12 rats in each group. * Significantly different ($p<0.05$) from respective CTRL. # Significantly different ($p<0.05$) from respective basal. $ Significantly different ($p<0.05$) from CTRL and HS using repeated measures two-way ANOVA.

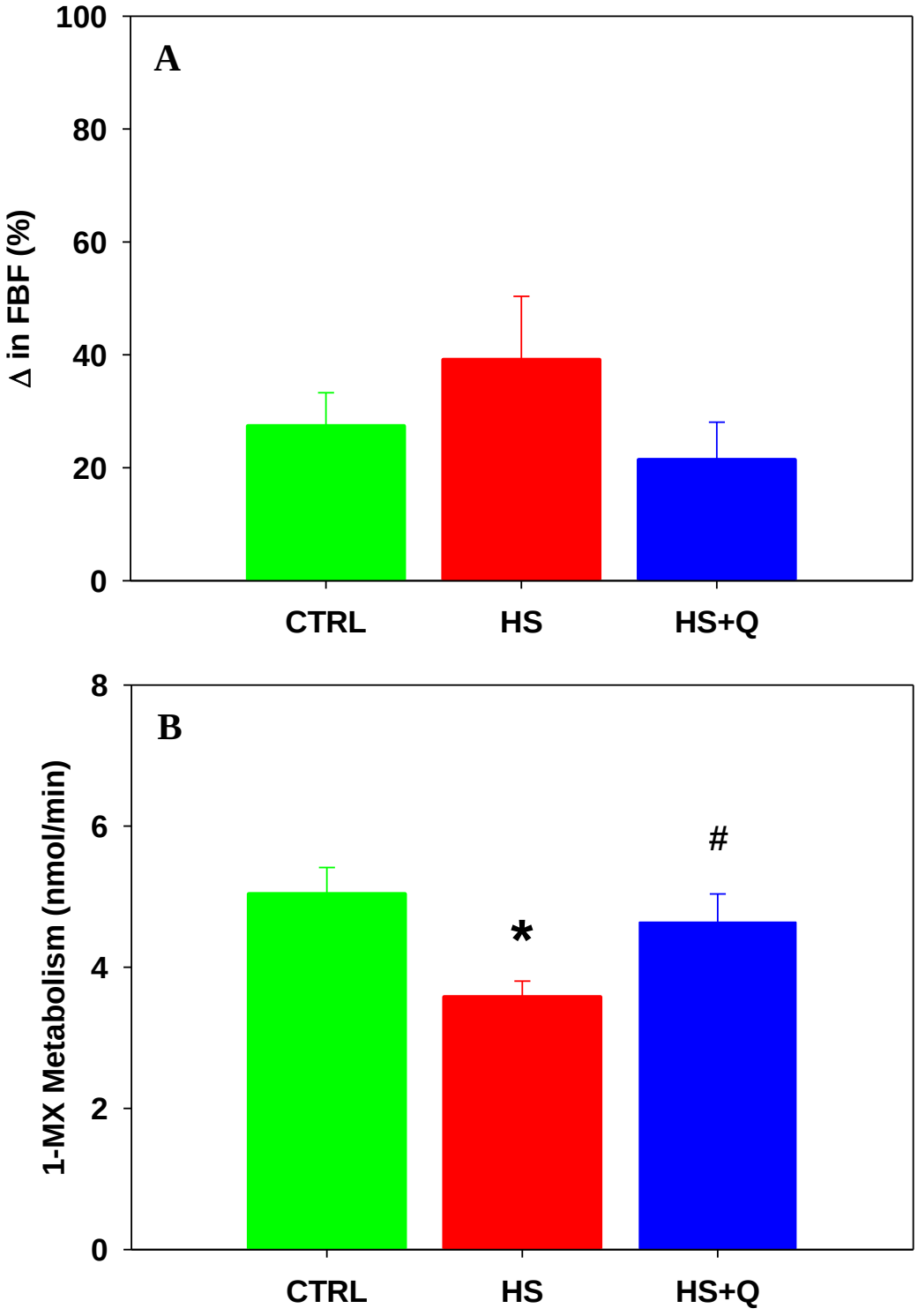

Figure 5.9. Vascular actions of insulin *in vivo*.

Insulin mediated change in FBF (A) and hindlimb 1-MX metabolism (B) in CTRL, HS and HS+Q rats. Data are means ± SEM for n=8-12 rats in each group. * Significantly different ($p<0.05$) from CTRL. # Significantly different ($p<0.05$) from HS using one-way ANOVA.

5.4. Discussion

The current study produced three main findings. Firstly, ACE inhibition in HS fed rats reduced hindleg vascular sensitivity to AngII-mediated constriction. Secondly, quinapril treatment of HS fed rats significantly improved whole body insulin sensitivity *in vivo*. Thirdly, quinapril treatment of HS fed rats significantly augmented insulin-stimulated microvascular perfusion and skeletal muscle glucose uptake *in vivo*. Taken together these data indicate that reducing the production/activity of AngII by inhibiting ACE in the HS fed rat augments insulin-mediated microvascular recruitment and significantly improves whole body and skeletal muscle insulin resistance.

Treatment of HS fed rats with quinapril resulted in a marked attenuation of AngII-mediated vasoconstriction when compared to untreated HS rats. While the mechanism responsible for this improvement was not investigated in the present study, previous reports have suggested that as well as reducing formation of AngII, ACE inhibitors also reduce AT1R expression in the vasculature [362-364, 366]. Schmeisser and colleagues (2004) showed that inhibiting ACE resulted in a dose-dependent decrease in AT1R receptor expression in endothelial cells while expression of AT2R receptors was unaffected [364]. Moreover, Liang and Leenen (2007) demonstrated that ACE inhibition markedly reduced the level of AT1R expression in the aorta of HS fed rats [362]. In addition, Hara and colleagues (2001) found that quinapril treatment reduced the expression of the AT1R in deoxycorticosterone acetate-salt hypertensive rats [363]. Since AT1 receptors mediate AngII constriction in the vasculature, this AT1R down-regulating effect of ACE inhibitors may explain the reduced responsiveness of AngII-mediated constriction in the muscle vasculature of the HS+Q rats in the current study.

Surprisingly, quinapril treatment of HS fed rats partially restored the basal FBF compared to untreated HS rat without altering the mean arterial blood pressure. This resulted in a reduction in FVR in the HS+Q group compared to the untreated HS group. Following 2 hrs of insulin infusion, FBF increased by a similar extent in all groups, thus resulting in a reduction in the FVR in each group compared to their respective basal FVR. Moreover, quinapril treatment of HS fed rats also augmented insulin-stimulated

microvascular recruitment in muscle compared to the untreated HS rats. Thus, in the present study treating HS fed rats with quinapril resulted in significant improvement in both total flow at basal and augmentation of insulin-mediated microvascular recruitment in skeletal muscle. While the direct mechanisms responsible for improvement insulin-mediated haemodynamics were not assessed in the current study the improvement are likely to be mediated by partial correction of endothelial dysfunction.

Indeed, improvement in endothelial function following ACE inhibition has previously been reported in a number of animal models [367-370]. In part, this effect can be attributed to the classic action of ACE inhibitors to reduce AngII formation while simultaneously preventing the breakdown of bradykinin [371-373], thus favouring a state of enhanced vasodilation. Other reports have demonstrated significant improvements in eNOS activity and subsequent improvements in NO-mediated vasodilation following ACE inhibition in normotensive [374] and hypertensive rats [375, 376]. Furthermore, HS feeding has also been shown to increase the production of ROS, which reduce NO bioavailability, via an AngII-AT1R mediated mechanism [163, 291, 377, 378]. With previous reports showing reduced AT1R expression/activity following ACE inhibition [362-364, 366] inhibiting ACE in HS fed rats likely results in a shift in balance away from AngII-mediated constriction to favour NO-mediated vasodilation via AT2R and/or bradykinin. In light of these previous findings the improvement in macrovascular and microvascular function in the current study following ACE inhibition may be attributable to an improvement in endothelial function via increased NO bioavailability.

As with insulin's vascular actions, whole body insulin sensitivity during hyperinsulinaemia in HS+Q rats was improved compared to untreated HS fed rats. Moreover, no difference in liver glucose output during hyperinsulinaemia was detected between HS and HS+Q groups. Taken together these data would suggest that the increase in whole body insulin sensitivity during hyperinsulinaemia in the HS+Q group was not related to an improvement in liver insulin sensitivity but rather an increase in glucose disposal. This conclusion is supported by an improvement in insulin-mediated muscle glucose uptake in the HS+Q treated rats compared to the untreated HS rats. Since this was associated with an improvement in insulin-mediated microvascular recruitment

in muscle, quinapril treatment may have improved muscle glucose disposal by increasing delivery of glucose and insulin to the myocyte. However, it should be noted that HS fed rats have also been shown to exhibit direct myocyte insulin resistance [327]. Furthermore, other reports have demonstrated a direct improvement in insulin-mediated myocyte glucose uptake following ACE inhibition [255, 365]. Therefore, there exists the possibility that treatment of HS fed rats with quinapril may have improved not only the microvascular defect but also myocyte insulin resistance. This is the first study to demonstrate that ACE inhibition improves both microvascular function in muscle and also muscle glucose uptake in HS fed rats. Although myocyte insulin sensitivity was not directly characterised in the present study, previous reports have demonstrated that insulin-mediated microvascular recruitment in muscle accounts for approximately 40-50% of insulin-mediated muscle glucose disposal [100, 167, 177]. Therefore, it would be reasonable to conclude that the improvement in insulin-mediated muscle glucose uptake, following quinapril treatment in the current study, may also be at least in part due to correction of the microvascular defect in HS fed rats.

While the direct mechanism responsible for the improvement in insulin sensitivity following ACE inhibition was not investigated in the current study, a reduction in AngII activity looms as a likely candidate. Indeed a number of studies have demonstrated that increased AngII activity is detrimental to insulin signalling in the vasculature [163, 202] and insulin-mediated muscle glucose uptake [199, 200, 203, 291, 379]. This AngII-mediated development of insulin resistance has been largely attributed to increased production of ROS via NADPH oxidase, which has in turn been shown to inhibit insulin-mediated GLUT-4 translocation in myocytes and thus reduces muscle glucose uptake [200, 203, 294]. Furthermore, insulin-mediated microvascular recruitment, which contributes to enhanced muscle glucose uptake [100, 167, 177], has been shown to be at least in part NO dependent [100]. Therefore, it would be reasonable to assume that inhibition of ACE in HS fed rats in the current study augments insulin signalling in both vascular and muscle cells by directly attenuating AngII formation and activity, and thus reducing ROS production. However, determining the effects on myocyte insulin sensitivity would be essential in distinguishing between the vascular and metabolic insulin sensitisation following ACE inhibition in HS fed rats.

Apart from reducing AngII formation, ACE also acts to breakdown bradykinin. Therefore, it is possible that the effects of ACE inhibition on insulin sensitivity in the

current study may be mediated by increased bradykinin activity. Indeed, some reports have suggested that the insulin-sensitising effect of ACE inhibition in muscle can be largely attributed to enhancement of bradykinin, which in turn can increase GLUT-4 translocation and thus augment insulin-mediated myocyte glucose uptake [255, 365, 380]. However, the effect of bradykinin to increase insulin-mediated muscle glucose uptake *in vivo* appear to be restricted to the disease state. Henriksen and colleagues (1998) reported that chronic bradykinin infusion improves whole body insulin sensitivity and enhances skeletal muscle glucose uptake in obese Zucker rats [303]. In contrast the authors reported that bradykinin had no significant effect on either whole body insulin sensitivity or muscle glucose uptake in healthy, lean Zucker rats [303]. Similarly, Mahajan and colleagues (2004) used a local bradykinin infusion into a single hindleg *in vivo* and found that while it increased total FBF, no effect was observed on insulin-mediated microvascular recruitment or insulin-mediated muscle glucose uptake [307]. Thus, while bradykinin may not be an important determinant of insulin-mediated muscle glucose uptake in normal healthy rats, in insulin resistant states where ACE activity may be up-regulated, improved bradykinin activity may contribute to the improvement in insulin sensitivity. Since the HS fed rat has been reported to exhibit increased ACE activity (and thus presumably bradykinin breakdown) [381], it is possible that the improvement in insulin sensitivity in the present study may in part be due to increased activity of bradykinin following quinapril treatment of HS fed rats.

5.4.4. Conclusions

The primary aim of this study was to determine whether inhibition of ACE could improve insulin-mediated microvascular perfusion and muscle glucose uptake in the insulin resistant HS fed rat. The results of this study indicate that in the HS fed rat, where AngII sensitivity/action is increased, quinapril treatment reduced vascular AngII sensitivity and improved macrovascular function in skeletal muscle. Moreover, quinapril also enhanced whole body insulin sensitivity associated with improvement in both insulin-stimulated microvascular recruitment and muscle glucose uptake. While the direct mechanisms responsible for the improvement in insulin sensitivity were not investigated in the present study, the effects of quinapril to reduce AngII action within the hindleg vasculature would indicate that the improvement in insulin sensitivity may in part be the result of decreased vascular AngII action.

CHAPTER 6

ANGIOTENSIN CONVERTING ENZYME INHIBITION DOES NOT IMPROVE INSULIN ACTION IN A TYPE 2 DIABETIC RAT MODEL

6.1. Introduction

The incidence of insulin resistance and T2D is on the rise largely due to increasing obesity rates. In humans, these conditions often co-exist with cardiovascular disease and are often referred to as the metabolic syndrome [382, 383]. A number of clinical studies have identified that either ACE inhibition or ARB treatment in subjects with metabolic syndrome can improve insulin sensitivity [271, 272, 384, 385], while others have found negligible effects [279, 280, 282, 283, 386]. The exact mechanisms behind the improvement in insulin sensitivity remain unclear. Some reports have suggested that increased skeletal muscle insulin sensitivity following RAS inhibition is likely involved [285, 387]. Others have suggested that endothelial dysfunction is in part responsible for the development of the metabolic syndrome [388, 389] and that RAS inhibition may improve endothelial function, and thus augment insulin sensitivity [284, 390-393]. In chapter 5 of this thesis, inhibiting ACE in the HS fed rat significantly improved insulin-mediated microvascular recruitment in muscle and this was associated with improvement in both whole body and skeletal muscle insulin sensitivity. Whether this insulin sensitising effect of ACE inhibition also improves insulin sensitivity, specifically insulin-mediated microvascular recruitment, in a model of the metabolic syndrome is unclear and is the aim of the current study.

The obese Zucker (OZ) rat is a commonly used model of insulin resistance and T2D that has been found to exhibit obesity, hypertension, moderate hyperglycaemia and severe hyperinsulinaemia [394]. The OZ rat develops obesity as a result of a recessive mutation in the gene encoding the leptin receptor, resulting in hyperphagia [233]. By 4-5 weeks of age the OZ rats are significantly heavier than lean littermates and quickly develop both liver and muscle insulin resistance [240, 395]. Previous reports have suggested that one of the main causes of reduced skeletal muscle glucose uptake in the OZ rat is reduced expression and insulin-stimulated translocation of GLUT-4 in myocytes [396, 397]. Moreover, myocyte GLUT-4 expression and activity are reportedly enhanced following either ACE inhibition [254] or ARB treatment [358] in OZ rats, thus implicating increased AngII activity as a contributor to skeletal muscle insulin resistance in OZ rats.

As well as defects in the insulin-signalling pathway in myocytes, OZ rats also develop vascular alterations. Studies in isolated vessels have shown that the ability of insulin to reduce vasoconstriction in OZ rats is impaired. Zemel and colleagues (1991) reported that isolated aorta from OZ rats were more sensitive to phenylephrine induced vasoconstriction and insulin's ability to attenuated this response was reduced [245]. Additionally, Walker and colleagues (1997) demonstrated that insulin was able to attenuate norepinephrine induced vasoconstriction in mesenteric arteries of lean rats but not OZ rats [246]. Using the anaesthetised rat preparation, Wallis and colleagues (2002) reported that OZ rats exhibit attenuation of insulin-mediated microvascular recruitment in muscle and suggested that this may also in part contribute to the reduced insulin-mediated muscle glucose uptake [242].

Inhibition of AngII activity has been reported to improve muscle glucose uptake in OZ rats [254-256]. Henrkisen and colleagues (1995 and 2001) found that OZ rats treated with an ACE inhibitor or an ARB displayed significant improvement in insulin-mediated muscle glucose uptake compared to untreated OZ [254, 255]. However, in both studies insulin-mediated muscle glucose uptake was assessed using the *in vitro* muscle incubation preparation. In that technique insulin and glucose delivery to the myocyte is by diffusion and not via the vasculature as is the case *in vivo*. Whether ACE inhibition improves muscle glucose uptake *in vivo* by improving vascular delivery of glucose and insulin to the myocyte is unclear. There is evidence, however, that ACE inhibition in OZ rats improves acetylcholine-mediated vasodilation in aorta with intact endothelium [398], suggesting that inhibition of ACE in OZ rats can also improve endothelial function. Whether this also results in the improvement of insulin's vascular actions in skeletal muscle of OZ rats has not been previously investigated. However, Clerk and colleagues (2007) reported that long-term treatment of Zucker Diabetic Fatty rats (ZDF; a model of late stage T2D in which the pancreas fails to produce sufficient amounts of insulin) with quinapril improved whole body glucose disposal and insulin-mediated microvascular recruitment in muscle [324]. Unfortunately the authors of this previous study did not investigate whether ACE inhibition also improved insulin-mediated muscle glucose uptake in the ZDF rats.

In light of these previous findings, the aim of the present study was to determine whether ACE inhibition, using quinapril, can improve insulin-mediated microvascular recruitment and insulin-mediated muscle glucose uptake in the OZ rat.

6.2. Methods

6.2.1. Animals

Female lean Zucker (LZ; *Fa*/?) and obese Zucker (OZ; *fa/fa*) rats aged 10-12 weeks were obtained from Monash Animal Services (Monash University, Melbourne, Vic, Australia). On arrival, the OZ rats were split into two groups and one group was given quinapril (OZ+Q, 1 mg/kg/day; Sigma Aldrich) in their drinking water for 4 weeks. This dose and length of treatment of quinapril was demonstrated in chapter 5 to improve both muscle glucose uptake and microvascular recruitment in the HS-fed rat model. All animals were provided with standard chow and water *ad libitum*.

6.2.2. Anaesthetised rat experiments

Surgery was performed as outline in chapter 2, section 2.3.1. Following surgical preparation, 1 hr was allowed for FBF and mean arterial blood pressure to stabilise, at which time a 2 hr infusion of insulin (20mU/min/kg) was initiated. Since insulin stimulates glucose disposal, a 30% glucose solution (wt./vol.) was infused at a variable rate to maintain fasting blood glucose concentrations over the course of the experiment. For this reason, arterial blood glucose levels were assessed every 10 min in the first hour, and every 15 min in the second hour using a glucose analyser (YSI 2300) and the glucose infusion rate (GIR) was adjusted accordingly. See Fig. 6.1for a more detailed protocol. Plasma biochemistry, whole body glucose kinetics (Ra and Rd), muscle glucose uptake and skeletal muscle microvascular perfusion were assessed as outlined in chapter 2, sections 2.3.2-2.3.5.

