Hemodynamic and metabolic changes in muscle in relation to insulin action

by

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STATEMENT

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

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ABBREVIATIONS

ANOVA	Analysis of variance
BK	Bradykinin
BP	Blood Pressure
BSA	Bovine serum albumin
CEU	Contrast enhanced ultrasound
CR	Capillary recruitment
cGMP	Cyclic guanosine mono phosphate
cAMP	Cyclic adenosine mono phosphate
CTX	Charybdotoxin
2-DG	2-deoxyglucose
EDHF	Endothelium-dependent hyperpolarization factor
EDL	Extensor Digitorum Longus
ELISA	Enzyme linked immuno-sorbent assay
FBF	Femoral blood flow
FFA	Free fatty acid
GIR	Glucose infusion rate
GLUT	Glucose transporter
GU	Glucose uptake
GLUT4	Insulin-responsive glucose transporter
HGU	Hindleg glucose uptake
HR	Heart rate
HPLC	High performance liquid chromatography
IBT_X	Iberiotoxin
IGF-1	Insulin-like growth factor-1
IMCR	Insulin-mediated capillary recruitment
IMGU	Insulin-mediated glucose uptake
K_{ATP}	ATP-dependent potassium channels
K_{Ca}	Calcium-dependent potassium channels

L-NAME	N^{ω}-L-nitro-argininemethyl ester
L-NNA	N^{G}-nitro-L-arginine
L-NMMA	N-monomethyl-L-arginine
MAP	Mean arterial pressure
MC	Methacholine
1-MU	1-methylurate
1-MX	1-methylxanthine
NIDDM	Non-insulin dependent diabetes mellitus (type 2 diabetes)
NO	Nitric oxide
NOS	Nitric oxide synthase
PDE	Phosphodiesterase
Plan	Plantaris
RG	Red gastrocnemius
R'g	Glucose uptake
R.U.	Resistance units
SEM	Standard error of mean
Sol	Soleus
TEA	Tetraethylammonium chloride
Tib	Tibialis
TNF-α	Tumor necrosis factor-α
Vd	Volume of distribution
VR	Vascular resistance
VSMC	Vascular smooth muscle cell
WG	White gastrocnemius
XO	Xanthine oxidase

PREFACE

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

LIST OF PUBLICATIONS DIRECTLY ARISING FROM THIS THESIS

Mahajan H, Richards SM, Rattigan S, Clark MG, 2003. T-1032, a cyclic GMP phosphodiesterase-5 inhibitor, acutely blocks physiologic insulin-mediated muscle haemodynamic effects and glucose uptake *in vivo*. *Br J Pharmacol*. Dec;140(7):1283-91.

Mahajan H, Richards SM, Rattigan S, Clark MG, 2004. Local methacholine but not bradykinin potentiates insulin-mediated glucose uptake in muscle *in vivo* by augmenting capillary recruitment. *Diabetologia*, in press.

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Mahajan H, Kolka CM, Newman JMB, Rattigan S, Richards SM, Clark MG, Vascular and metabolic effects of methacholine in muscle. In preparation for *J. Physiol*. 2005.

POSTERS AT SCIENTIFIC MEETINGS

Mahajan H, Richards SM, Rattigan S, Clark MG, 2003. T-1032, a cyclic GMP phosphodiesterase inhibitor blocks physiologic insulin-mediated muscle hemodynamic effects and glucose uptake *in vivo*. Diabetes 52 Suppl. 1: A541, Abstract 2347-PO.

Mahajan H, Richards SM, Rattigan S, Clark MG, 2004. Local methacholine but not bradykinin potentiates insulin-mediated capillary recruitment and glucose uptake in muscle *in vivo. Diabetes* 53 Suppl. 2: A369, Abstract 1538-P.

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ABSTRACT

It is widely accepted that insulin increases total blood flow to muscle. This lab has demonstrated in a number of studies that insulin also recruits capillary flow in muscle by an unknown mechanism. This hemodynamic response to insulin is linked to its metabolic effects as it increases the access of glucose and insulin to muscle cells. It is possible that insulin may act on endothelial or vascular smooth muscle cells to release a vasodilator (NO, adenosine, prostaglandins or endothelium-dependent hyperpolarizing factor) causing capillary recruitment.