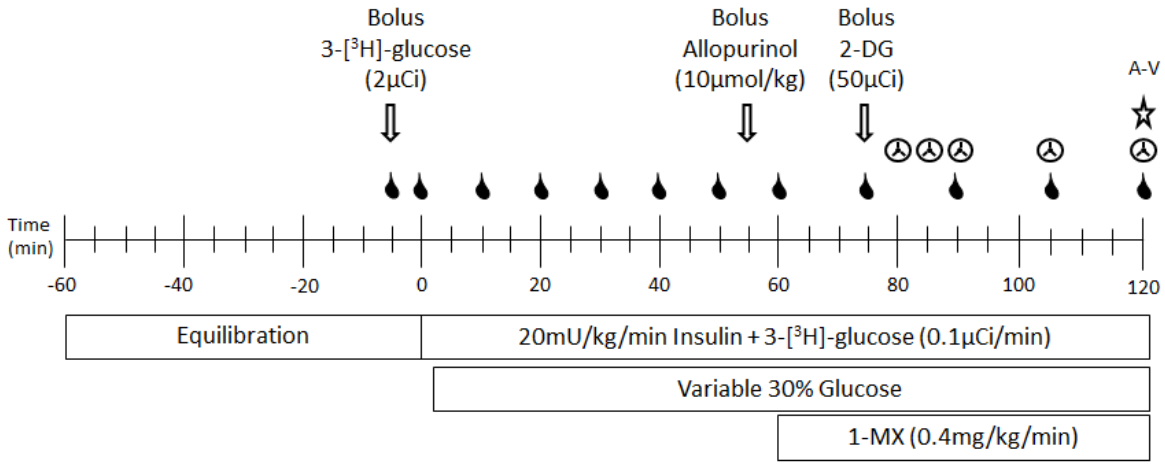

Figure 6.1. Experimental protocol.

After surgical preparation and 60 min of equilibration a continuous infusion of insulin (20mU/min/kg) was commenced and continued for 120 min. A 30% (wt./vol.) glucose infusion was initiated shortly after the commencement of the insulin infusion to maintain baseline glycaemia as assessed by arterial blood glucose sampling (). After 55 min of insulin infusion, a bolus of allopurinol (10µmol/kg) was administered. At 60 min, infusion of 1-methylxanthine (1-MX, 0.4mg/kg/min) was initiated and maintained until the end of the experiment. At 75 min a bolus of 2-deoxy-D-[1-^{14}C]-glucose (2-DG; 20µCi) was administered. Radioactive plasma samples () were collected at 80, 85, 90, 105 and 120 min to determine the plasma 2-DG clearance. At the conclusion of the experiment arterial and femoral vein plasma samples ( ) from were collected for the determination of hindleg glucose uptake and 1-MX metabolism. Immediately following sacrifice, calf muscle (gastrocnemius, plantaris and soleus group) was freeze clamped in liquid nitrogen and stored at -80°C.

6.2.3. Data and Statistics

Data are presented as the means ± SEM and statistics were performed using Sigma-Stat (Systat Software Inc, 2004). Comparisons between LZ, OZ and OZ+Q were performed by one-way ANOVA. Comparison of time-series measurements in each group was performed by two-way repeated measures ANOVA. When a significant difference of $p<0.05$ was found, pairwise comparisons by Student-Newman-Keuls test was used to assess treatment differences.

6.3. Results

6.3.1. Effects of quinapril treatment on the physical and biochemical characteristics of obese Zucker rats

Quinapril treatment of OZ rats did not affect any basal measures compared to untreated OZ rats. Table 6.1 shows the physical characteristics of the untreated LZ, OZ and quinapril-treated OZ rats. No difference in body weight was detected between OZ and OZ+Q rats; however, both were markedly heavier compared to LZ rats ($p<0.001$). Uterine horn fat, heart and liver weights were not different between OZ and OZ+Q rats. However, in both OZ groups, all were significantly increased when compared to LZ rats ($p<0.001$). During anaesthesia, mean arterial pressure, basal FBF and basal FVR were not different between the three groups. Compared to LZ rats, OZ rats displayed significantly elevated fasting plasma glucose ($p<0.001$), fasting plasma lactate ($p<0.01$) and fasting plasma insulin concentrations ($p<0.001$). No differences were detected between OZ and OZ+Q rats with regard to fasting plasma glucose, lactate or insulin concentrations.

	LZ (n=5)	OZ (n=9)	OZ+Q (n=9)
Body weight (g)	190 ± 8	357 ± 8*	372 ± 5*
Uterine horn fat (g)	1.03 ± 0.09	6.80 ± 0.28*	7.37 ± 0.19*
Heart (g)	0.64 ± 0.03	0.92 ± 0.03*	0.92 ± 0.03*
Liver (g)	1.07 ± 0.08	2.84 ± 0.24*	2.81 ± 0.13*
Mean arterial pressure (mmHg)	94 ± 3	102 ± 4	103 ± 3
Basal FBF (mL/min)	0.48 ± 0.07	0.39 ± 0.06	0.48 ± 0.07
Basal FVR (mmHg.min/mL)	208 ± 30	301 ± 48	240 ± 33
Fasting plasma glucose (mmol/L)	6.89 ± 0.17	12.47 ± 0.82*	12.83 ± 0.63*
Fasting plasma insulin (pmol/L)	187 ± 21	6650 ± 1170*	7490 ± 850*
Fasting plasma lactate (mmol/L)	0.68 ± 0.07	2.69 ± 0.36*	2.81 ± 0.26*

Table 6.1. Physical and biochemical characteristics of rats following 4 week quinapril treatment.

Measures were collected immediately prior to the commencement of the insulin clamp. Uterine horn fat, hearts and the right lobe of the liver were excised and weighed at the conclusion of each experiment. FBF; femoral artery blood blow, FVR; femoral artery vascular resistance. Data are means ± SEM for n=5-9 rats in each group. * Significantly different ($p<0.05$) from LZ using one-way ANOVA.

6.3.2. Effects of quinapril on whole body glucose metabolism

Quinapril treatment of OZ rats did not affect the fasting blood glucose concentration and did not improve the GIR during insulin infusion. During the hyperinsulinaemic clamp procedure, blood glucose levels were clamped to basal levels. Thus, blood glucose levels throughout the insulin clamp were significantly elevated in both OZ and OZ+Q rats compared to the LZ rats at all time-points (Fig. 6.2.A, $p<0.05$). Quinapril treatment of OZ rats for 4 weeks did not significantly alter the basal blood glucose concentration compared to untreated OZ rats. Whole body glucose disposal (Fig. 6.2.B; GIR) during insulin infusion was decreased approximately 5-fold in OZ (4.0 ± 0.7 mg/kg/min) and OZ+Q (2.7 ± 0.7 mg/kg/min) rats when compared to the LZ rats (23.0 ± 2.3 mg/kg/min, $p<0.001$ vs. both OZ and OZ+Q). No difference in GIR was detected between OZ rats and OZ+Q rats ($p=0.121$).

Quinapril treatment of OZ rats did not improve liver insulin sensitivity and did not affect whole body glucose disposal. Liver glucose output (Fig. 6.3.A; Ra) during insulin infusion was significantly suppressed in LZ rats compared to OZ (2.03 ± 0.75 vs. 5.22 ± 0.78 mg/kg/min, $p=0.028$) and OZ+Q rats (2.03 ± 0.75 vs. 7.83 ± 0.91 mg/kg/min, $p<0.001$). OZ+R rats displayed slightly higher Ra during insulin infusion compared to untreated OZ rats ($p=0.033$). The rate of glucose disappearance (Fig. 6.3.B; Rd) was significantly higher in LZ rats compared to OZ (26.2 ± 2.5 vs. 10.0 ± 0.5 mg/kg/min, $p=0.002$) and OZ+Q rats (26.2 ± 2.5 vs. 10.6 ± 0.8 mg/kg/min, $p=0.003$), while no difference was detected between OZ and OZ+Q rats ($p=0.510$).

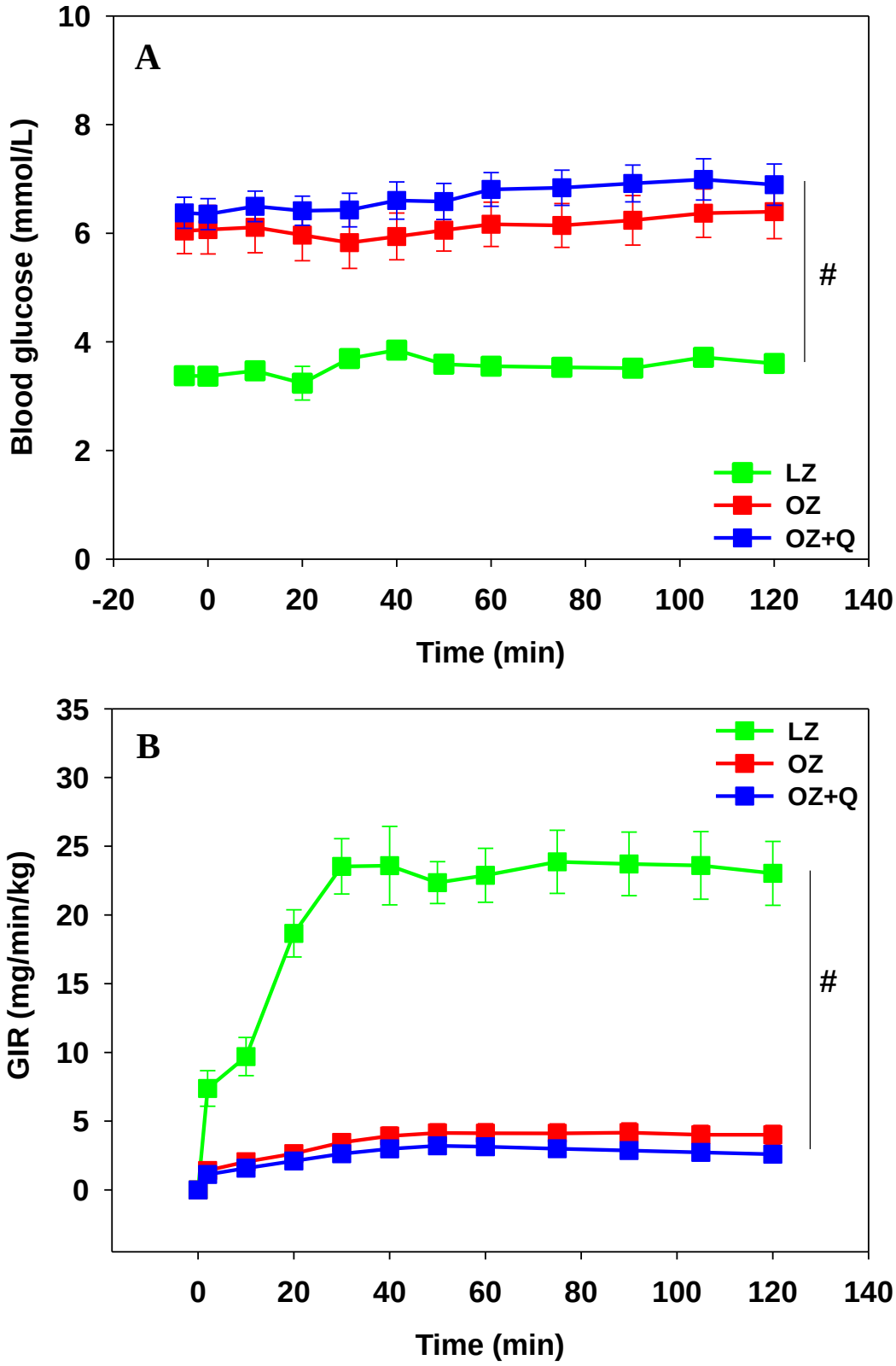

Figure 6.2. Quinapril effects on whole body insulin sensitivity.

Blood glucose (A) and glucose infusion rate (GIR; B) during insulin clamp of LZ, OZ and OZ+Q rats. Data are means ± SEM for n=5-9 rats in each group. # Significantly different ($p<0.05$) compared to OZ and OZ+Q using two-way repeated measures ANOVA.

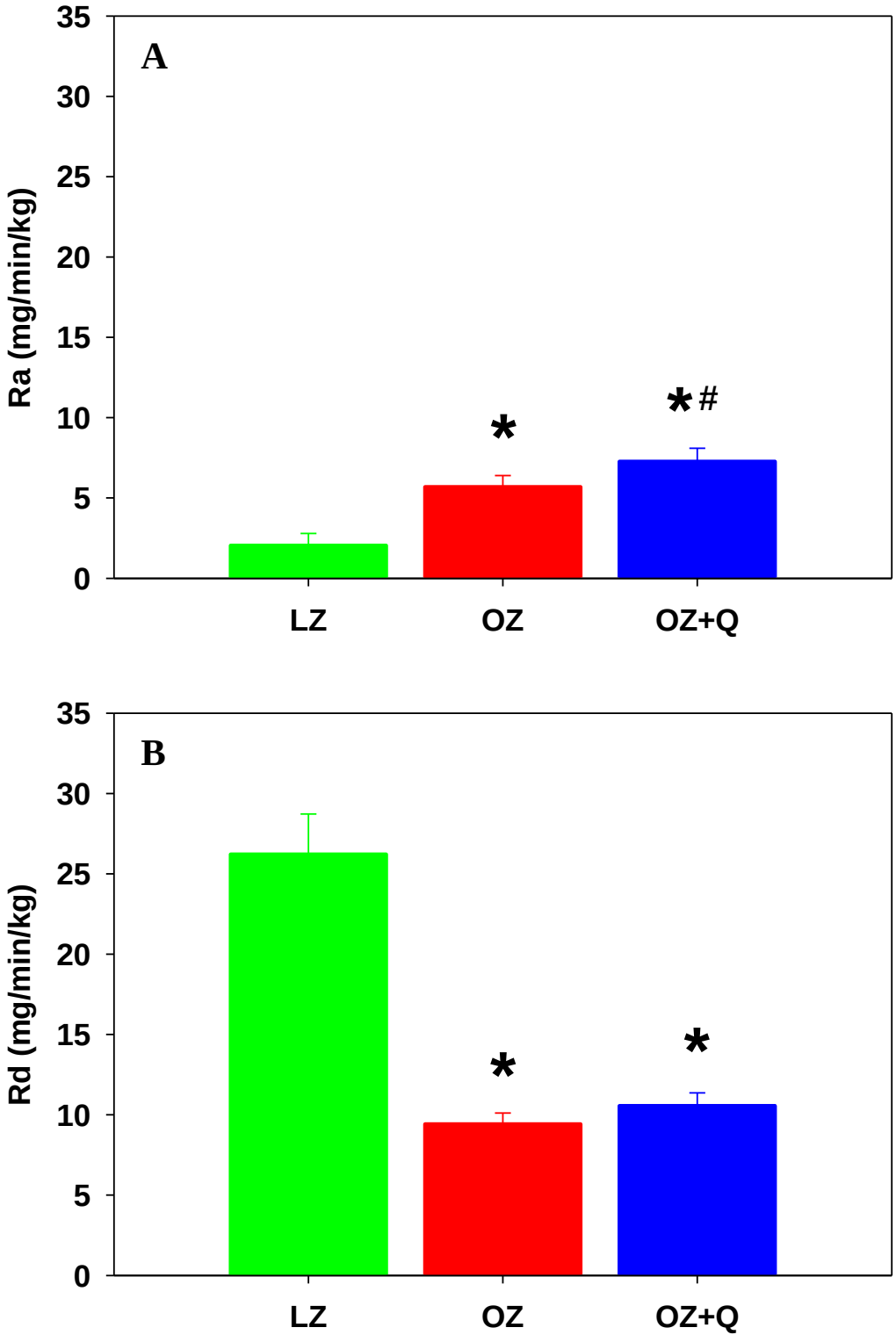

Figure 6.3. Quinapril effects on whole body glucose kinetics.

Rate of glucose appearance (Ra; panel A) and disappearance (Rd; panel B) at the conclusion of 2 hrs of insulin infusion in LZ, OZ and OZ+Q rats. Data are means ± SEM for n=5-9 rats in each group. * Significantly different ($p<0.05$) from LZ using one-way ANOVA. # Significantly ($p<0.05$) different from OZ rats using one-way ANOVA.

6.3.4. Effects of quinapril on hindleg and muscle specific glucose uptake

Obese Zucker rats exhibited markedly reduced insulin-mediated hindleg and muscle glucose uptake compared to LZ rats and quinapril treatment of OZ rats did not improve either of these measures. During insulin infusion, hindleg glucose uptake was higher in LZ rats compared to OZ (Fig. 6.4.A; 0.77 ± 0.16 vs. 0.14 ± 0.02 µmol/min, p=0.002).Treatment of OZ rats with quinapril did not enhance insulin-mediated hindleg glucose uptake compared to untreated OZ rats (0.14 ± 0.02 vs. 0.18 ± 0.03 µmol/min, p=0.282). Untreated OZ rats displayed a significant (p=0.003) decrease in insulin-mediated muscle glucose uptake (Fig. 6.4.B; R`g) compared to LZ rats (26.6 ± 3.8 vs. 5.5 ± 0.6 mg/kg/min). Quinapril treatment of OZ rats did not improve R`g compared to untreated OZ rats (5.5 ± 0.6 vs. 5.6 ± 0.5 mg/kg/min, p=0.860) and remained significantly reduced compared to LZ rats (p=0.003).