The aim of this thesis was to look at possible mechanisms underlying insulin's hemodynamic and metabolic action in muscle. This was examined during hyperinsulinemic euglycemic clamps in anesthetized rats. To test the agents affecting insulin action at the local muscle level, a novel technique was developed wherein the epigastric artery (a branch of the femoral artery) was cannulated and test substances were infused into one leg to avoid any systemic effects; the contralateral leg served as control. Femoral artery blood flow and metabolism of exogenously infused 1-methylxanthine (1-MX) as an index of capillary recruitment were measured in both legs for comparison.

There is some evidence that insulin's hemodynamic action on muscle is mediated by nitric oxide-cGMP pathway. T-1032, a phosphodiesterase-5 inhibitor, was infused systemically, to see whether NO-dependent insulin-mediated capillary recruitment in muscle could be enhanced by inhibiting cyclic GMP degradation. T-1032, however, produced an acute insulin resistance. In addition, NO production was enhanced using two endothelium-dependent nitro-vasodilators methacholine and bradykinin. Methacholine infused systemically caused MAP to fall and blood glucose to rise, resulting in a lower GIR. Local infusion of methacholine but not bradykinin in one leg significantly increased capillary recruitment and insulin-mediated glucose uptake.

Furthermore, a NOS inhibitor, L-NAME, infused locally in one leg had no effect on insulin action in muscle. Systemic L-NAME infusion partially blocked the insulin-mediated capillary recruitment without any effect on insulin-mediated glucose uptake. On the other hand, local infusion of calcium-dependent potassium channel (K_{Ca}) blocker TEA in one leg, almost completely blocked insulin's effects on capillary recruitment and attenuated insulin-mediated glucose uptake.

Collectively these findings indicate that the action of insulin on muscle is the net result of a combination of effects. There is evidence for involvement of systemic NO and local K_{Ca} channels in insulin-mediated capillary recruitment. Hence, modulation of either of these components could potentially alter the hemodynamics and metabolism in muscle.

CHAPTER 1

INTRODUCTION

It is well established that insulin has an overall effect to promote cellular uptake and storage of metabolic fuels, but it is the recognition of the key role that insulin plays in vascular physiology and pathophysiology, which has spurred an explosion of investigation of its vascular effects during the past 15 years. There has been an enormous interest in insulin action in muscle due to the fact that it is impaired in diabetes. There is a marked decrease in insulin-mediated glucose uptake in obesity, type II diabetes and in patients with essential hypertension [1].

Interest in the vascular actions of insulin was stimulated by the seminal research of Alain Baron and coworkers which focused on insulin, its effects on total flow and glucose uptake in skeletal muscle. Their observations have been extended by Rattigan and Clark [2] with emphasis on insulin's microvascular action in muscle.

1.1 Importance of microvascular blood flow in muscle

Skeletal muscle comprises 40% of total body mass in man [3] and represents the largest vascular bed of the body- one of the most important hemodynamic circuits in the systemic circulation [4].

The distribution of flow within the capillary network of muscle has been assumed to be regulated by pre-capillary sphincters, whose all-or-none behaviour determines the functional surface area [5]. Lindbom [6] examined the microvasculature of rabbit tenuissimus muscle by intravital microscopy and demonstrated that stepwise reduction of perfusion pressure during exposure to the low oxygen environment resulted in marked compensatory arteriolar dilatation. They suggested that the flow to the muscle capillaries was controlled by the terminal arterioles, although it was also dependent on the resistance

of the more proximal arteriolar segments [6]. The terminal arterioles are reported to have the highest tone *in vivo* [7], have dense sympathetic innervations [8] and can readily close their lumen [7, 9].

Locally, the vasorelaxant substances synthesized by vascular endothelium are endothelium-derived nitric oxide, endothelium-derived hyperpolarizing factor and prostacyclin as well as the potent vasoconstrictors, angiotensin II and endothelin [10]. Imbalance between endothelial mediators has been implicated in type II diabetes, hypertension and atherosclerosis [11].

1.2 Effects of insulin on skeletal muscle blood flow

It is well accepted that insulin causes GLUT4 translocation, thus increasing the facilitated diffusion of glucose into the myocytes. Since this process is the rate limiting step for insulin-stimulated glucose uptake into the muscle cell, it is important that insulin and glucose must be delivered to muscle cells for insulin to interact with insulin receptor on plasma membrane and to increase glucose uptake. To accomplish this, it has been suggested that insulin increases the blood flow to muscle thus improving the substrate delivery (of glucose) as well as increasing its own access. Recent data from Vincent et al [12] suggest that insulin has two separate hemodynamic actions, first, to cause capillary recruitment, and second, to increase the total blood flow to muscle. The increase in capillary recruitment is independent of total flow [13] (discussed in section 1.4).

Baron and his colleagues [14-19] pioneered the concept that insulin acts as a vasodilator and can thereby control access of glucose as well as insulin to skeletal muscle. The total flow increase by insulin was demonstrated in lean, obese and type II diabetics by Baron and coworkers [20]. The dose response curve for insulin action to increase leg blood flow in insulin resistant obese patients showed a marked right shift, with a 2 to 3-fold rise in EC50 (140μU/ml) as compared to leans. A 17-fold rise (700μU/ml) was required to double the leg blood flow in type II diabetic patients. They have shown that insulin decreases vascular resistance in skeletal muscle, that this vasomodulating action is

skeletal muscle-specific, occurs at physiological [insulin] and is impaired in obese, type II diabetic and hypertensive patients.

The effect of glucose on insulin-mediated increases in blood flow in muscle has also been investigated [21]. Vasodilatation was reported to be greater when glucose infusion was given with insulin rather than with insulin alone [22]. In a separate study, Baron et al [23] demonstrated that this change in blood flow is not the effect of hyperglycemia *per se*. By inducing a state of insulinopenia with somatostatin infusion in subjects after an overnight fast, and clamping the glucose concentration at different levels, they demonstrated that hyperglycemia *per se* does not affect the blood flow. In contrast, Veen et al [24] have reported that glucose has a dose-dependent vasodilating effect in forearm, that is probably via a direct action of glucose and is not modified by local hyperinsulinemia.

Other researchers have also shown varying increases in blood flow in response to insulin stimulation [21, 25-27]. This insulin-mediated increase in blood flow has been shown both in humans as well as rats in various experimental situations. However, some groups failed to find a change in flow in response to insulin [28-30]. The mode of infusion may have been important as vasodilatation is greater with systemic than with local infusion of insulin. Cardillo et al 1998 [31], demonstrated in humans that systemic, but not local hyperinsulinemia induced vasodilation in the forearm.

Since the hemodynamic effects of insulin are most pronounced at supra-physiological concentrations of insulin or with physiological insulin administered over extended periods, the relevance of total blood flow increase by physiological insulin in skeletal muscle has remained controversial. It remains to be proved whether this increase in total blood flow has any physiological significance in the context of an increase in muscle glucose uptake. Thus skepticism has remained regarding the importance of blood flow reductions *per se* mediating insulin resistance *in vivo.*

1.3 Insulin-mediated blood flow and glucose uptake

How might total flow affect glucose uptake?

It has been hypothesized that insulin's vasodilatory and metabolic actions are functionally coupled [32]. Glucose uptake is limited either by cellular permeability to glucose or flow, as glucose uptake is calculated based on Fick principle which takes into account both the arterio-venous glucose difference (extraction) and total limb blood flow. As stated by Baron [18] in the fixed capillary area model, in the absence of a permeability barrier, the extraction of glucose will be 100%, with the limiting factor being the flow, meaning that an increase in flow could increase the glucose uptake. If, however, the permeability is low, an increase in the flow will have no effect on the rate of glucose uptake. The true physiological situation is probably intermediate between these two extreme situations. It is apparent that an increase in blood flow increases the velocity (of blood flow), thereby decreasing the transit time of glucose through capillaries so in effect, the extraction of glucose from capillaries to muscle cells is decreased, therefore, an increase in flow will result in a very small increase in glucose uptake.