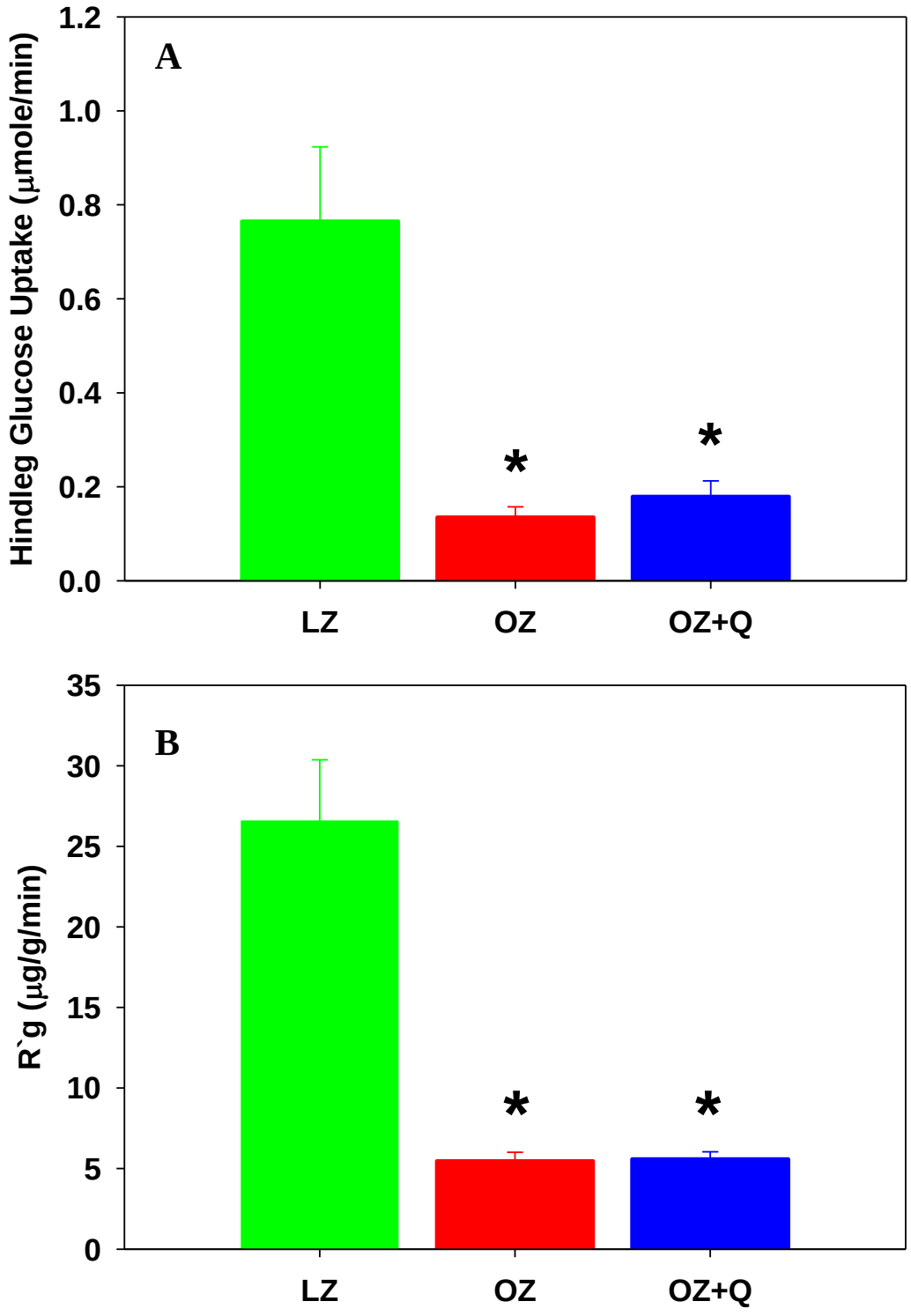

Figure 6.4. Effects of quinapril on hindleg and muscle specific glucose uptake.

Hindleg glucose uptake (A) and muscle specific glucose uptake (B) following 2 hrs of insulin infusion in LZ, OZ and OZ+Q rats. Data are means ± SEM for n=5-9 rats in each group. * Significantly different ($p<0.05$) from LZ using one-way ANOVA.

6.3.5. Quinapril effects on insulin's vascular action in obese Zucker rats

While no difference in basal FVR was detected between the three groups, all groups exhibited reduced FVR in response to 120min of insulin infusion. Basal FVR was not different between OZ and OZ+Q rats (Fig. 6.5; 301 ± 48 vs. 240 ± 33 mmHg.min/mL, p=0.181). OZ and OZ+Q rats had slightly increased FVR compared to LZ rats but neither group reached statistical significance (208 ± 30 vs. 301 ± 48, p=0.167 and 208 ± 30 vs. 240 ± 33 mmHg.min/mL, p=0.544, respectively). Following 2 hrs of insulin infusion FVR was reduced in LZ (208 ± 33 vs. 137 ± 38 mmHg.min/mL, p=0.013), OZ rats (301 ± 48 vs. 214 ± 31 mmHg.min/mL, p<0.001) and OZ+Q rats (240 ± 33 vs. 189 ± 32 mmHg.min/mL, p=0.032) compared to their respective basal FVR.

As with basal FVR, basal FBF was not different between the three groups. However, while insulin stimulated a greater increase in FBF in LZ rats compared to OZ rats, no improvement in the insulin-mediated increase in FBF was detected following quinapril treatment of OZ rats. Following 2 hrs of insulin infusion FBF in LZ rats was increased by 74% (Fig. 6.6.A; 0.48 ± 0.07 vs. 0.86 ± 0.09 mL/min, p<0.001). While insulin infusion increased FBF by 30% in OZ (0.39 ± 0.07 vs. 0.48 ± 0.07, p=0.128) and 18% in OZ+Q rats (0.48 ± 0.07 vs. 0.53 ± 0.07 mL/min, p=0.429) neither reached statistical significance compared to their respective basal FBF. The insulin-mediated change in FBF was increased in LZ compared to OZ (p=0.009) and OZ+Q rats (p=0.011). Insulin-stimulated change in FBF was similar between OZ and OZ+Q rats (p=0.654).

Similar to FBF, insulin stimulated a greater increase in 1-MX metabolism in LZ rats compared to OZ rats and quinapril treatment of OZ rats did not improve insulin-mediated 1-MX metabolism. Metabolism of 1-MX following 2 hrs of insulin infusion was significantly higher in LZ rats compared to OZ rats (Fig. 6.6.B; 4.39 ± 0.60 vs. 2.09 ± 0.22 nmol/min, p=0.005). Metabolism of 1-MX was not different between OZ+Q rats compared to untreated OZ rats (2.09 ± 0.22 vs. 2.59 ± 0.53 nmol/min, p=0.381) and remained significantly lower compared to LZ rats (p=0.013). No difference in the arterial concentration of 1-MX was detected between the three groups (LZ; 31.4 ± 4.4 vs. OZ; 34.3 ± 4.9 vs. OZ+Q; 33.2 ± 1.8 µmol/L, p=0.891). However, compared to LZ rats, oxypurinol concentrations were higher in both OZ and OZ+Q rats (6.3 ± 0.4 vs. 10.5 ± 0.8 vs. 10.3 ± 0.5 µmol/L, p<0.001, respectively) rats but were not different between OZ and OZ+Q rats (p=0.729).

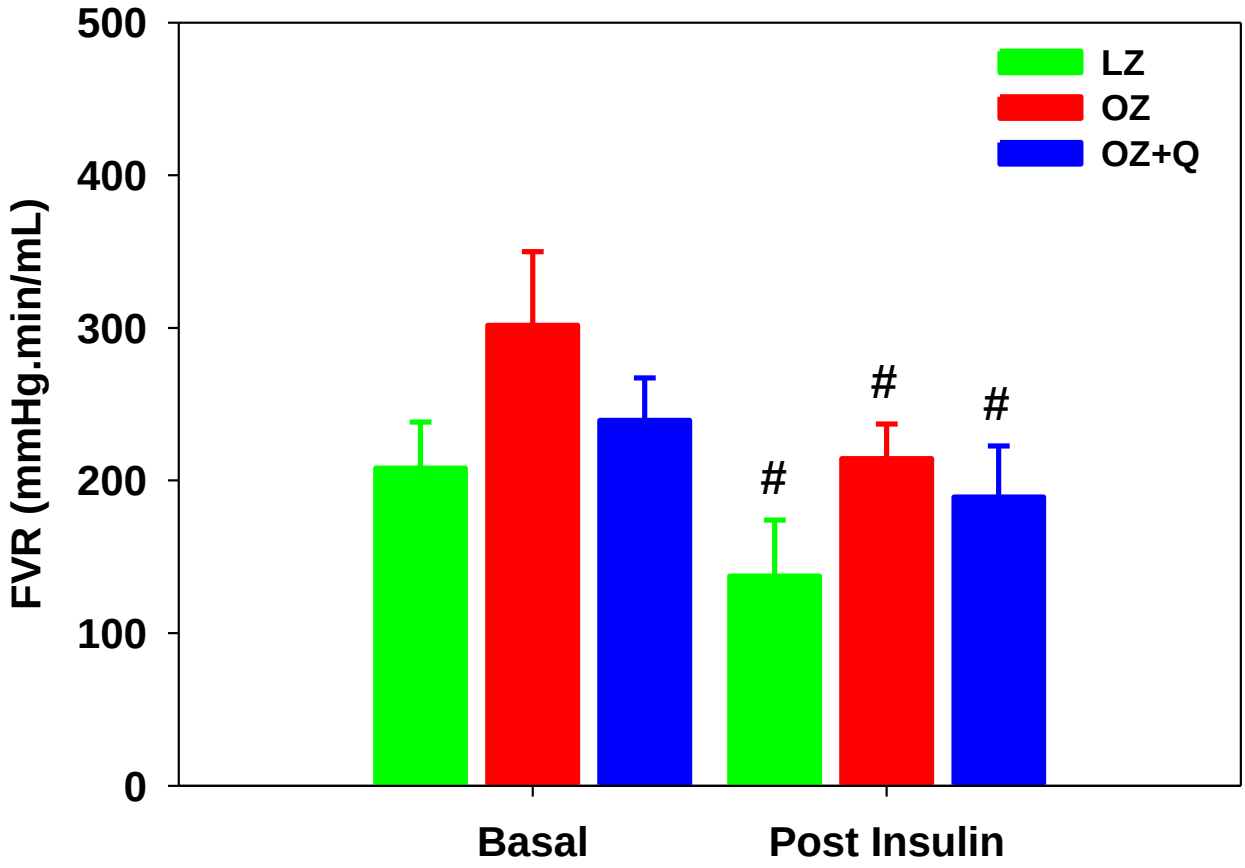

Figure 6.5. FVR at basal and following 120 min of insulin infusion.

Basal and post-insulin clamp FVR in LZ, OZ and OZ+Q rats. Data are means ± SEM for n=5-9 rats in each group. # Significantly different ($p<0.05$) from respective basal using repeated measures two-way ANOVA.

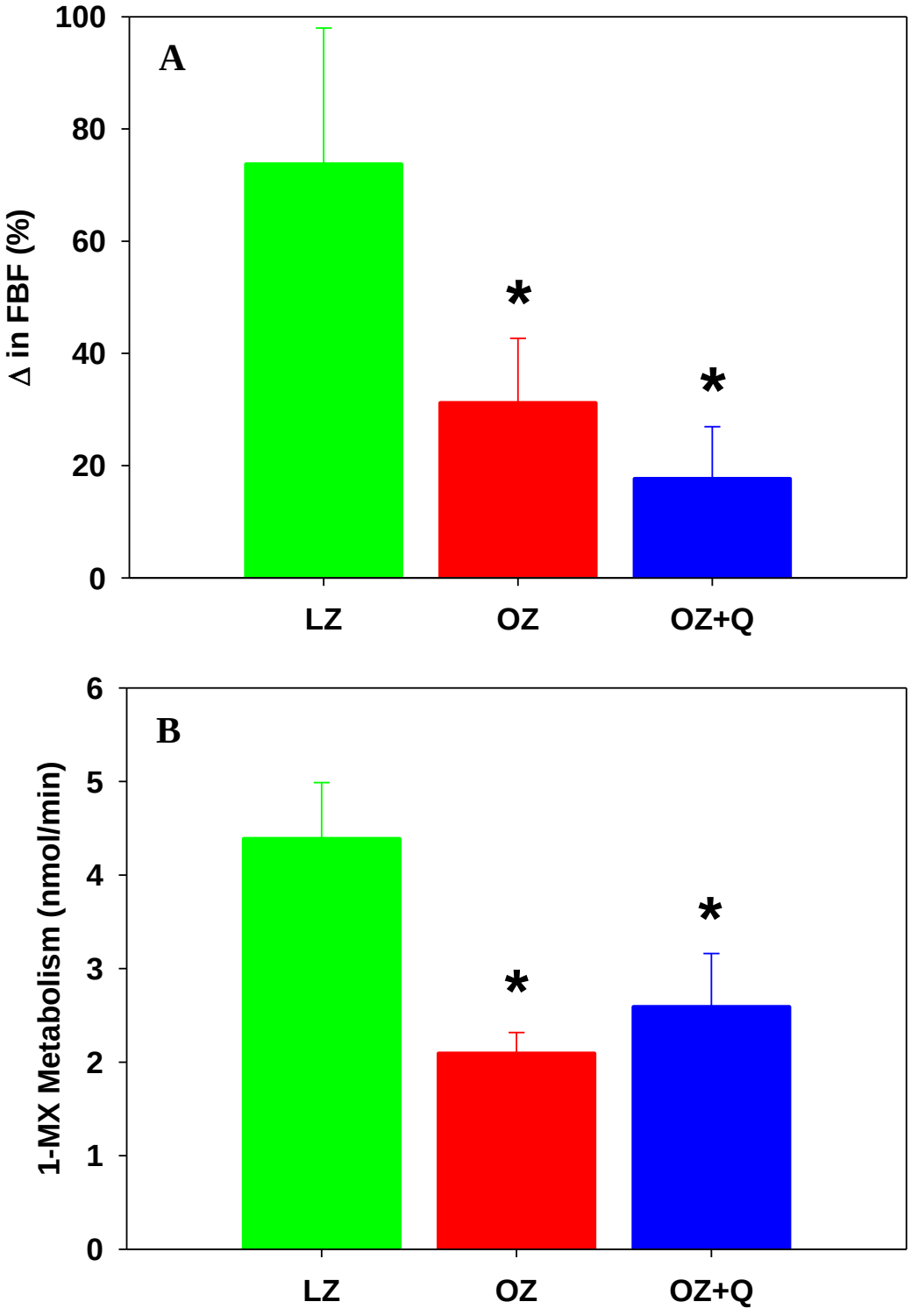

Figure 6.6. Vascular actions of insulin in LZ, OZ and OZ+Q rats.

Insulin-mediated change in FBF (A) and hindlimb 1-MX metabolism (B) in LZ, OZ and OZ+Q rats. Data are means ± SEM for n=5-9 rats in each group. * Significantly different ($p<0.05$) from LZ using one-way ANOVA.

6.4. Discussion

This study produced two main findings. Firstly, 4 weeks of quinapril treatment of OZ rats did not improve whole body or muscle insulin sensitivity. Secondly, quinapril treatment did not improve insulin-mediated microvascular recruitment in muscle compared to untreated OZ rats. Thus, 4 weeks of ACE inhibition in OZ rats was not beneficial at improving insulin sensitivity when assessed *in vivo*.

A previous report by Wallis and colleagues (2002) demonstrated that OZ rats displayed significant attenuation in insulin-mediated microvascular recruitment, whole body and skeletal muscle glucose disposal compared to LZ rats [242]. These previous findings were confirmed by the results of the present study. Surprisingly, treatment of OZ rats with quinapril for 4 weeks did not improve any measure of insulin sensitivity assessed in OZ rats in the present study. Indeed, no improvement in whole body glucose disposal, muscle glucose uptake or insulin's macrovascular and microvascular actions was detected following quinapril treatment of OZ rats. Therefore, the data from the current study would seem to suggest that inhibition of ACE in OZ rats does not correct or is unable to improve the underlying cause(s) of insulin resistance. The data from the current study is in contrast to previous reports where improvement in insulin sensitivity was detected following RAS inhibition [254, 324, 358]. The discrepancies between these findings regarding insulin sensitivity may be the result of differences in, i) methods used to assess the insulin response, ii) the difference in the animal models utilised or iii) dosage and length of ACE inhibition.

Henriksen and colleagues (1995 and 2001) have reported that insulin-mediated muscle glucose uptake can be augmented in OZ rats by inhibition of either ACE or the AT1R [254, 358]. In contrast, the findings in the present study suggest that inhibition of ACE does not improve insulin-mediated muscle glucose uptake in the OZ rat. The difference between these findings may result from the different methods utilised to determine insulin-mediated muscle glucose uptake. In both previous studies Henriksen and associates determined insulin-mediated muscle glucose uptake using the incubated muscle preparation. The advantage of this *in vitro* technique, which has been repeatedly

utilised in the literature, is that it allows for direct assessment of insulin-mediated glucose uptake by the myocyte, independent of any potential haemodynamic contributions. However, in the case of the OZ rats, this *in vitro* technique may overestimate the improvement insulin sensitivity in muscle since both the current study and the study by Wallis and colleagues (2002) have demonstrated that OZ rats display marked attenuation in insulin-mediated microvascular recruitment and also muscle glucose uptake *in vivo* [242]. Since quinapril treatment did not improve either insulin-mediated microvascular recruitment or muscle glucose uptake *in vivo*, the data from the present study is in contrast to that of the previous *in vitro* studies and suggests that muscle glucose uptake is not improved by 4 week quinapril treatment of OZ rats.

The animal models used may also be another point of difference between the current and previous studies. Henriksen and colleagues used the OZ rat from Harlan World Headquarters, USA while the OZ rats in the current study were obtained from Monash University animal services, Australia. While both are obese Zucker rats, it would seem that the OZ rats in the current study and the study by Wallis and colleagues (2002), who also used the Monash OZ rats [242], are significantly more insulin resistant than the rat strain used by Henriksen and colleagues. This is evidenced by approximately a 6 fold difference in fasting plasma insulin (~1100 pmol/L vs. ~6500 pmol/L) and a 2 fold higher fasting plasma glucose (~6.5 mmol/L vs. ~12.5 mmol/L) concentration in the OZ rats in the present study compared to the previous studies at a relatively similar age. Therefore, it is possible that the difference in effect on insulin sensitivity following RAS inhibition may due to the relative difference in the underlying insulin resistance of the OZ rats between the studies.

In the present study insulin-mediated microvascular recruitment was found to be significantly attenuated in the OZ rats compared to LZ rats and quinapril treatment of OZ rats did not improve this. This data is in contrast to a previous report by Clerk and colleagues (2007) who showed that quinapril treatment of ZDF rats augmented microvascular recruitment during hyperinsulinaemia [324]. While both rats are models of the metabolic syndrome they are representative of different stages of T2D. The ZDF rat is similar to the OZ rat in that it develops obesity and insulin resistance at an early age.

However, at the same age as the OZ rats in the current study, the ZDF model displays late-stage T2D and is characterised by impaired pancreatic insulin secretion and thus exhibits relatively low fasting plasma insulin concentrations (~200pmol/L) accompanied by severe hyperglycaemia (~20mmol/L) [399]. In contrast, the OZ rats in the present study are more representative of mid-stage T2D with compensatory hyperinsulinaemia (i.e. pancreatic insulin secretion is significantly increased), illustrated by severely increased fasting plasma insulin concentrations (~6500 pmol/L) and moderate hyperglycaemia (~12mmol/L). Thus, in relative terms, the ZDF rats would tend to be more insulin sensitive than the OZ rats. Indeed, not only was the insulin infusion rate required to induce whole body glucose disposal significantly higher in the present study (20mU/kg/min) compared to the study by Clerk and colleagues (3mU/kg/min), the subsequent GIR required to maintain euglycaemia was significantly lower in the present study (~4mg/kg/min vs. ~7mg/kg/min). Furthermore, the ACE inhibitor intervention was also considerably longer (16 weeks vs. 4 weeks) in the ZDF rat study compared to the OZ rats in the present study. Thus, the relative difference in insulin sensitivity or decreased length of ACE intervention may account for some of the differences regarding improved insulin sensitivity between the current and previous studies following quinapril treatment.