In vitro studies in isolated perfused rat hindleg have reported glucose delivery to be an important determinant of muscle glucose uptake. Schultz et al [33] demonstrated that increase in glucose uptake occurred both with an increase in blood flow at constant glucose concentrations and with an increase in glucose delivery at constant blood flow. Their study looked at the basal glucose uptake more from delivery point of view as there was no insulin added to the perfusate. Grubb and Snarr [34] demonstrated in perfused rat muscle preparations that both the glucose concentration and the flow rate have influence on the glucose uptake by skeletal muscle at a fixed insulin concentration which gave muscle a near maximal glucose permeability. They have shown that increasing the flow beyond the *in vivo* resting flow at constant arterial glucose resulted in a hyperbolic relationship between blood flow and glucose uptake.

Also in some *in vivo* studies an increase in glucose uptake was found with an increase in flow [35-37]. Buchanan et al [36] reported that the increase in blood flow led to an increase in glucose uptake when angiotensin II (AII) infusion was superimposed on a hyperinsulinemic euglycemic clamp (physiological and supra-physiological insulin concentration) although the insulin concentration was significantly higher in the AII + insulin group than in the insulin or AII group alone.

In contrast to the aforementioned studies, others have observed no association between insulin-mediated increases in total flow and glucose uptake. Yki-Jarvinen and Utriainen [29, 38] showed that insulin-mediated increases in total flow correlated poorly with muscle glucose uptake at different insulin doses. Furthermore, Scherrer [39] also reported a dissociation of the hemodynamic and metabolic actions of insulin. They demonstrated that nitric oxide synthase inhibition with L-NMMA prevented the insulin-induced (1 mU/kg/min) calf vasodilation but had no effect on whole body insulin-stimulated glucose uptake. This dissociation was also observed in studies by Vollenweider et al [40]. They found that an acute reduction in insulin sensitivity induced by fat infusion did not alter the vascular response to insulin (86 μU/min/kg).

Thus it seemed that the increase in blood flow *per se* was not responsible for the increase in glucose uptake *in vivo*.

This is further supported by a number of studies showing that vasodilators that augment total limb blood flow do not enhance insulin action nor do they overcome insulin resistance. For example, it was found that intra-arterial infusion of sodium nitroprusside, an endothelium independent vasodilator, did not increase glucose uptake in either normal or insulin resistant, hypertensive patients despite causing similar increases in blood flow [41, 42]. Similarly, a significant increase in blood flow stimulated by bradykinin had no effect on glucose uptake in either normal or obese patients [21, 43, 44]. Adenosine, another vasodilator that increases limb blood flow, had no effect on glucose uptake in patients with essential hypertension [45].

Despite these contrary reports, Baron and others [46-51] maintain that defects in insulin-mediated increases in blood flow were coupled with impaired glucose uptake in obesity, hypertension, aging, type I and type II diabetes. Baron et al conclude that a defect in insulin-mediated increase in blood flow was responsible for part of the insulin resistance in muscle of type II diabetics [52, 53].

Baron and coworkers [54] reported that an intra-arterial infusion of N-monomethyl-L-arginine (L-NMMA, which blocks NO synthesis in endothelial cells), during a hyperinsulinemic euglycemic clamp (120 or 300 $mU/m^2/min$) resulted in 50% decrease in leg blood flow and a 21% decrease in glucose uptake vs. steady state hyperinsulinemia [54]. On the other hand, superimposed intra-femoral infusion of endothelial-dependent vasodilator methacholine, increased the blood flow by 105% and increased the glucose uptake by 49%.

Sarabi et al [55] demonstrated using intra-brachial infusion of methacholine, a significant increase in blood flow and glucose uptake in hypertensive insulin resistant patients. This increase in glucose uptake was not seen with sodium nitroprusside, which is an endothelium independent vasodilator though it increased the blood flow to same extent as methacholine.

Overall it is clear that not all the vasoactive agents which result in an increase in total blood flow have effects on glucose uptake. Apart from methacholine, other vasodilators are ineffective despite the fact that they increase blood flow to muscle. An increase in total blood flow, as occurred with sodium nitroprusside, adenosine or bradykinin was not sufficient to lead to an increase in glucose uptake. So, why has the increase in blood flow not affected the glucose uptake? Is it the selective distribution of blood flow between metabolically active myocytes and rather quiescent connective tissue and adipose tissue, which is important, or in other words might methacholine dilate vessels in such a way that blood flow is directed towards the more metabolically active skeletal muscle tissue resulting in glucose uptake?

These published discrepancies may be resolved if redistribution of blood flow occurs between the nutritive and non-nutritive routes under the influence of agents such as insulin and methacholine.

1.4 Dissociation of total flow and microvascular recruitment - insulin-mediated capillary recruitment

All the studies quoted above measured the changes in total blood flow but did not assess flow distribution within muscle. At any given time all the capillaries in the muscle are neither closed nor open. Precapillary arterioles undergo vasomotion [56] presumably to prevent an anaerobic state from occurring [18]. By causing capillary recruitment, insulin can increase the volume of distribution for itself and for glucose. Insulin can achieve this by decreasing pre-capillary arteriolar tone, and redistributing blood flow from non-nutritive to nutritive vessels, resulting in more homogenous perfusion by opening previously quiescent capillaries, a process termed "functional capillary recruitment".

Evidence for such capillary recruitment has been demonstrated in a study by Rattigan et al [57]. Blood flow distribution within muscle was determined by measuring the metabolism of blood-borne 1-methylxanthine (1-MX), an exogenous substrate for xanthine oxidase (XO) found in the capillaries. Use of this exogenous reporter substrate, 1-MX as a marker of capillary flow through skeletal muscle *in vivo* is novel. Immuno-histochemical techniques have demonstrated that in rodent and human skeletal muscle, XO is mainly concentrated in capillary endothelial cells, with much less in the endothelium of large arteries, vascular smooth muscle and skeletal myocytes [58, 59]. 1-MX is converted to a single product 1-methylurate (1-MU). The merits of using 1-MX are: it is not vasoactive and does not alter the hemodynamics, is solely metabolized by xanthine oxidase, and, both 1-MX and 1-MU can be readily detected by HPLC [60].

It was shown by utilizing this 1-MX method that insulin (10 mU/min/kg) in addition to increasing blood flow, acted to recruit new capillaries (significantly increased l-MX

metabolism as compared to saline). However, when total blood flow was similarly increased by epinephrine infusion it did not significantly alter l-MX metabolism as compared to saline. Thus the results suggested [57] that it is possible to have increased total blood flow without capillary recruitment.

The dissociation between total flow and capillary recruitment may help to explain the data from human studies quoted in the previous section where a number of vasodilators similarly increased the flow but differentially affected the glucose uptake. The total flow increase in these circumstances would have occurred preferentially in the non-nutritive route. Thus, both total flow and flow distribution need to be quantitated in studies to determine the effects of a vasoactive agent on insulin action.

Other methods which have subsequently been employed to measure capillary recruitment are contrast-enhanced ultrasound and laser Doppler flowmetry. Contrast-enhanced ultrasound, (CEU) is a less invasive method (than the 1-MX method) in which labeled albumin containing microbubbles are infused systemically to identify perfusion through insulin-sensitive muscle tissue. These microbubbles are detected by an ultrasound probe which is positioned over the area of interest. A signal is recorded when a pulse of high-frequency ultrasound waves destroy all the bubbles within the probe field. The signal emitted from their destruction is recorded as video intensity. The area is then replenished with microbubbles from the systemic circulation until the next pulse of ultrasound. The interval between these pulses is progressively prolonged to allow more extensive replenishment of the microbubbles in the microcirculation [61, 62]. The contribution from larger vessels can be eliminated by subtracting the signal obtained at early time points when only the large vessels have re-filled. The residual video intensity is the measure of the microvascular blood volume or capillary perfusion. Using both l-MX and CEU techniques, capillary recruitment by insulin has been demonstrated [63].

1.5 Effect of insulin-mediated capillary recruitment on glucose uptake

By causing capillary recruitment, insulin boosts its own delivery to target tissues and plays an integral part in its main action, the promotion of glucose disposal. Insulin maximizes the glucose uptake by the most efficient coupling of capillary blood flow, myocyte permeability (GLUT4 translocation) and capillary surface area [64].