Excluding methodological differences and difference between the rat models, another point of difference between the current and previous studies regarding improvement in insulin sensitivity following ACE inhibition is the age at which the treatment was initiated. Henriksen and colleagues (1995) used 8 week old OZ rats and treated them with a high dose of captopril (50 mg/kg/day) for 2 weeks [254], while Clerk and colleagues (2007) used 5 week old ZDF rats and treated them for 16 weeks with quinapril (0.3 mg/kg/day)[324]. In contrast, the current study used 11-12 week old OZ rats and treated them for 4 weeks with quinapril (1.0 mg/kg/day). Although it is difficult to directly compare the doses of different types of ACE inhibitors with outcomes in different rats, the dose and duration of quinapril treatment in the current study was sufficient to overcome the microvascular and muscle insulin resistance in the HS-fed rat model in the previous chapter of this thesis. However, this dose and duration of quinapril did not correct these defects in the OZ rat. At the stage quinapril treatment was initiated, it is likely that the OZ rats were more severely insulin resistant than the HS fed rats. This

is evidenced by the significantly elevated fasting plasma insulin concentration in the OZ rats in the current study compared to these previous reports where ACE inhibition was found to be beneficial, including the HS fed rat. Thus, treatment with quinapril from an earlier age (i.e. less insulin resistant age) in OZ rats may be required for any beneficial effects to be seen. It is also possible that the dose of quinapril or length of treatment may not have been sufficient in the current study to similarly improve microvascular recruitment and/or muscle glucose uptake as seen previously. Whether a longer intervention period or increased dose of quinapril would yield more positive results regarding insulin sensitivity in the current OZ rat model is not known.

Based on the phenotype of the OZ rats at the time of intervention in the present study, current results may not be surprising. Indeed, a number of clinical studies have also failed to find significant effects following inhibition of AngII action in certain patient populations [280-283]. This concept will be further explored in the final discussion (chapter 8) of this thesis.

6.4.1. Conclusions

The primary aim of this study was to assess whether 4 week quinapril treatment of OZ rats could similarly improve insulin sensitivity as seen in the HS fed rat in the previous chapter. The results from the current study illustrate that inhibition of ACE did not improve skeletal muscle insulin sensitivity in the OZ rat. Therefore, these data would suggest that ACE inhibition may not be an appropriate target for improving muscle insulin sensitivity *in vivo* in models of well-established T2D. Therefore, it may be reasonable to conclude that ACE inhibition may only be effective at relatively early stages of insulin resistance (such as the HS fed rat) where the microvascular defect may impair delivery of insulin and glucose to the myocyte, without the presence of overt myocyte insulin resistance. For this reason, it is important to determine whether microvascular dysfunction occurs early and precedes the development of myocyte insulin resistance. As explained in chapter 1 of this thesis, microvascular and myocyte insulin resistance often co-exist. While studies of acutely induced microvascular dysfunction also lead to reduced muscle glucose uptake, these studies do not model the

normal disease progression. An animal model that displays a purely microvascular defect in skeletal muscle that leads to insulin resistance *in vivo* has not been previously developed or characterised. Such a model would represent one of the earliest events in the aetiology of insulin resistance and demonstrate that microvascular insulin resistance can occur prior to myocyte insulin resistance.

CHAPTER 7

MODERATE INCREASES IN DIETARY FAT INDUCE MICROVASCULAR INSULIN RESISTANCE IN SKELETAL MUSCLE *IN VIVO*

7.1. Introduction

In chapters 3 and 4 of this thesis two different control diets were utilised to compare/contrast the effects of the HS diet on insulin and AngII sensitivity. In chapter 3 a semi-purified diet was used, and in chapter 4 a standard laboratory chow was used. It was initially assumed that there would be no difference between the two control diets regarding insulin sensitivity. However, it was noticed that during the euglycaemic hyperinsulinaemic clamp, rats fed the laboratory chow displayed reduced whole body insulin sensitivity (GIR; 19.4 ± 0.4 vs. 18.5 ± 1.1 mg/kg/min) and muscle glucose uptake (R`g; 24.0 ± 0.7 vs. 20.9 ± 3.2 µg/kg/min) compared to the rats fed the semi-purified diet. The main difference between these diets is the amount of fat. The semi-purified diet contains 7% fat wt./wt. whereas the chow contains 9% fat wt./wt. Given that increases in dietary fat have been associated with the development of insulin resistance, this preliminary data indicates that the moderate increase in dietary fat may have contributed to a reduced insulin responsiveness in the chow (9% fat) fed rats. If true, this would suggest that insulin sensitivity may be more sensitive to moderate increases in dietary fat than previously thought.

High fat diets have repeatedly been shown to induce a pre-diabetic state in rodents that is characterised by mild hyperinsulinaemia without any significant effects on circulating glucose concentrations [222, 223, 400, 401]. Kraegen and colleagues (1986 and 1991) have shown that feeding rats a high fat diet (~36% fat wt./wt.) for 3-4 weeks results in the development of both skeletal muscle and liver insulin resistance when compared to chow (~6% fat wt./wt.) fed rats [223, 347]. In the latter of these two studies the authors reported that hepatic insulin resistance developed following only 3 days of high fat feeding and that in contrast, skeletal muscle insulin resistance was present following 21 days of fat feeding [347]. Thus, the authors proposed that a loss of liver insulin sensitivity was an early event in the development of insulin resistance following high fat feeding. However, other reports have demonstrated that relatively short periods of high fat feeding, ranging from days to weeks, are sufficient to provoke muscle insulin resistance [227, 402, 403]. Kim and colleagues (1996) reported that skeletal muscle insulin resistance developed after 4 days of high fat feeding (~36% fat wt./wt.) when compared to rats fed chow (~6% fat wt./wt.) [403]. The authors also demonstrated that

further increasing the length of the feeding intervention also increased the extent of insulin resistance in skeletal muscle [403]. Thus, the latter study suggests that skeletal muscle insulin sensitivity may also be affected at early stages of high fat feeding and may contribute to the development of insulin resistance.

Whilst the mechanisms responsible for the development of insulin resistance during high fat feeding appear to be multifactorial [134, 169, 404, 405], a recent report by St-Pierre and colleagues (2010) has demonstrated a possible role for defects in insulin-mediated microvascular recruitment in skeletal muscle [227]. In their study the authors found that feeding rats a high fat diet (~36% wt./wt.) for 4 weeks resulted the development of both hepatic and skeletal muscle insulin resistance compared to chow (~5% fat wt./wt.) fed controls [227]. Moreover, this was associated with marked impairment in insulin-mediated microvascular recruitment in muscle [227]. For this reason, the authors proposed that the attenuation of insulin-mediated microvascular recruitment following high fat feeding may contribute to the reduced insulin-mediated muscle glucose uptake.

Additionally, there is evidence that impairment in vascular function may be an early consequence of increased dietary fat. Naderali and Williams (2001) reported that 2 days of high fat feeding (~17% fat wt./wt.) resulted in attenuation of carbamylcholine-mediated vasodilation in 3rd order mesenteric arteries compared to chow (~9% fat wt./wt.) fed animals [224]. Moreover, the authors reported that this vascular dysfunction was independent of changes in body weight, fat mass or glucose and insulin concentrations and was only associated with increased circulating FFA and triglyceride concentrations [224]. In addition, other reports have demonstrated that increasing FFAs alone leads to the development of endothelial dysfunction and induce insulin resistance in muscle [193-195]. Interestingly, Watanabe and colleagues (2005) demonstrated that FFA-induced endothelial dysfunction could be prevented by inhibiting either ACE or the AT1R [198]; suggesting a possible role for increased AngII activity in the development of FFA-induced endothelial dysfunction. Taken together, these studies raise the possibility that increased fat intake may negatively affect insulin's vascular actions in muscle which may in turn contribute to the development of muscle insulin resistance.

However, the amount of dietary fat needed to provoke both muscle and microvascular insulin resistance is unknown.

In light of the findings in the previous chapters of this thesis, the aim of the current study was to determine whether moderately increasing dietary fat induces insulin resistance in rats. Specifically, to characterise the effects of increasing dietary fat from 5% to 9% fat (wt./wt.) on insulin-mediated microvascular recruitment and muscle glucose uptake *in vivo*. Since previous reports have identified a possible role for AngII in high fat-FFA-induced endothelial dysfunction and insulin resistance, a secondary aim of the current study was to investigate whether these rats exhibit increased vascular sensitivity to AngII-mediated constriction.

7.2. Methods

7.2.1. Animals

Male Sprague Dawley rats (4 weeks of age) were obtained from the University of Tasmania Central Animal Facility. On arrival, the rats were split into two groups and provided either a low fat (4.8% fat wt./wt.; 5%F, Specialty Feeds) or moderately elevated fat (9% fat wt./wt.; 9%F, Ridley Agri Products) diet *ad libitum* for 3-4 weeks (Table 7.1). Following an overnight fast, animals were subjected to either an *in vitro* perfused rat hindleg protocol or an *in vivo* anaesthetised rat protocol.

	5%F	9%F
Protein	19.4 %	22.0 %
Carbohydrate	70.7 %	65.8 %
Fat	4.8 %	9.0 %
Total Mono-unsaturated Fats	38.5 %	36.6 %
Total Poly-unsaturated Fats	43.8 %	42.1 %
Total Saturated Fats	17.3 %	20.4 %
Crude Fibre	5.1 %	3.2%
Total Digestible Energy	14.0 MJ/Kg	13.2MJ/Kg

Table 7.1. Macronutrient composition of the 5%F and 9%F diets as wt./wt.

The fatty acid profiles represent the contribution of each subset of fat to the overall fat content of the respective diets.

7.2.2. Anaesthetised rat experiments

7.2.2.1. Experimental procedure

Surgery was performed as outlined in chapter 2 section 2.3.1. Following surgical preparation, 1 hr was allowed for FBF and mean arterial blood pressure to stabilise, at which time a 2 hr infusion of either saline (10µL/min) or insulin (10mU/min/kg) was initiated. Since insulin stimulates glucose disposal, a 30% glucose solution (wt./vol.) was infused at a variable rate to maintain fasting blood glucose concentrations over the course of the experiment. For this reason, arterial blood glucose levels were assessed every 10 min in the first hour, and every 15 min in the second hour using a glucose analyser (YSI 2300) and the glucose infusion rate was adjusted accordingly. See Fig. 7.1 for a more detailed protocol. Plasma biochemistry, whole body glucose kinetics (Ra and Rd), muscle glucose uptake and microvascular recruitment in muscle were assessed as previously outlined in chapter 2, sections 2.3.2-2.3.5.

HOMA-IR was calculated using the following equation [406]:

$$HOMA - IR = \frac{Fasting\ Plasma\ Glucose\ \left(\frac{mg}{dL}\right) X\ Fasting\ Plasma\ Insuiln\left(\frac{mg}{dL}\right)}{22.53}$$

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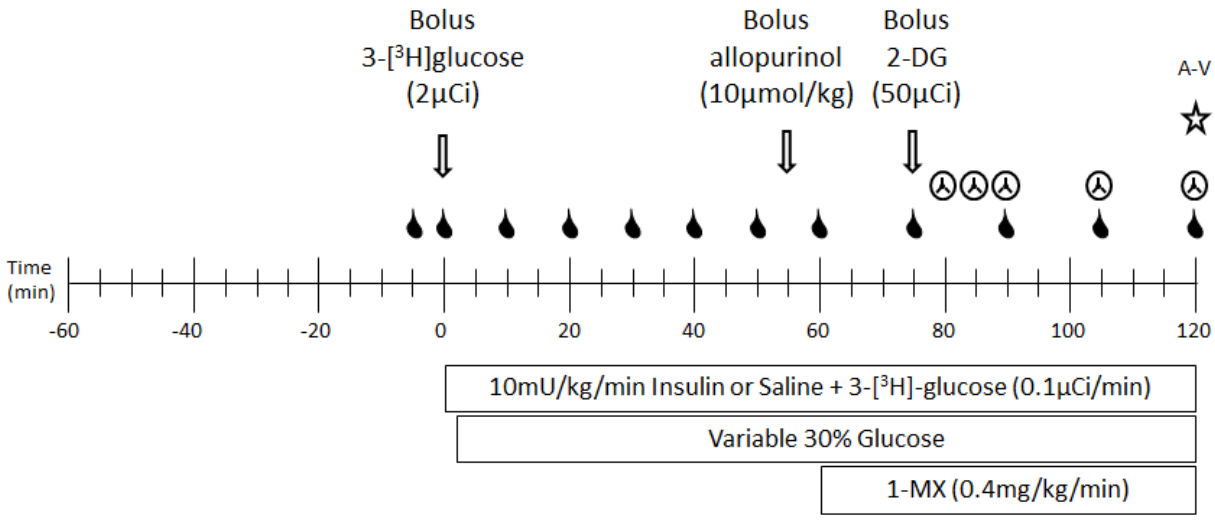

Figure 7.1. Experimental protocol for hyperinsulinaemic euglycaemic clamp.

After surgical preparation and 60 min of equilibration, a continuous infusion of saline (10µL/min) or insulin (10mU/min/kg) was commenced and continued for 120 min. A 30% (wt./vol.) glucose infusion was initiated shortly after the commencement of the insulin infusion to maintain baseline glycaemia as assessed by arterial blood glucose sampling (). After 55 min of saline or insulin infusion, a bolus of allopurinol (10µmol/kg) was administered. At 60 min, infusion of 1-MX (0.4 mg/kg/min) was initiated and maintained until the end of the experiment. At 75 min a bolus of 2-deoxy-D-[1-^{14}C]-glucose (2-DG; 20µCi) was administered. Radioactive plasma samples () were collected at 80, 85, 90, 105 and 120 min to determine the plasma 2-DG clearance. At the conclusion of the experiment arterial and femoral vein plasma samples ( ) were collected for the determination of hindleg glucose uptake and 1-MX metabolism. Immediately following sacrifice, calf muscle (soleus, gastrocnemius and plantaris group) was freeze clamped in liquid nitrogen and stored at -80°C.

7.2.3. Perfused rat hindleg experiments

7.2.3.1. AngII dose curve procedure

Surgical procedure and experimental setup were performed as previously outlined in chapter 2, sections 2.4.2 and 2.4.3. Following surgery and commencement of the perfusion, total flow through one hindleg was adjusted to a constant flow rate of 8ml/min for a 220g rat (equivalent to 0.4mL/min/g muscle). Following a 30 min equilibration period (to allow washout of red blood cells from the hindleg) an AngII dose curve was commenced to assess the vascular responsiveness of the hindleg. AngII was infused into the hindleg at 0.1, 0.3, 1.0, 3.0 and 10nM in 10 min stepwise increments and is shown in more detail in Fig. 7.2.A.

7.2.3.2. Muscle insulin sensitivity in the perfused hindleg procedure

The isolated perfused hindlimb preparation was utilised to assess insulin-mediated muscle glucose uptake independent of the vascular actions of insulin. Thus, this approach assesses myocyte insulin sensitivity.

Following 30 min of equilibration, saline or insulin (15nM final concentration; Humulin) infusion was initiated at 1:200 of the total flow for 60 min. Radiolabelled glucose (20nCi/mL; 2-deoxy-D-[1-^{14}C]-glucose, 2-DG) was infused during the last 30 min of the saline or insulin infusion and was used to measure muscle glucose uptake (R`g). At the conclusion of the experiment the calf muscle (soleus, gastrocnemius and plantaris group) was excised, quickly frozen in liquid nitrogen and kept at -80°C, see Fig. 7.2.B. Assessment of muscle glucose uptake was performed as outlined in chapter 2, section 2.3.4.

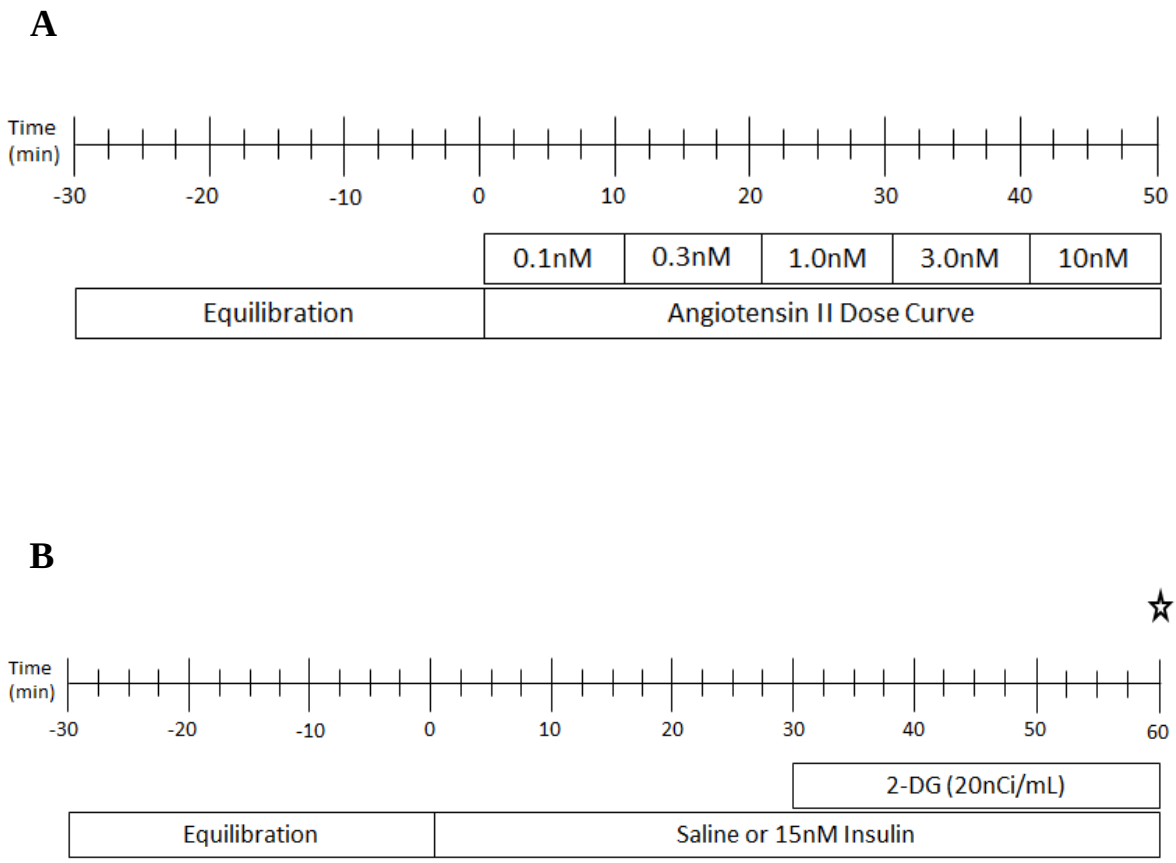

Figure 7.2. Experimental protocols for perfused hindleg experiments.