Studies where insulin-mediated capillary recruitment has been found to be impaired in conjunction with impaired muscle glucose uptake have been documented by our lab. Rattigan and colleagues have shown that α-methyl serotonin, a vasoconstrictor that inhibits insulin-mediated increases in both total flow and capillary recruitment *in vivo* impairs ~50% of insulin-mediated glucose uptake in the same hindlimb muscles [65]. Moreover, tumor necrosis factor-α (TNF-α), which is elevated in various insulin resistant states, completely blocks both the hemodynamic actions of insulin, and approximately 50% of the insulin-mediated glucose uptake [66]. Another such situation is following acute administration of Intralipid® and heparin to raise circulating free fatty acids which has a similar effect [67]. Based on these studies it can be concluded that insulin-mediated capillary recruitment as measured by 1-MX metabolism correlates well with insulin-mediated glucose uptake but shows no significant correlation with total limb flow [65]. These findings support the hypothesis that microvascular action of insulin enhances perfusion of muscle, by redistribution of blood flow from the non-nutritive to nutritive route independently of changes in total flow.

The increase in glucose uptake that takes place is secondary to insulin-induced capillary recruitment. This is supported by the study which used CEU and 1-MX metabolism to show that at physiological insulin concentrations, insulin-mediated capillary recruitment occurs within 10 min and increases tissue perfusion, prior to increases in total blood flow [68]. In addition, [12] insulin-mediated capillary recruitment (5 minutes) preceded insulin's effect to increase glucose uptake.

Thus, the microvascular action of insulin by vasodilatation of preterminal arterioles appears to be important for its metabolic action.

1.6 Nutritive vs. non nutritive routes

Dissociation of total flow from capillary recruitment can possibly occur if we address the notion of two flow routes in muscle.

Work using the isolated, pump-perfused rat hindlimb supports the idea that there are two flow routes in muscle [69-73]. One is termed nutritive because it has the highest potential for nutritive exchange with muscle cells, and may consist of long tortuous capillaries in direct contact with myocytes. The other one is termed non-nutritive for muscle but nourishes the tendon, septa and possibly adjoining adipocytes, and may consist of slightly wider, but shorter capillaries although the exact anatomy of the two beds is unknown [74]. It has been proposed that the non-nutritive route acts as a reservoir which can rapidly redistribute flow to the nutritive route during periods of high metabolic demand, such as exercise and also after insulin stimulation following a meal, where glucose storage can occur in muscle without the need for an immediate increase in bulk blood flow [20].

The agents which increase metabolism in the constant-flow pump-perfused skeletal muscle by vasoconstriction have been termed type A and they include low dose norepinephrine, angiotensin II, vasopressin and low frequency sympathetic nerve stimulation. They are thought to constrict non-nutritive feed arterioles, redirecting flow to the nutritive route and increasing the oxygen consumption. Type A vasoconstrictors require extracellular calcium for their action and can be blocked by calcium channel blockers. In contrast, another group of vasoconstrictors termed Type B, decrease the metabolism by decreasing perfusion of the nutritive route. They include serotonin, high dose norepinephrine and the high frequency sympathetic nervous system [69, 75].

The presence of the non-nutritive flow route may explain why certain vasodilators (adenosine, bradykinin, sodium nitroprusside) increase blood flow without affecting insulin-mediated glucose uptake.

1.7 Possible mechanisms of insulin-mediated capillary recruitment

The mechanism of insulin-mediated capillary recruitment remains unresolved to date. Dilation of fourth order arterioles in response to insulin has been reported [76]. McKay et al [77] have reported an increase in insulin sensitivity (vasodilatation) with decreasing vessel size. There are number of possible mechanisms by which insulin can cause vasodilatation in small arterioles.

First, insulin may act directly on vascular smooth muscle cells (VSMC) to cause vasodilatation via an endothelium independent mechanism. Insulin has been shown to relax the isolated carotid arteries, whether the endothelium is present or not [78]. Insulin can act directly on smooth muscle causing hyperpolarization as described in section 1.12b and decrease cytosolic calcium concentration in aortic smooth muscle cells [79-83]. In patients with insulin resistance increased intracellular calcium has been reported in platelets and vascular smooth muscle cells [84].

Second, it can involve production of another vasoactive molecule possibly NO, which can act in a paracrine manner [85-88]. The source could be endothelial cells, VSMC or skeletal muscle, since all 3 have insulin receptors and a capacity for synthesising NO because NOS is present in all 3 tissues [89-92]. Adenosine may also be involved as Abbink demonstrated [93] in human forearm that insulin-induced vasodilation was mediated by the release of adenosine. Increased production of prostacyclin may also play a role in vascular effects of insulin [94-96].

Third, insulin may cause cellular hyperpolarization by increasing calcium concentration in endothelial cells leading to endothelium-dependent hyperpolarization of underlying smooth muscle cells, inhibiting voltage-activated calcium channels and vasorelaxation.

Fourth, it may result from metabolic vasodilatation [97, 98], analogous to exercise-induced capillary recruitment where increased oxygen demand in muscle myocytes facilitates local blood flow, by releasing molecules such as adenosine, H^+, K^+ or lactate to cause vasodilatation. Here, insulin-mediated glucose uptake might lead to synthesis of a vasodilator.

Fifth, insulin can also cause direct or indirect antagonism of vasoconstrictors produced in endothelium [99, 100].

Sixth, insulin has also been suggested to act by an α–, β-adrenergic mechanism [101, 102].

1.8 Role of Nitric oxide in insulin's action

Nitric oxide (NO) is now accepted to be a major mediator of insulin action to cause arterial smooth muscle relaxation [52, 103, 104]. NO is identified as an endothelium-derived relaxing factor because of the similarities in physical, chemical and physiological characteristics between the endogenous substance and authentic NO [105], and also from the evidence derived from the use of arginine analogues that act as competitive inhibitors of NOS, and from gene-deletion studies [86, 87]. Within muscle, NOS is present in both myocytes (nNOSμ, a variant of nNOS) [106-108] and endothelium (eNOS). However, Zeng [109, 110] demonstrated that insulin receptors are present on endothelial cells and that insulin stimulates phosphorylation and activation of protein kinase B (Akt), which then phosphorylates and activates eNOS [111, 112]. It is still unknown which NOS mediates insulin's action in muscle since both nNOS and eNOS deficient mice are insulin-resistant [113, 114]. However, it is difficult to assess the results of gene-deletion studies since gene-deletion may lead to compensatory developmental changes.

Numerous *in vitro* studies have indicated the involvement of NO in insulin action in different cells. While insulin acts directly on endothelium [109, 110], human VSMC have also been demonstrated to express constitutive NOS, which is activated by insulin to increase cGMP, an effect blocked by L-NMMA [115]. In first order arterioles isolated from rat cremaster muscle, removal of endothelium or N^G-nitro-L-arginine (L-NNA,

inhibitor of NOS) treatment completely abolished insulin-mediated vasodilation [116]. Bertuglia [117] observed that the increase in arteriolar diameter by insulin in hamster cheek pouch microcirculation was significantly reduced by L-NMMA. It has been suggested that insulin can inhibit vascular contraction by acting on vascular endothelium increasing the endothelial calcium and releasing nitric oxide, which decreases smooth muscle calcium [118].

It has been observed that insulin (100nM) stimulation of glucose uptake in human vascular smooth muscle cells was abolished by L-NAME, a NOS inhibitor and by 1H-[1,2,4]oxadiazolo[4,3-a]quinozalin-1-one (ODQ), a selective soluble guanylate cyclase inhibitor [119]. On the other hand, Balon et al reported that NOS inhibition did not diminish 2-DG transport in rat skeletal muscle even by maximally stimulating concentrations of insulin *in vitro* [90].