After surgical preparation and 30 min of equilibration, (A) AngII infusion was initiated through the infusion port at 1:100 of the total flow rate through the hindleg. An increasing AngII dose curve was used to assess the sensitivity of the vasculature to AngII mediated constriction and perfusion pressure was recorded continuously using Windaq® software. (B) Saline or insulin (15nM final concentration) infusion was initiated at 1:200 of the total flow for 60 min. Radiolabelled glucose (20nCi/mL; 2-deoxy-D-[1-^{14}C]-glucose, 2-DG) was infused during the last 30 min of the saline or insulin infusion. At 60 min calf muscle was excised and quickly frozen in liquid nitrogen and kept at -80°C.

7.2.4. Data and Statistics

Data are presented as the means ± SEM and statistics were performed using SigmaStat (Systat Software Inc, 2004). Comparisons between 5%F and 9%F fed rats were made using un-paired Student's t-test. Comparison of time-series measurements in each group was performed by two-way repeated measures ANOVA. When a significant difference of $p<0.05$ was detected, pairwise comparisons by Student-Newman-Keuls test was used to assess treatment differences. Linear regression was used to assess the relationships between insulin-mediated 1-MX metabolism and muscle glucose uptake, and insulin-mediated change in FBF and muscle glucose uptake.

7.3. Results

7.3.1. Physical and biochemical characteristics of rats following 3-4 week feeding intervention

The physical and clinical chemistry of 5%F and 9%F are shown in Table 7.2. At the conclusion of the dietary intervention there was no difference in body weight ($p=0.885$) or epididymal fat mass ($p=0.717$) between the 5%F and 9%F fed animals. The fasting plasma glucose concentration was not different ($p=0.778$) between the two dietary groups; however the 9%F group displayed an elevated fasting plasma insulin concentration ($p=0.033$) when compared to the 5%F group. Consequently the calculated HOMA-IR (an index of insulin resistance) was higher ($p=0.024$) for the rats maintained on the 9%F diet compared to the 5%F diet. The fasting plasma FFA concentration was increased by 45% in the 9%F group ($p<0.001$). No differences in basal mean arterial pressure ($p=0.502$) or FBF ($p=0.216$) during anaesthesia were detected between the two groups.

	5%F	9%F	p value
Body weight (g)	237 ± 8	238 ± 7	0.885
Epididymal fat pad (g)	1.18 ± 0.08	1.27 ± 0.10	0.717
Mean arterial pressure (mmHg)	103 ± 3	101 ± 2	0.502
Basal FBF (mL/min)	0.94 ± 0.04	0.88 ± 0.05	0.216
Fasting plasma glucose (mmol/L)	6.77 ± 0.17	6.74 ± 0.16	0.778
Fasting plasma insulin (pmol/L)	84 ± 11	129 ± 19	0.033
HOMA-IR	3.77 ± 0.56	5.74 ± 0.87	0.024
Fasting plasma FFA (mmol/L)	0.60 ± 0.02	0.87 ± 0.07	<0.001

Table 7.2. Physical and biochemical characteristics of rats following 3-4 week feeding intervention.

Measures were collected immediately prior to the commencement of the saline infusion or insulin clamp. Epididymal fat pads were excised and weighed at the conclusion of the experiments. FBF; femoral artery blood blow, FFA; free fatty acids. Data are means ± SEM for n=10-18 rats in each group. Comparisons between groups were made using un-paired Students t-test.

7.3.2. Effects of 9%F diet on whole body insulin sensitivity

Rats fed the 9%F diet displayed whole body and skeletal muscle insulin resistance, but not liver insulin resistance *in vivo*. No differences in blood glucose concentrations were detected during the 2 hr infusion of either saline or insulin between 5%F and 9%F groups (Fig 7.3.A). Insulin infusion for 2 hrs increased the plasma insulin concentration to a similar level in 5%F (1170 ± 110 pmol/L) and 9%F (1370 ± 100 pmol/L) fed rats and no significant ($p=0.202$) difference between the two was detected. However, the glucose infusion rate (GIR) required to maintain glycaemia during insulin infusion was higher in the 5%F group compared to the 9%F group by 20 min (Fig. 7.3.B; $p<0.05$). At the end of the 120 min insulin clamp, the GIR was approximately 12% lower (21.2 ± 0.3 vs. 18.9 ± 0.1 mg/kg/min; $p=0.020$) in the 9%F group compared to the 5%F group. No significant difference in insulin-mediated suppression of liver glucose output (Ra; 2.93 ± 0.91 vs. 1.39 ± 0.57 mg/kg/min; $p=0.113$) was detected between the 5%F and 9%F groups during insulin infusion (Figure 7.4.A). Similarly, no significant difference in the rate of glucose disappearance (Rd; 5%F; 22.5 ± 4.0 vs. 9%F; 21.3 ± 2.2 mg/kg/min, $p=0.501$) was detected between the 5%F and 9%F groups during insulin infusion (Fig. 7.4.B).

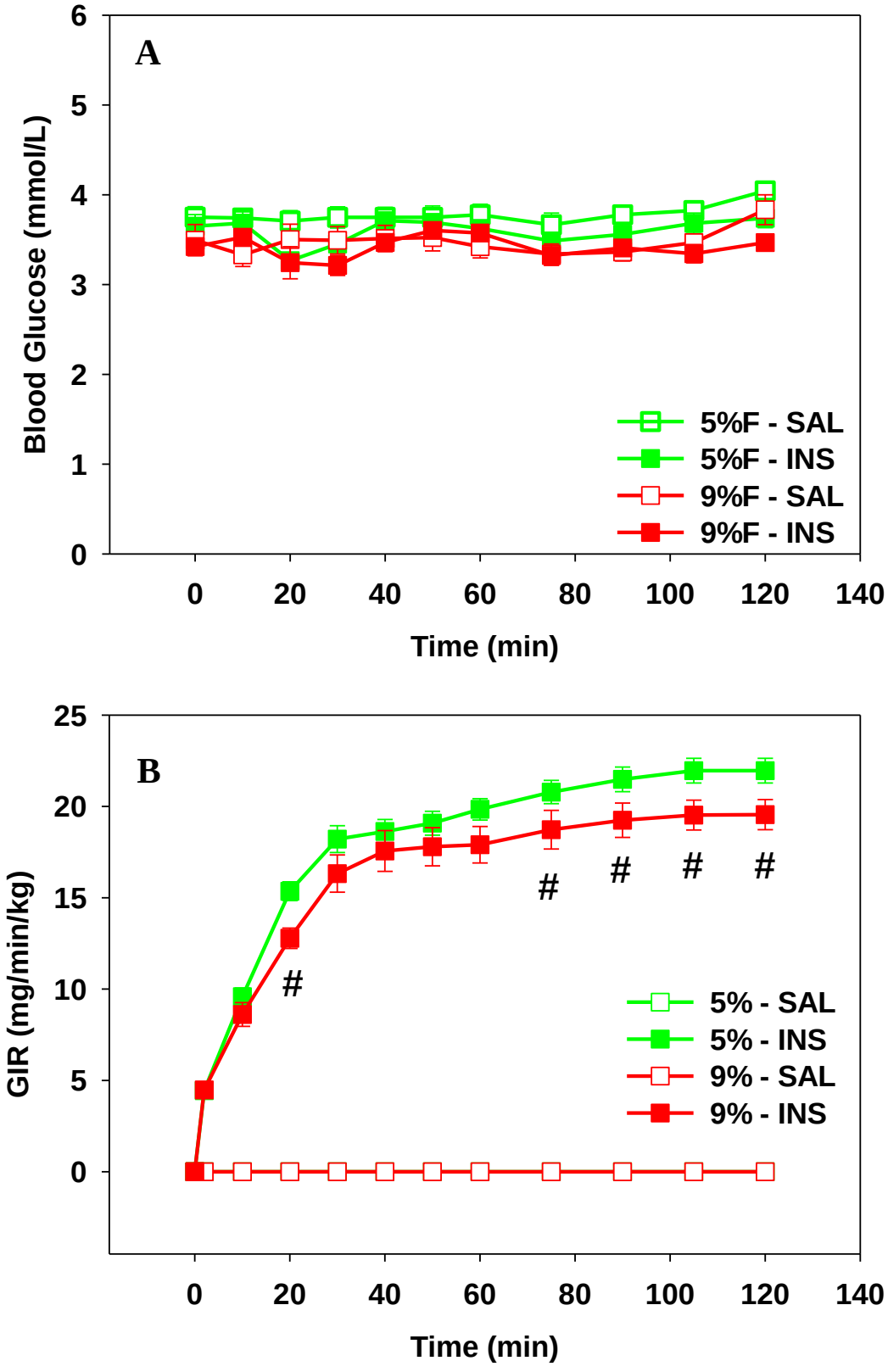

Figure 7.3. Dietary effects on whole body insulin sensitivity.

Blood glucose (A) and glucose infusion rate (GIR; B) during saline infusion or insulin clamp of 5%F and 9%F rats. Data are means ± SEM for n = 8-10 rats in each group. # Significantly different ($p<0.05$) from 5%F-INS using two-way repeated measures ANOVA.

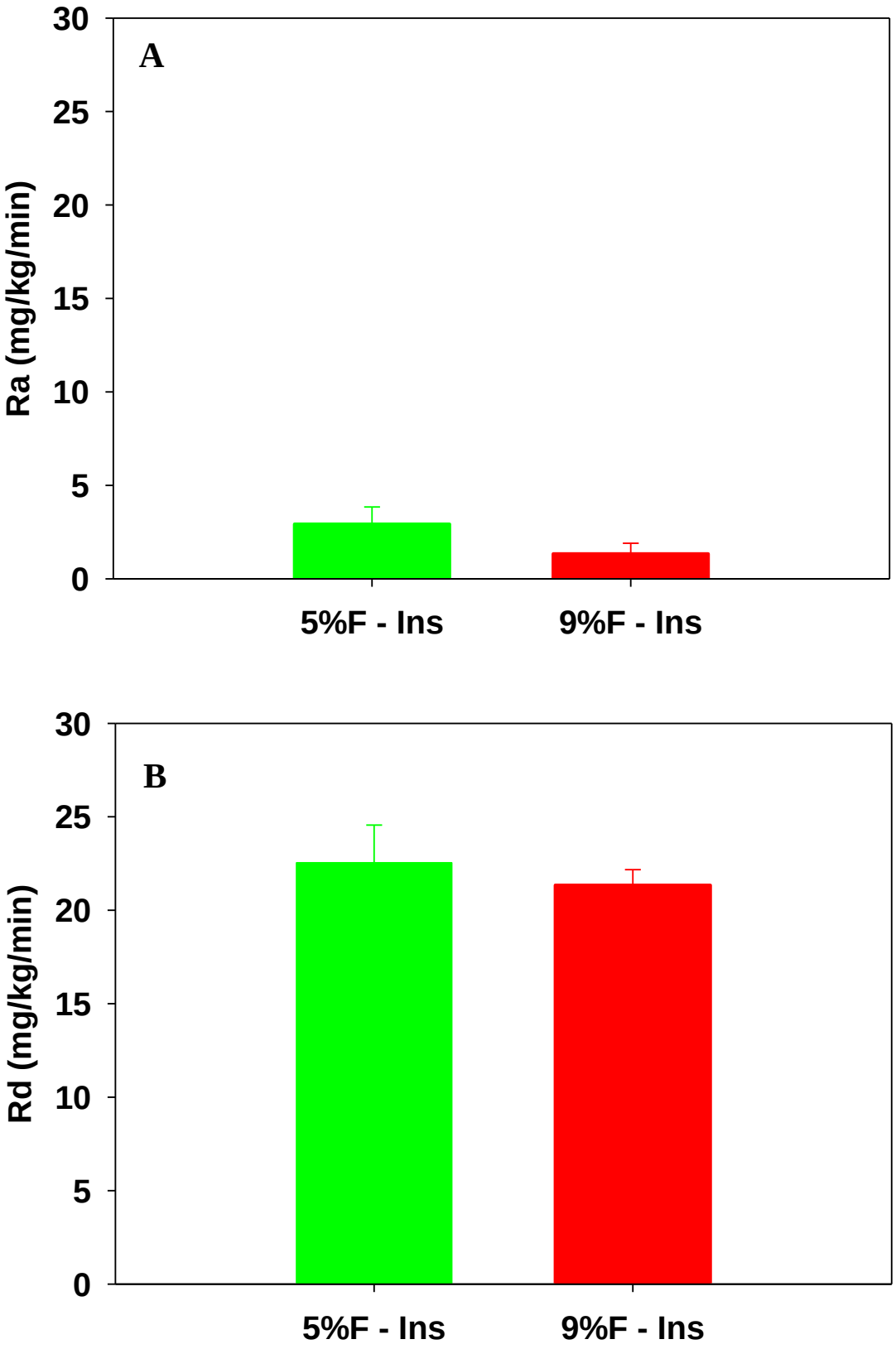

Figure 7.4. Dietary effects on whole body glucose kinetics *in vivo*.

Rate of glucose appearance (A; Ra) and disappearance (B; Rd) at the conclusion of the insulin clamp in 5%F and 9%F rats. Data are means ± SEM for n = 5-8 rats in each group.

7.3.4. Effects of the 9%F diet on insulin's vascular actions *in vivo*

Neither basal nor the insulin-stimulated change in FBF was different between 5%F and 9%F groups. Compared to basal, 2 hrs of insulin infusion increased FBF in both 5%F (0.92 ± 0.04 vs. 1.18 ± 0.05 mL/min; $p<0.001$) and 9%F groups (0.82 ± 0.07 vs. 1.11 ± 0.10 mL/min: $p<0.001$) to a similar extent. The insulin-stimulated increase in FBF was not different between the 5%F and 9%F fed rats (Fig. 7.6.A; 25.0 ± 7.8 vs. 30.9 ± 8.7 %; $p=0.517$).

Whilst no difference in 1-MX metabolism was detected during saline infusion between the two groups, 120 min of insulin infusion revealed a significant reduction in 1-MX metabolism in the 9%F group compared to the 5%F group. No difference in metabolism of 1-MX was detected following 2 hrs of saline infusion (Fig. 7.6.B; 2.97 ± 0.33 vs. 2.83 ± 0.34 nmol/min; $p=0.776$) between 5%F and 9%F fed rats. Compared to their respective saline controls, insulin infusion increased 1-MX metabolism by 89% in the 5%F group (2.97 ± 0.33 vs. 5.62 ± 0.25 nmol/min; $p<0.001$) and by 44% in the 9%F group (2.83 ± 0.34 vs. 4.08 ± 0.41 nmol/min; $p=0.010$). Metabolism of 1-MX following insulin infusion was 38% higher ($p=0.002$) in the 5%F group compared to the 9%F group. Therefore, the 5%F group had elevated insulin-stimulated microvascular perfusion compared to the 9%F group. There were no significant differences between the arterial 1-MX (22.2 ± 1.3 vs. 24.4 ± 1.2 µmol/L; $p=0.226$) or oxypurinol (7.59 ± 0.38 vs. 7.18 ± 0.33 µmol/L; $p=0.419$) concentrations between the 5%F and 9%F groups respectively indicating that both groups received the same amount of 1-MX and had the same degree of xanthine oxidase inhibition.

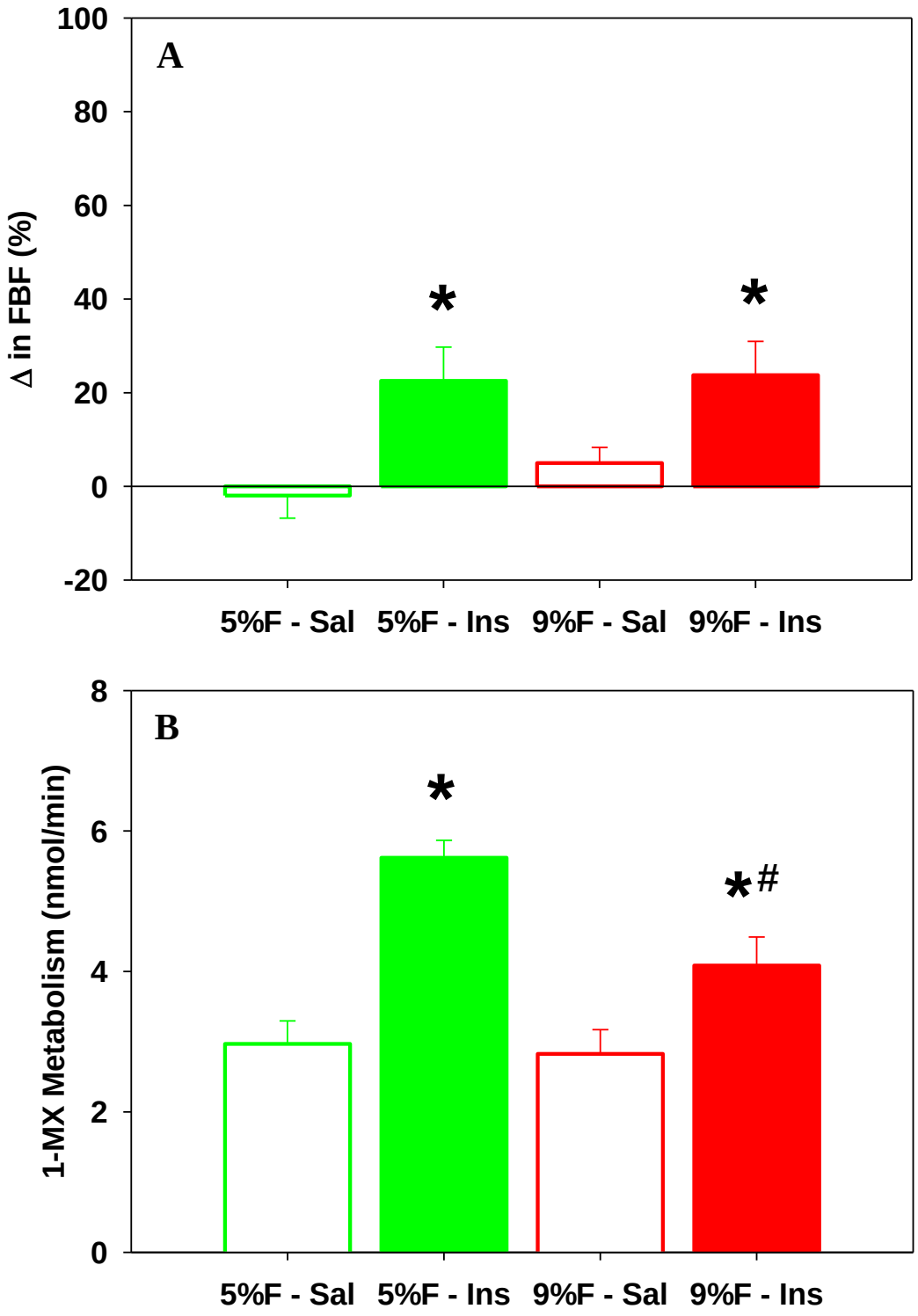

Figure 7.6. Vascular effects of saline and insulin *in vivo*.

Saline infusion or insulin clamp mediated change in FBF (A) and 1-MX metabolism (B) in 5%F and 9%F rats. Data are means ± SEM for n=8-10 rats in each group.
* Significantly different ($p<0.01$) from respective Sal, # Significantly different ($p<0.01$) from 5%F-Ins using two-way repeated measures ANOVA.

7.3.3. Effects of 9%F diet on hindleg and muscle glucose uptake *in vivo*

The 9%F fed rats exhibited significant attenuation of insulin-mediated hindleg and skeletal muscle glucose uptake *in vivo*. Following 2 hrs of insulin infusion, hindleg glucose uptake (Fig. 7.5.A; HGU) increased in both 5%F (0.11 ± 0.02 vs. 0.87 ± 0.07 µmol/min; $p<0.001$) and 9%F (0.11 ± 0.02 vs. 0.51 ± 0.06 µmol/min; $p<0.001$) groups compared to their respective saline controls. However, compared to the 5%F group, the 9%F group displayed significant attenuation in insulin-stimulated HGU ($p<0.001$). The rate of muscle specific glucose uptake (Fig. 7.5.B; R`g) during hyperinsulinaemia was increased in both 5%F (3.0 ± 0.2 vs. 29.4 ± 1.6 µg/g/min; $p<0.001$) and 9%F (2.5 ± 0.3 vs. 20.1 ± 1.6 µg/g/min; $p<0.001$) fed rats compared to their respective saline controls. However, insulin-mediated R`g was reduced by approximately 45% ($p<0.001$) in the 9%F group compared to the 5%F group. No differences in HGU ($p=0.941$) or R`g ($p=0.800$) were detected between the two saline groups.

7.3.5. The relationship between insulin's vascular and metabolic actions in muscle *in vivo*

The relationships between insulin's vascular and metabolic actions in muscle *in vivo* are shown in Figs. 7.7 and 7.8. Individual data for either 1-MX metabolism or change in FBF were plotted against muscle glucose uptake (R`g) from 5%F and 9%F fed rats following 2 hrs of insulin infusion. In addition, data from the control diet (7%F, which represents an intermediate fat content) which was used in chapter 3 was also included in the correlations to give more statistical power to the correlation. The data demonstrate that there is a strong positive relationship between insulin-stimulated 1-MX metabolism and R'g (Fig. 7.7; $r=0.505$, $p=0.004$) *in vivo*. In contrast, there is no relationship between insulin-stimulated FBF and R'g (Fig. 7.8; $r=0.085$, $p=0.655$) *in vivo*. Thus, insulin's microvascular (and not macrovascular) actions are mirrored by changes in muscle glucose disposal *in vivo* following increased dietary fat intake.

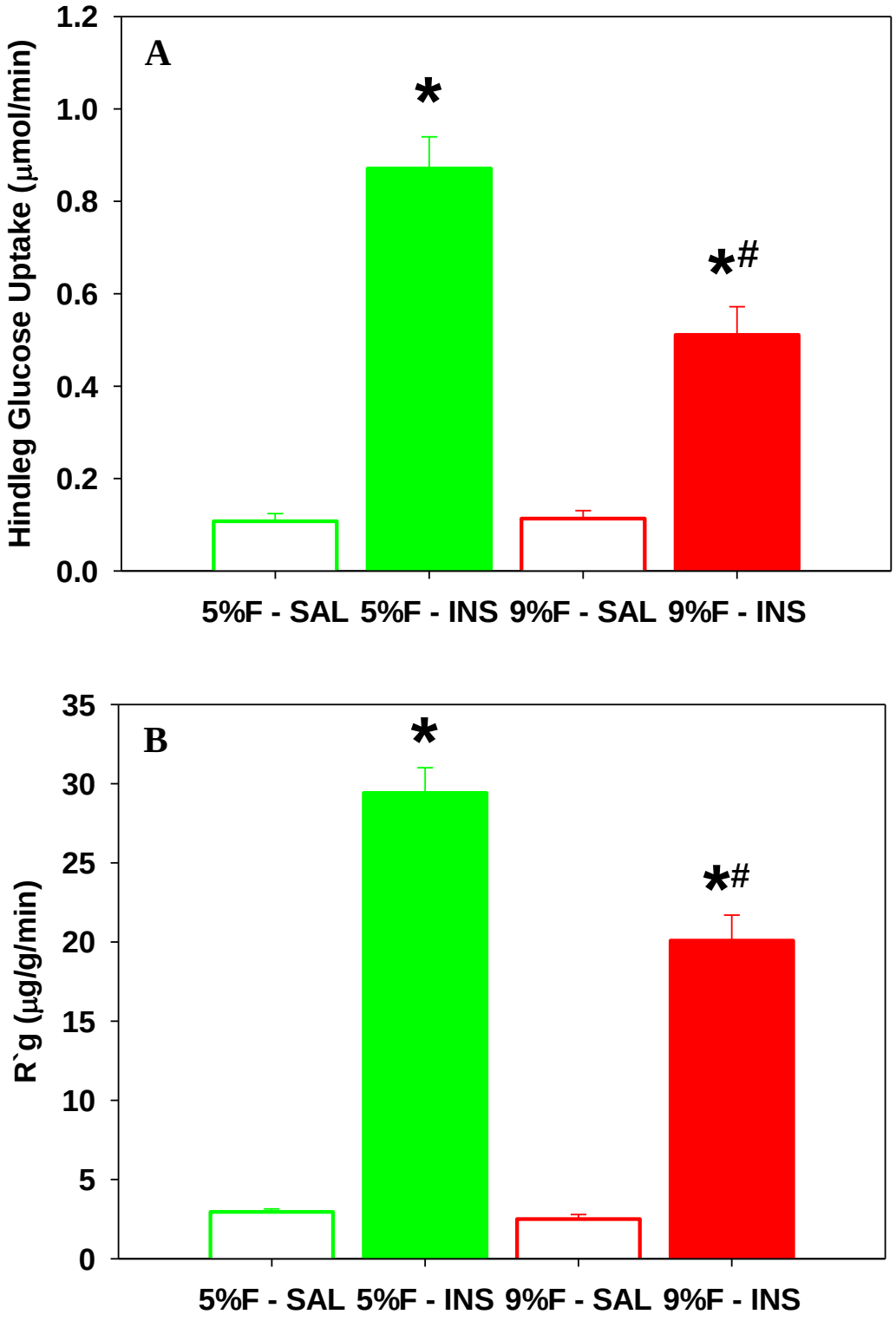

Figure 7.5. Hindleg and muscle specific glucose uptake *in vivo*.

Hindlimb glucose uptake (A) and muscle specific glucose uptake (B) during saline infusion or insulin clamp in 5%F and 9%F rats. Data are means ± SEM for n=8-10 rats in each group. * Significantly different ($p<0.001$) from respective Sal, # Significantly different ($p<0.001$) from 5%F-Ins using two-way repeated measures ANOVA.

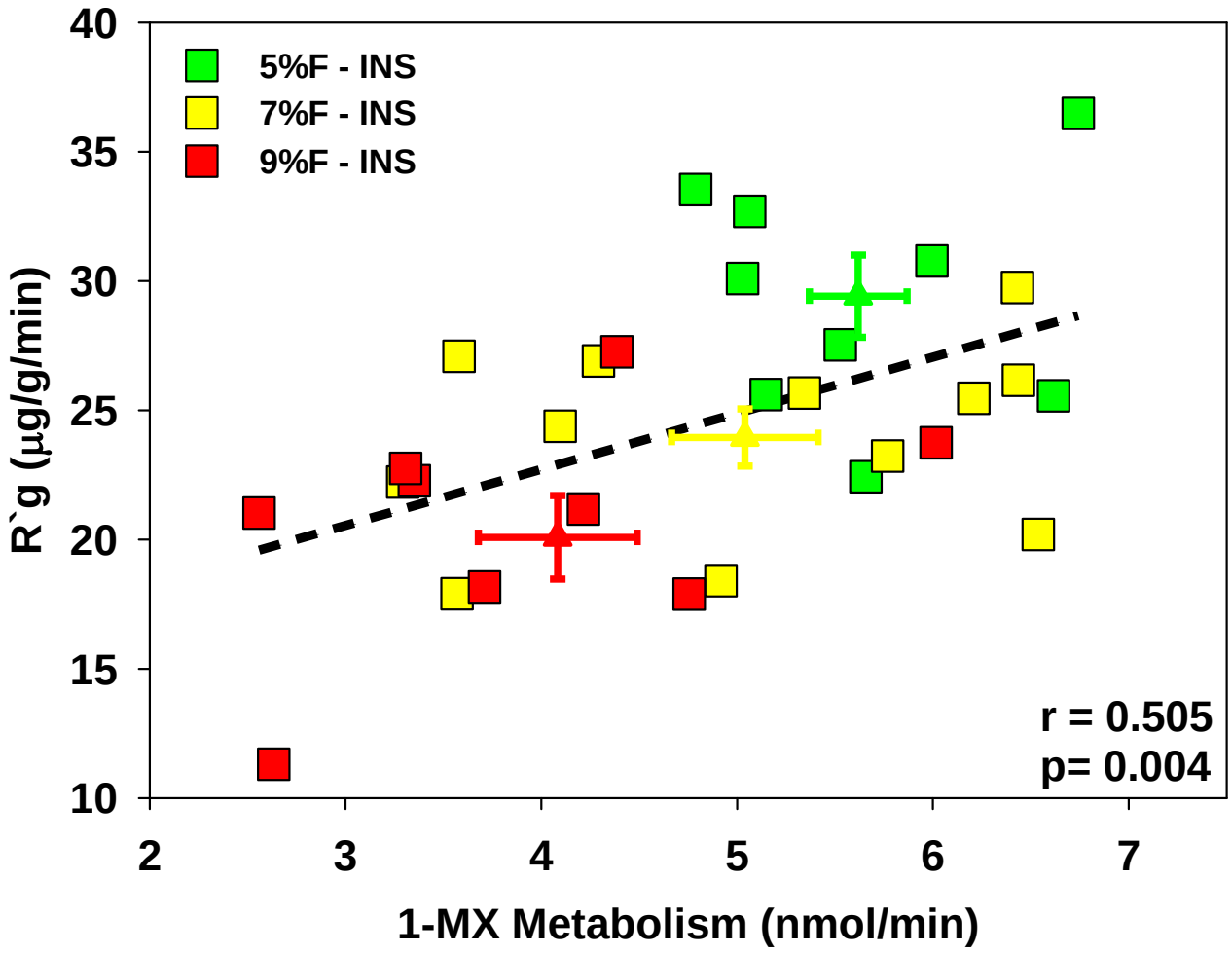

Figure 7.7. Relationship between insulin-mediated 1-MX metabolism and R`g in muscle *in vivo*.

Relationship between R`g and 1-MX metabolism in 5%F, 7%F and 9%F rats following 2hr insulin clamp. Individual values are shown for n = 30 rats. The group means ± SE are shown for 5%F (▲), 7%F (▲) and 9%F (▲) fed rats.

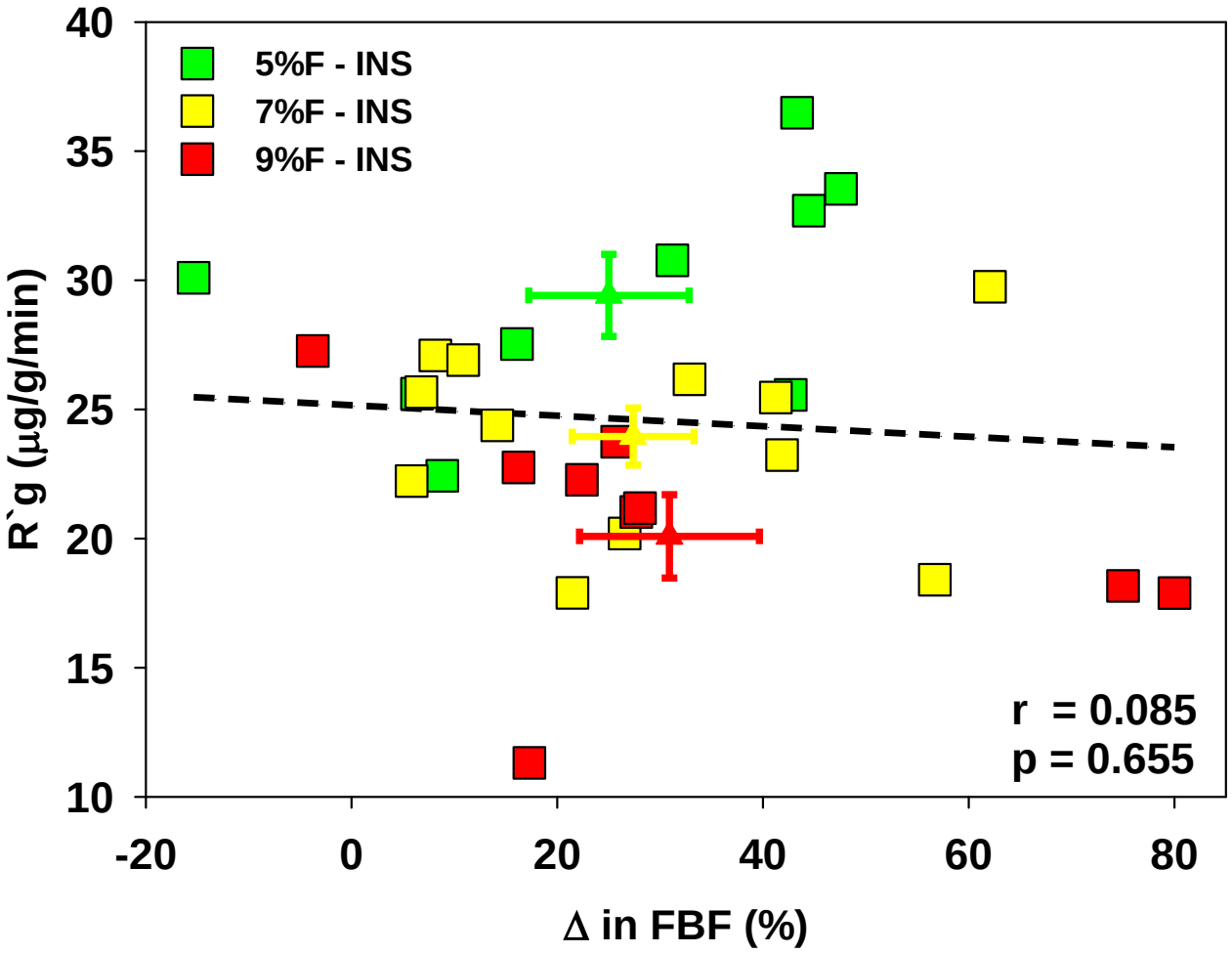

Figure 7.8. Relationship between insulin-mediated change in FBF and R`g in muscle *in vivo*.

Relationship between R`g and 1-MX metabolism in 5%F, 7%F and 9%F rats following 2hr insulin clamp. Individual values are shown for n = 30 rats. The group means ± SE are shown for 5%F (▲), 7%F (▲) and 9%F (▲) fed rats.

7.3.6. Effects of 9%F diet on muscle glucose uptake in the perfused rat hindleg preparation

In order to determine whether 9%F fed rats developed direct myocyte insulin resistance, insulin-mediated glucose uptake was determined using the perfused rat hindleg preparation. This preparation allows for assessment of insulin-mediated glucose uptake without the contribution of other factors such as the vascular actions of insulin.

No differences in muscle glucose uptake (Fig. 7.9; R`g) were detected between 5%F and 9%F groups following either saline or insulin infusion. In the 5%F group, 15nM insulin increased R`g by approximately 7 fold compared to saline (5.3 ± 0.7 vs. 35.2 ± 4.7 mg/g/min; $p<0.001$). Insulin increased R`g to a similar extent in the 9%F group compared to saline (6.5 ± 1.0 vs. 35.2 ± 3.0 mg/g/min; $p<0.001$). No difference in R`g was detected following either saline ($p=0.796$) or insulin ($p=0.987$) infusion between 5%F and 9%F groups.

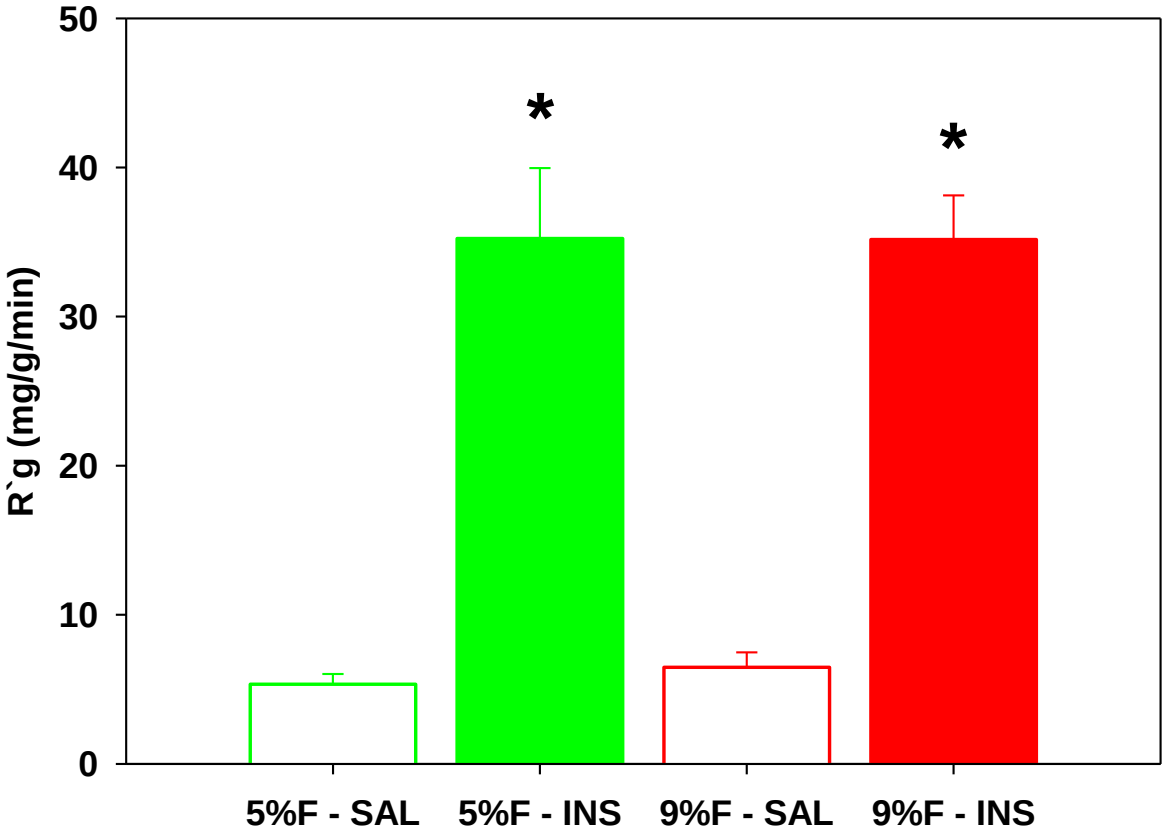

Figure 7.9. Muscle glucose uptake in the isolated perfused rat hindleg.

Muscle glucose uptake (R`g) in the constant flow perfused hindleg of saline and insulin (15nM) treated 5%F and 9%F rats. Data are mean ± SEM for n=6-8 rats in each group. * Significantly different ($p<0.001$) from respective saline using two-way ANOVA.

7.3.7. Effects of 9%F diet on AngII-mediated vasoconstriction in the perfused rat hindleg preparation

The responsiveness of skeletal muscle vasculature to AngII was assessed in 5%F versus 9%F rats using the constant flow perfused rat hindleg preparation. The basal perfusion pressure was not different between 5%F and 9%F fed rats (33.1 ± 0.3 vs. 32.8 ± 1.2 mmHg, p=0.841). The time course for the AngII dose curve is shown in Fig. 7.10. AngII increased perfusion pressure dose-dependently in the hindlegs of both 5%F and 9%F fed rats. Perfusion pressure in both groups peaked approximately 4 min following the initiation of each dose of AngII. For this reason the perfusion pressure developed at 4 min was chosen to compare AngII mediated constriction and is shown in Fig. 7.11.

Infusion of AngII increased perfusion pressure in a dose-dependent fashion in both 5%F and 9%F groups. A significant increase in perfusion pressure from basal was detected following infusion of 3nM AngII (12.5 ± 2.5 mmHg, $p<0.001$) in the 9%F group and further increased following 10nM infusion (22.7 ± 3.5 mmHg, $p<0.001$). Similarly, perfusion pressure increased significantly following infusion of 3nM AngII (14.5 ± 1.9 mmHg, $p<0.001$) in the 9%F group and further increased following 10nM infusion (25.9 ± 2.4 mmHg, $p<0.001$). However, no difference between 5%F and 9%F groups was detected during either 3.0nM (p=0.387) or 10nM (p=0.181) AngII.

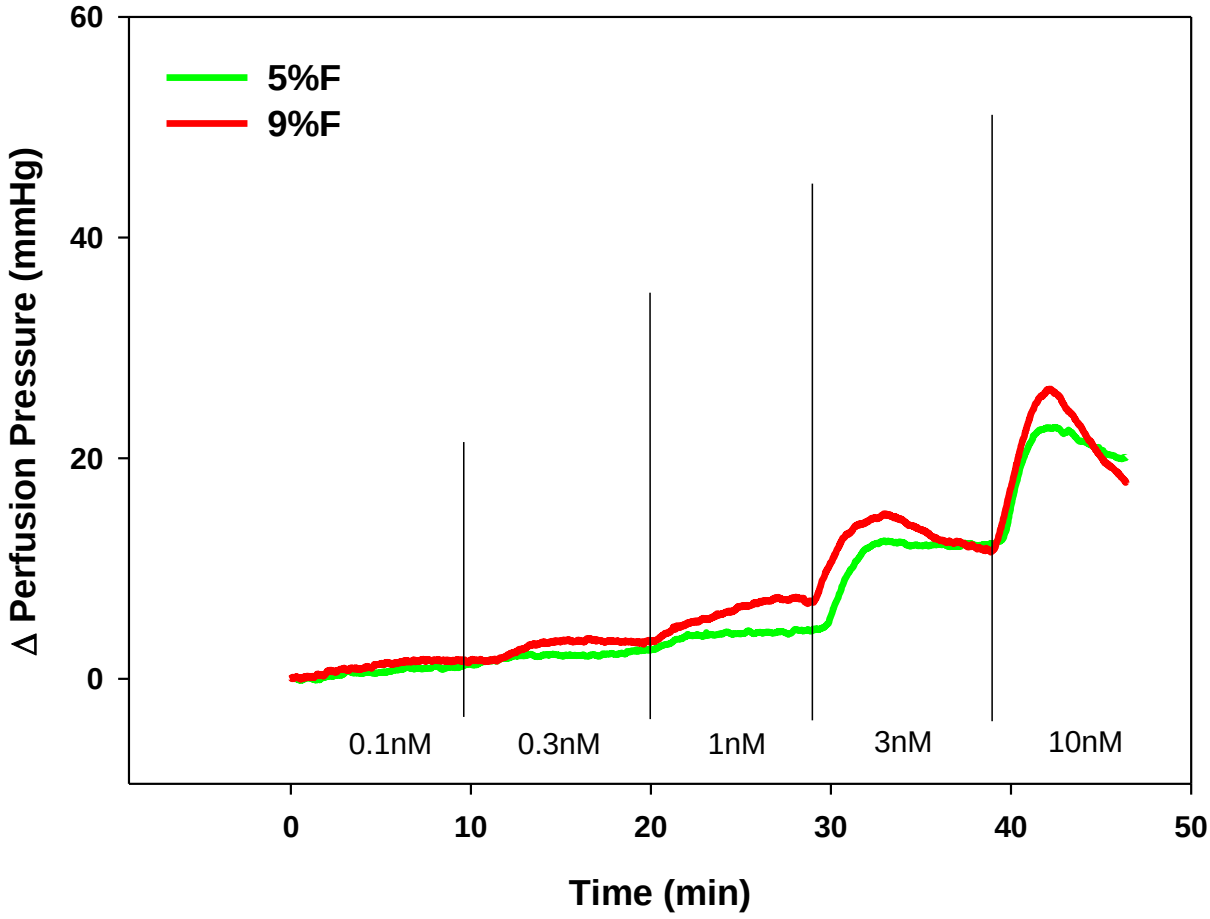

Figure 7.10. Time course of changes in perfusion pressure in response to AngII dose curve in perfused hindlegs of rats fed 5%F or 9%F diets.

Time course of AngII mediated changes in perfusion pressure with increasing dose of AngII. AngII concentration increased in 10 min step-wise increments as indicated. Data are means for n=5 rats in each group. SE bars were omitted for clarity.

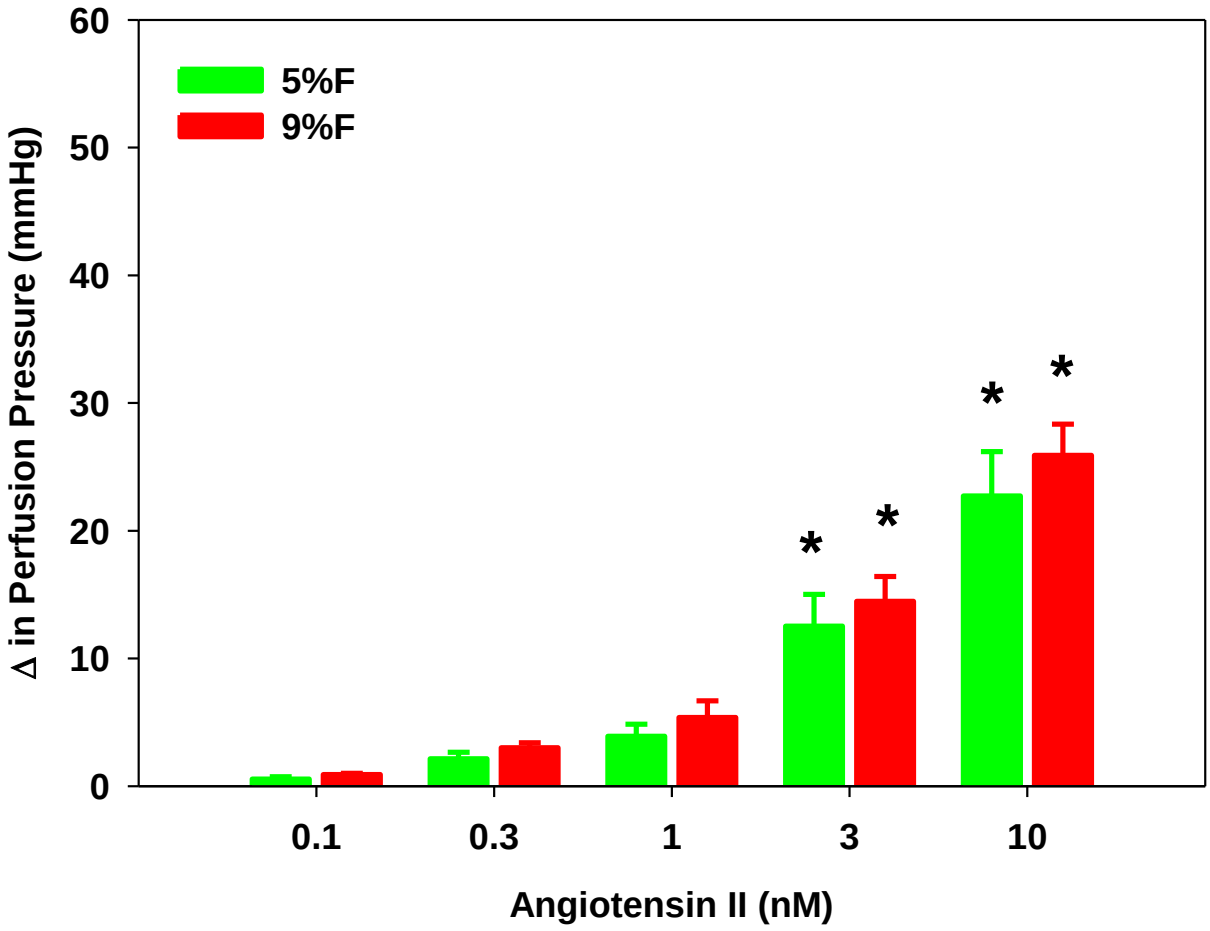

Figure 7.11. Maximum perfusion pressure changes in response to AngII dose curve in perfused hindlegs of rats fed 5%F or 9%F diets.

The maximum perfusion pressure obtained for each dose of AngII in perfused hindlegs of 5%F and 9%F fed rats. Data are means ± SEM for n=5 rats. * Significantly different ($p<0.05$) from the respective basal within the same dietary group using two-way repeated measures ANOVA.

7.4. Discussion

The present study demonstrated that moderate increases in dietary fat (from 4.8 to 9% wt./wt.) caused whole body and microvascular insulin resistance, but not liver insulin resistance. Importantly, this animal model does not display overt myocyte insulin resistance as demonstrated by normal insulin-stimulated muscle glucose uptake when assessed using the isolated perfused rat hindleg preparation, where insulin and glucose is delivered to the muscle independent of changes in muscle blood flow. Because this animal model has markedly impaired insulin-mediated microvascular recruitment *in vivo*, this consequently led to impairment of insulin-stimulated glucose uptake in skeletal muscle due to impaired delivery of insulin and glucose to the myocyte. Thus, this is the first animal model that displays a microvascular defect in skeletal muscle that directly contributes to muscle insulin resistance *in vivo*. Therefore, the 9%F model represents one of the earliest events in the aetiology of insulin resistance and demonstrates that microvascular insulin resistance can occur prior to myocyte insulin resistance.

The data from the present study demonstrates for the first time that feeding rats a moderately elevated fat diet for 3-4 weeks induces insulin resistance. These findings are consistent with previous studies showing reduced insulin sensitivity following fat feeding of rats [221, 223, 227, 347]. Storlien and colleagues (1986) demonstrated that high fat feeding of rats for 4 weeks induced widespread *in vivo* insulin resistance with approximately a 50% decrease in GIR [221]. More recently St-Pierre and colleagues (2010) reported that feeding rats a high fat diet resulted in approximately a 40% decrease in GIR at two different doses of insulin [227]. However, both these previous studies used diets containing 36% fat (wt./wt.) to induce insulin resistance. The current study is the first to demonstrate that only moderate increases in dietary fat, which may represent a more physiologically relevant increase, are sufficient to reduce insulin sensitivity *in vivo*.

High fat feeding has also been associated with the development of liver insulin resistance [221, 227, 347]. Kraegen and colleagues (1991) reported that liver insulin resistance developed early (3 days of 36% fat feeding), and preceded muscle insulin resistance following high fat feeding of rats [347]. These data are in contrast to the data of the

current study where feeding rats a 9%F diet did not affect insulin's ability to suppress liver glucose output. This discrepancy may be due to the difference in the level of dietary fat, the level of hyperinsulinaemia used to determine insulin sensitivity, or a combination of these factors. Indeed, the GIR required to maintain euglycaemia was only 12% lower compared to the control diet in the current study, while the other studies reported approximately a 50% decrease in GIR following high fat intervention [221, 227, 347]. Therefore, it would not be unreasonable to assume that the 9%F fed rat in the current study is a model of less severe insulin resistance compared to the previous studies where 36% fat was used [221, 227, 347], and therefore may not exhibit hepatic insulin resistance. Thus, the current study highlights the possibility that liver insulin resistance may occur later than muscle insulin resistance.

Another point of difference between the current and previous studies is the concentration of insulin infused during the clamp procedure. Insulin was infused at a rate of 10mU/kg/min in the present study to obtain a plasma concentration of approximately 1200pmol/L. This concentration of insulin was almost double the previous studies where a final concentration reached was approximately 700pmol/L [221, 347]. Therefore, it is possible that the insulin concentration used in the current study may be sufficiently high to overcome and thus mask any negative effects of the 9%F diet on liver insulin sensitivity. Indeed, St-Pierre and colleagues (2010) demonstrated that in the 4 week high fat fed rat (36% fat wt./wt.) liver insulin resistance was more apparent following 3mU/kg/min (~600pmol/L) infusion of insulin compared to 10mU/kg/min (~1200pmol/L) infusion of insulin [227]. Whether a lower infusion rate of insulin, such as 3mU/kg/min, would yield different results regarding liver insulin sensitivity in 9%F fed rats is not known.

Nevertheless, the present study demonstrates that insulin-mediated microvascular recruitment and *in vivo* muscle glucose uptake were significantly attenuated in the 9%F fed rats compared to the 5%F fed controls. However, the 9%F diet did not affect insulin-mediated muscle glucose uptake when assessed *in vitro* using the perfused rat hindleg preparation. Together, these data suggest that the development of muscle insulin resistance *in vivo*, following 9%F feeding, is due to a loss of insulin-mediated

microvascular recruitment rather than myocyte insulin resistance. Furthermore, the data also imply that insulin-mediated microvascular recruitment is highly sensitive to changes in dietary fat and that insulin-mediated microvascular recruitment correlates strongly with insulin-mediated muscle glucose uptake *in vivo*. Thus, the current study demonstrates for the first time that moderate increases in dietary fat can negatively affect insulin-mediated microvascular recruitment, which in turn is associated with a reduction in insulin-mediated muscle glucose uptake *in vivo*. Importantly, these data indicate that the development of skeletal muscle insulin resistance *in vivo* can be the result of a purely microvascular defect in muscle.

Accumulating evidence has implicated that defects in insulin-mediated microvascular recruitment contribute to the development of myocyte insulin resistance *in vivo*. Acutely, infusions of serotonin [167], TNFα [177], Intralipid [195], L-NAME [100] and glucosamine [208] in otherwise healthy animals have all been shown to attenuate insulin-mediated microvascular recruitment and significantly reduce insulin-mediated muscle glucose uptake. Importantly these effects on muscle insulin sensitivity *in vivo* appear to be independent of myocyte insulin resistance since most [89, 126, 178], but not all [206] of these interventions do not induce myocyte insulin resistance. Chronic models of insulin resistance including the high fat fed rat (36% fat wt./wt.) [227], OZ rat [242] and the HS fed rat (chapter 3) also display marked attenuation in insulin-mediated microvascular recruitment and insulin-mediated muscle glucose uptake, however, these have also been associated with the development of myocyte insulin resistance [197, 327, 397]. Therefore, the data from the current study, while consistent with these previous reports regarding microvascular recruitment and muscle glucose uptake *in vivo*, demonstrates for the first time that microvascular defects in skeletal muscle can directly impair insulin-mediated glucose disposal.

Long-term high fat (≥20% wt./wt.) feeding has previously been reported to induce insulin resistance in both microvasculature and skeletal muscle *in vivo* and to cause direct myocyte insulin resistance when assessed *in vitro* [197, 227]. The mechanisms responsible for the development of insulin resistance in this model have not been fully elucidated. However, some reports have identified increased accumulation of lipids in

tissues and elevated circulating FFA concentrations may contribute to development of high fat-induced insulin resistance [187, 189, 190, 197, 407, 408]. In muscle, increased circulating FFAs results in increased formation and accumulation of FFA metabolites such as fatty acyl Co-A, ceramides and diacylglycerol which can inhibit the insulin signalling pathway and thus reduce insulin sensitivity [187, 190, 408, 409]. As well as inhibiting insulin signalling in muscle these FFA metabolites have also been reported to inhibit insulin signalling in endothelial cells [407]. For this reason increased circulating FFA concentrations have also been implicated in the development of endothelial dysfunction in humans and animals and thus may contribute to the development of insulin resistance [182, 193, 407]. Since the 9%F diet increased FFA concentrations in the current study, a mediating factor in the development of microvascular insulin resistance may be increased FFAs.

Indeed, Clerk and colleagues (2002) demonstrated that increasing the FFA concentration by infusing Intralipid for 4 hrs in rats attenuated insulin-mediated microvascular recruitment and reduced insulin-mediated muscle glucose uptake *in vivo* [195]. Similar results have been found in healthy human subjects following FFA infusion [196]. The findings of the current study are consistent with these previous reports since 9%F fed rats exhibited approximately a 50% increase in circulating FFAs and a 38% decrease in insulin-mediated microvascular recruitment compared to 5%F fed rats. A recent report by Han and colleagues (2009) also reported that acute FFA-induced myocyte insulin resistance could be ameliorated following FFA washout *in vitro* [197]. Similar data were obtained in the present study when insulin-mediated muscle glucose uptake was assessed using the perfused hindleg preparation. Taken together, these results indicate that the microvascular insulin resistance in the 9%F fed rat may be mediated by increased circulating FFAs, which in turn directly alter microvascular function.

It has previously been reported that high fat diets may result in the up-regulation of the RAS, specifically increased sensitivity to AngII [232, 410, 411], and thus may contribute to the development of insulin resistance [163, 285, 412]. Additionally, vascular sensitivity to AngII-mediated constriction is reportedly enhanced following 4 week high fat (36% fat wt./wt.) feeding and is associated with increased oxidative stress [232, 410].

However, feeding rats a 9%F diet for 3-4 weeks did not enhance vascular AngII sensitivity compared to 5%F fed rats in the present study. The discrepancies between the previous and current studies are most likely due to the difference in the amount of dietary fat. Both previous studies used a 4 week intervention with 36% fat diet (wt./wt.) to induce insulin resistance and the rats displayed significant increases in body weight, fat mass and blood glucose and insulin concentration [232, 410]. In contrast the rats in the current study only displayed mild hyperinsulinaemia. Moreover, local infusion of AngII into one leg of 9%F fed rats (chapter 4 of this thesis) did not significantly affect insulin-mediated microvascular recruitment or glucose uptake when compared to the control leg. Thus, it would be reasonable to assume that the 9%F fed rat does not display altered vascular AngII activity/sensitivity. Therefore, the development of insulin resistance in the 9%F is most likely not associated with increased RAS activity in the vasculature. Interestingly, however, Watanabe and colleagues (2005) demonstrated that attenuation of either ACE activity or the AT1R prevented acute FFA-induced endothelial dysfunction [198]. Since no evidence of increased vascular AngII activity was detected in the present study, it is unlikely that treating the 9%F fed rats with an ACE inhibitor, such as quinapril, would improve insulin sensitivity.

7.4.5. Conclusions

This is the first study to demonstrate that moderately increasing dietary fat (from 4.8% to 9% fat) results in the development of whole body and skeletal muscle insulin resistance *in vivo*. The 9%F fed rat displayed no signs of liver or myocyte insulin resistance and only exhibited attenuation of insulin-mediated microvascular recruitment. Insulin-stimulated microvascular recruitment was found to be highly sensitive to changes in dietary fat and correlated strongly with muscle glucose uptake. The development of insulin resistance in the 9%F fed rat may be mediated by increased circulating FFA concentration but does not appear to have increased vascular RAS activity. The data from the current study are important since it for the first time position microvascular insulin resistance as an early defect that contributes to the development of muscle insulin resistance following relatively small increases in dietary fat.

CHAPTER 8

DISCUSSION

8.1. Key findings

The main hypothesis tested in the current thesis was that increased AngII-mediated vasoconstriction via the AT1R is detrimental to insulin-mediated microvascular recruitment in muscle and contributes to the development of skeletal muscle insulin resistance. To assess the contribution of increased AT1R activity on insulin action in muscle, the HS fed rat model was studied. The data from the present thesis demonstrates that HS fed rats exhibit significant attenuation of insulin-mediated microvascular action in muscle and this was associated with a marked increase in vascular sensitivity to AngII-mediated constriction. In addition, the data from chapter 4 demonstrated that local infusion of AngII into hindleg vasculature of HS fed rats resulted in further attenuation of insulin-mediated microvascular recruitment in skeletal muscle. Conversely, quinapril treatment (which has been previously reported to reduce both AngII and AT1R) significantly improved insulin sensitivity in the HS fed rat and prevented the decrease in insulin-mediated microvascular recruitment. This was associated with reduced vascular AngII sensitivity. However, quinapril treatment (at the same dose and length of time as used in the HS fed rats) of type 2 diabetic OZ rats did not improve insulin's metabolic or vascular actions.

An unexpected finding of the current thesis was that moderate increases in dietary fat (~ 2-fold) induce muscle insulin resistance with origins that are purely microvascular. Collectively, the data from the current thesis reinforce the important link between microvascular recruitment and insulin action in skeletal muscle, and for the first time positions impairment of microvascular recruitment as an early event that can directly contribute to the development of muscle insulin resistance. However, given the data in chapter 7, this does not appear to be dependent on increased vascular AngII sensitivity and suggests that the origin microvascular insulin resistance may be multifactorial.

8.2. General discussion

Ogihara and colleagues (2001) previously demonstrated that feeding rats a HS diet results in the development of whole body and skeletal muscle insulin resistance [327].

The findings of chapter 3 of the current thesis are consistent with this previous report. Indeed, HS fed rats developed whole body and skeletal muscle insulin resistance. However, this is the first study to demonstrate that HS feeding also attenuates insulin-mediated microvascular recruitment in muscle. Therefore the findings of chapter 4 reinforce the notion that defects in insulin's ability to increase microvascular perfusion in muscle contribute to the development of muscle insulin resistance HS fed rats. What is not clear from this study is whether direct myocyte insulin resistance also accompanies the defects in microvascular recruitment. Other investigators have shown that incubated muscles from HS fed rats exhibit reduced insulin-mediated muscle glucose uptake [327]. Therefore, whether the reduced insulin-mediated muscle glucose uptake *in vivo* in the present study is also a result of direct myocyte insulin resistance cannot be distinguished given the current data. However, since previous reports have demonstrated that acutely blocking insulin-mediated microvascular recruitment also reduces muscle glucose uptake by 40-50% [100, 167], it would be reasonable to conclude that the muscle insulin resistance *in vivo* can at least in part be accounted for by impairments in microvascular perfusion.

There is considerable evidence in the literature that the HS-induced insulin resistance may be partly attributable to up-regulation of AngII activity. Specifically, increased AT1R and/or decreased AT2R activity in resistance arteries of various tissues including skeletal muscle [163, 330, 338, 344]. The data obtained in chapter 4 of the current thesis would support these previous reports. Using the perfused rat hindleg preparation, HS fed rats were shown to exhibit markedly enhanced AngII sensitivity. Whilst AT1R levels in the vasculature of HS fed rats were not directly assessed in the present thesis, the marked increase in AngII-mediated vasoconstriction is indicative of increased AngII-stimulation of the AT1R. Thus, increased AT1R activity in the muscle vasculature may contribute to the attenuation of insulin's vascular actions in skeletal muscle.

Local AngII infusion into the hindleg vasculature of HS fed rats resulted in further attenuation of insulin-mediated microvascular recruitment and muscle glucose uptake. Interestingly, however, local AngII infusion in CTRL fed animals did not affect insulin's vascular or metabolic actions in skeletal muscle. Thus, these data suggest that acute

AngII infusion may only be detrimental to insulin's actions in states where the AT1R is increased and/or the AT2R is decreased (HS fed rat) and not when the expression of these receptors is normal (CTRL fed rat). This conclusion is supported by recent reports in which Chai and colleagues demonstrated that insulin-mediated microvascular perfusion of muscle can be differentially regulated by inhibition of either the AT1R (to increased insulin-mediated microvascular perfusion) or the AT2R (to decrease insulin-mediated microvascular perfusion) in skeletal muscle [108, 109]. Taken together, attenuation of insulin-mediated microvascular recruitment in muscle in HS fed rats may be the results of increased AngII activity via the AT1R.

The mechanisms involved in AngII-mediated attenuation of insulin's vascular actions in HS fed rats were not directly investigated in the present thesis. However, various other studies have demonstrated that AngII, acting via the AT1R, can directly inhibit the IRS/PI3K/Akt insulin signalling pathway in both vascular endothelial cells and myocytes [199, 201, 202, 294, 295]. Additionally, AngII has also been reported to increase oxidative stress in the vascular endothelium through increased NADPH-mediated ROS production [200, 291]. Thus, the increased vascular activity of AngII via the AT1R in HS fed rats may inhibit insulin's vascular actions in skeletal muscle by attenuating either endothelial cell insulin signalling or by increasing ROS production in the endothelium, or a combination of these factors. Furthermore, there is also the possibility that AngII may increase the production of other factors that can negatively influence insulin actions such as TNFα [291], interleukin-6 [297] and ET-1 [413]. However, at this time these are only speculative mechanisms and further work is required to elucidate which of these may be involved in the pathogenesis of AngII-mediated microvascular insulin resistance.

Importantly, however, the results in chapter 5 of this thesis demonstrated that inhibition of AngII activity using quinapril almost completely corrected the vascular and metabolic insulin resistance in HS fed rats. Furthermore, quinapril treatment reduced the sensitivity of the skeletal muscle vasculature to AngII in HS fed rats. Thus, the effects of quinapril to improve insulin's vascular actions in muscle may in part be the result of decreased AngII activity. However, while attenuation of insulin-mediated microvascular recruitment was almost completely improved following quinapril treatment of HS fed

rats, it cannot be determined that this directly led to improved insulin-mediated muscle glucose uptake. Indeed, other studies have found that ACE inhibitors and ARBs can directly improve insulin-mediated muscle glucose uptake in vitro [254, 358]. Therefore, determining the effects on myocyte insulin sensitivity would be essential in distinguishing between the vascular and metabolic insulin sensitisation following ACE inhibition in HS fed rats as observed in the present thesis.

In contrast to the HS fed rats, treatment of OZ rats with quinapril did not improve insulin sensitivity. The reasons for the discrepancies between these studies are not clear at this time. However, the vast differences between the two animal models themselves may be a contributing factor. While the OZ rats exhibit a number of the symptoms of already developed T2D such as obesity, severe hyperinsulinaemia and hyperglycaemia, the HS fed rats exhibit none of these traits. Therefore, these data would suggest that ACE inhibition may not be an appropriate target for improving muscle insulin sensitivity in models of well-established T2D. Nevertheless, ACE inhibition may be effective at relatively early stages of insulin resistance (such as the HS fed rat) where the microvascular defect may impair delivery of insulin and glucose to the myocyte, without the presence of overt myocyte insulin resistance.

The results obtained in chapter 7 of this thesis demonstrate for the first time that moderately increasing dietary fat (by approximately 2-fold) results in the development of whole body and skeletal muscle insulin resistance *in vivo*. The 9%F model did not display hepatic or myocyte insulin resistance, but exhibited marked attenuation of insulin-mediated microvascular recruitment in muscle. Furthermore, insulin-stimulated microvascular recruitment was found to be highly sensitive to changes in dietary fat and correlated positively with insulin-mediated muscle glucose uptake *in vivo*. Together, these data suggest that the development of muscle insulin resistance *in vivo*, following 9%F feeding, is due to a loss of insulin-mediated microvascular recruitment rather than myocyte insulin resistance. These data position microvascular insulin resistance as an early defect that directly contributes to the development of muscle insulin resistance following relatively small increases in dietary fat.

A number of previous studies have demonstrated that increasing plasma FFA levels alone is sufficient to induce microvascular insulin resistance in humans and animal models [195, 196, 414]. Since the 9%F fed rats exhibited approximately a 50% increase in fasting FFA compared to the 5%F fed rats, it would be reasonable to conclude that increased circulating FFA concentrations may contribute to the loss of insulin-mediated microvascular recruitment in the 9%F fed rats. Given that the vascular response to AngII was unaffected following the 9%F diet, it would seem unlikely that increased vascular AngII activity is involved in the development of microvascular insulin resistance in the 9%F fed model. Nevertheless, while increased FFA concentrations may be involved, further work is required to elucidate other potential mechanisms that may contribute to the development of microvascular insulin resistance in the 9%F model.

8.3. Implications

The overarching aim of this thesis was to investigate the effects of increased vascular AngII activity on the development of microvascular insulin resistance and to determine whether inhibition of ACE could correct insulin's microvascular actions in skeletal muscle. The dichotomy of results in the present thesis with regard to the effectiveness of ACE inhibition at augmenting insulin sensitivity *in vivo* may help explain some of the discrepancies regarding RAS inhibition and insulin action in humans. Previous clinical studies have found that ACE inhibitors and ARBs may be effective at improving insulin sensitivity in some [270-272] but not all individuals [280-283]. The discrepancies between these findings may be partly attributable to the underlying cause, and the degree of insulin resistance.

Improvement in insulin sensitivity following RAS inhibition has been reported in insulin resistant individuals with hypertension or cardiovascular disease [270-272]. However whether RAS inhibition is effective during states of insulin resistance and T2D without pre-existing cardiovascular disease appears to be complicated. The current thesis compared the effectiveness of ACE inhibition in two animal models at different stages of

the insulin resistance/T2D spectrum (Fig. 8.1). Quinapril treatment of HS fed rats, which exhibit both insulin resistance and a compromised vascular system, resulted in improved insulin sensitivity in muscle. However, quinapril treatment of OZ rats, a genetic model of established T2D, for the same length of time using the same dose did not improve insulin sensitivity *in vivo*. This finding would support previous clinical studies where RAS inhibition failed to improve insulin sensitivity in individuals with either severe hypertension and T2D [280, 281] or obese, non-hypertensive, insulin resistant subjects [282, 283]. Thus a potential paradigm emerges; inhibiting RAS to improve insulin sensitivity requires a specific time-frame where cardiovascular disease and insulin resistance co-exit, but without overt T2D (Fig. 8.1). Indeed, this would seem logical since a number of investigators have indicated a strong relationship between cardiovascular disease and insulin resistance in humans which has been linked to increased RAS activity [258, 284, 415]. Moreover, given that individuals with T2D exhibit severe metabolic dysfunction in almost all tissues and thus represent a significantly later stage of whole body insulin resistance, inhibition of the RAS alone may simply be insufficient to improve insulin action. Nevertheless, there appears to be a window of opportunity where inhibition of the RAS may be of significant benefit to individuals that exhibit early signs of the metabolic syndrome. The data from this thesis indicates that this may involve improvement in the microvascular actions of insulin in skeletal muscle to augment muscle glucose disposal. However, whether long term RAS inhibition prevents insulin resistance by preserving insulin's microvascular actions has not been previously investigated in humans.

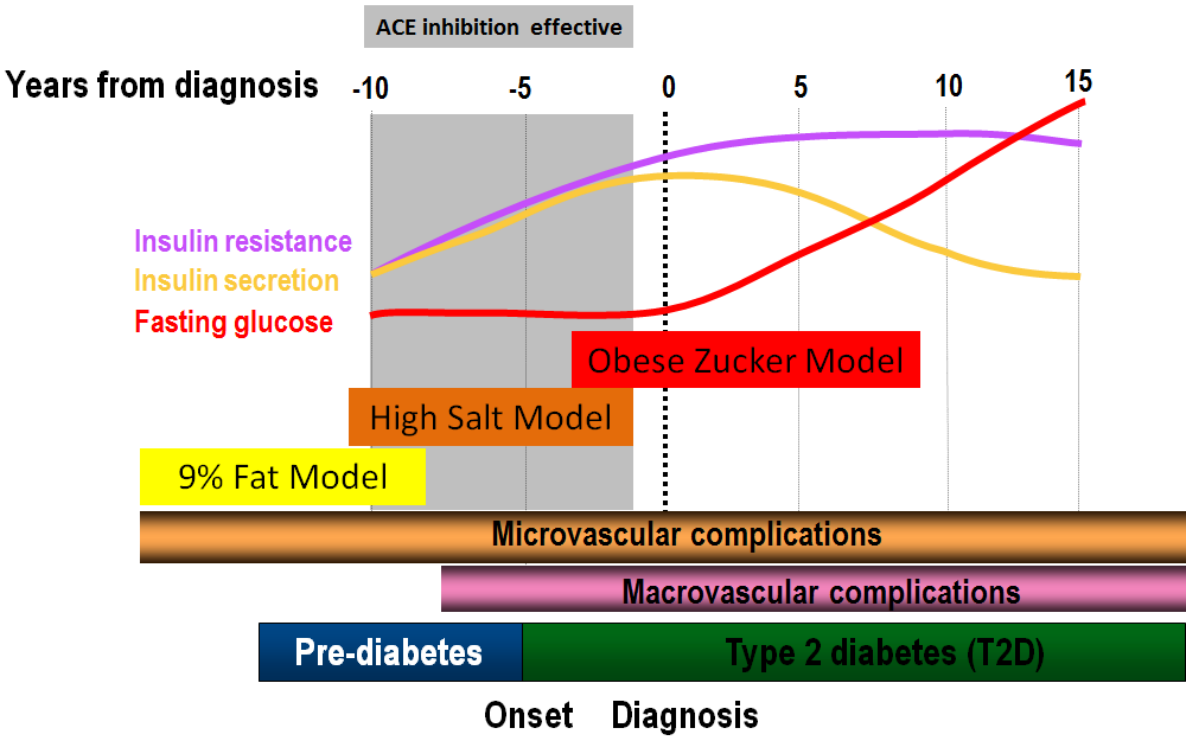

Figure 8.1. Aetiology of insulin resistance and type 2 diabetes in humans.

Figure 8.1 is graphic representation of the progression of insulin resistance leading to the development of T2D in humans. The three models used in the present thesis represent three distinct stages in the pathogenesis of insulin resistance and T2D. Importantly, all three models exhibited attenuation of insulin-mediated microvascular recruitment. The data from chapter 7 for the first time indicate that microvascular complications may be an early defect that can lead to the development of insulin resistance. The shaded area represents a possible time-frame where inhibition of ACE may be of benefit to augmenting insulin resistance. Modified from the CADRE Lecture Kit [143].

8.4. Limitations and future directions

One limitation of the current thesis is that all work regarding insulin's microvascular actions was performed in anaesthetised animals. The relevance of this technique has previously been questioned since others have shown that anaesthesia alone can affect blood flow [416]. While the effects of anaesthesia on insulin-mediated microvascular recruitment in muscle cannot be completely excluded, others have shown that insulin induces similar effects on the microvasculature in muscle of conscious humans [54, 61]. It is also encouraging that in states of insulin resistance, the ability of insulin to increase microvascular perfusion of muscle has been shown to be attenuated similarly in both anaesthetised animals [100, 195, 227] and in conscious humans [98, 196, 219]. Thus, while anaesthesia may affect blood flow *per se* it does not seem to impact greatly on the ability of insulin to increase microvascular perfusion of skeletal muscle.

As with most studies a number of questions have arisen from the current thesis that requires further investigation. While HS fed rats exhibited attenuation of insulin-mediated microvascular recruitment and skeletal muscle glucose uptake *in vivo*, the contribution of microvascular insulin resistance to the reduced insulin-mediated myocyte glucose uptake needs to be characterised. Similarly, whether the improvement in insulin-mediated muscle glucose uptake *in vivo* following quinapril treatment of HS fed rats is the result of improved insulin-mediated microvascular recruitment alone or also an enhancement of myocyte insulin sensitivity is not clear. Whether quinapril treatment of OZ rats from an earlier age, and presumably a less insulin resistant stage could be of benefit to insulin's vascular actions requires investigation. Finally, determining the mechanisms involved in the development of microvascular insulin resistance in the 9%F model may lead to potential therapeutic target aimed at preventing the progression of insulin resistance.

8.5. Conclusions

In order to characterize the involvement of the RAS in the development of microvascular dysfunction in skeletal muscle, the current thesis used three rat models of insulin

resistance that have different degrees of microvascular, myocyte and liver insulin resistance (Fig. 8.1). Firstly the 9%F fed rat, an early model of insulin resistance that exhibits a purely microvascular defect, implicates microvascular dysfunction as an important early event in the progression of insulin resistance. Secondly, the HS fed rat, a model that is characterised by the co-existence of insulin resistance and cardiovascular disease originating from increased vascular RAS activity. Thirdly, the OZ rat, a genetic model of T2D that is characterised by both macro- and microvascular insulin resistance and exhibits severe hyperinsulinaemia and hyperglycaemia. While the origin of the microvascular defect is likely to be different between these models, all three models are characterised by significant attenuation of insulin-mediated microvascular recruitment in skeletal muscle *in vivo*. Furthermore, the 9%F model exhibits what appears to be exclusively microvascular insulin resistance that precedes both myocyte and liver insulin resistance. This for the first time implicates microvascular dysfunction as an initiating factor in the development of muscle insulin resistance. The results from chapters 5 and 6 suggest that inhibition of ACE may only be effective at relatively early stages of insulin resistance (such as the HS fed rat) where the microvascular defect may impair delivery of insulin and glucose to the myocyte, without the presence of overt myocyte insulin resistance (such as the OZ rat). Collectively, the data from the current thesis suggest that while various mechanisms can attenuate insulin's microvascular actions in muscle, impairment of this action of insulin contributes to the pathogenesis of insulin resistance and may represent a new therapeutic target for the prevention of T2D in humans.

CHAPTER 9

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