There is also significant evidence available from *in vivo* studies that insulin's vasodilatory action in muscle is NO-dependent. Steinberg et al [52, 53] demonstrated that acute local NOS inhibition by L-NMMA (16 mg/min) during a euglycemic hyperinsulinemic clamp in healthy humans completely inhibited insulin-mediated vasodilatation and diminished insulin-mediated leg glucose uptake by ~25%. Another group of workers [120] found that systemic L-NAME infusion (30 mg/min/kg) during an insulin clamp (64 mU/kg/min) in rats significantly blunted whole body glucose disposal (-16%) and muscle 2-DG uptake (-30%) but L-NAME had no effect on basal or insulin-stimulated glucose uptake in isolated muscles.

Vincent and coworkers [63] using both 1-MX and contrast enhanced ultrasound reported that acute systemic infusion of L-NAME (50 μg/min/kg) in rats completely inhibited insulin-mediated capillary recruitment and diminished insulin-mediated glucose uptake (insulin clamp- 10 mU/min/kg).

A novel study by Shankar et al [121] demonstrated the effect of acute central inhibition of nitric oxide inhibition by intracerebroventricular infusion of L-NMMA in

rats which induced hyperglycemia, insulin resistance, defective insulin secretion and hypertension. Central L-NMMA reduced the glucose disposal rates by 22% during an insulin clamp (12 mU/min/kg).

In contrast to the above-mentioned studies several other workers did not observe a significant decrease in insulin sensitivity in muscle after NOS inhibition. It was observed by Scherrer et al [39] that in humans the insulin-induced increase in blood flow (1 mU/min/kg) could be abolished by inhibiting NOS synthesis using local infusion of L-NMMA (8μmol/min) without affecting the insulin-mediated glucose uptake. Sartori et al [122] demonstrated that infusion of L-NMMA (50 μg/min/kg) in denervated limb abolished the insulin-mediated vasodilatation, but did not have any detectable effect on whole body glucose uptake (insulin clamp- 1 mU/min/kg). Kohlman [123] found that acute systemic NOS inhibition by L-NAME (200 μg/min/kg) had no effect on glucose disposal during hyperinsulinemic euglycemic clamp in rats. Cardillo et al [31], also did not observe a significant decrease in insulin-mediated forearm glucose uptake (insulin clamp 120 mU/m^2/min) after NOS inhibition by local L-NMMA (1 mg/min).

It has been demonstrated that L-NMMA infusion into human forearm abolished the NO-dependent increase in blood flow in response to local insulin-like growth factor I (IGF-I) without affecting the insulin-like metabolic response of skeletal muscle tissue to IGF-I [124].

On the other hand L-NMMA can also increase insulin sensitivity as acute systemic administration of L-NMMA (3 mg/hr/kg) in humans increased the calf blood flow and increased the whole body glucose uptake thereby increasing insulin sensitivity (1.5 mU/min/kg) [125].

The effect of NOS inhibition has also been addressed in chronic studies [126, 127]. Balon et al [126] reported that chronic NOS inhibition increased peripheral insulin-mediated glucose uptake but blunted the insulin secretion in response to oral and

intravenous glucose tolerance tests. Swislocki et al [127]) did not notice insulin resistance after chronic NOS inhibition.

Thus, the aforementioned studies suggest that the hemodynamic actions of insulin are NO-dependent. On the other hand, an effect of NOS inhibition on insulin-stimulated glucose uptake is debatable. The published studies vary in terms of methodology such as route of administration, type, dose and duration of NOS inhibitor used. Thus, one of the aims of this thesis was to determine whether the systemic and local infusion of L-NAME during an insulin clamp affects insulin's hemodynamic and metabolic actions in muscle and whether they differ.

1.9 Insulin-mediated capillary recruitment by nitric oxide pathway

It has been speculated [39, 115] that the signaling cascade causing capillary recruitment and vasodilatation starts with stimulation of insulin receptors on the surface of endothelial cells of terminal arterioles. This stimulates an intracellular cascade including IRS1/2, PI3 kinase, PKB and phosphorylation of eNOS, which forms NO [128]. Arginine is converted to citrulline when NO is formed [129]. The reaction requires cofactors including tetrahydrobiopterin, calcium calmodulin, flavin adenine nucleotide (FAD), flavin mononucleotide (FMN), molecular O_2 and nicotinamide adenine dinucleotide phosphate (NADPH) as co-substrates (Fig. 1, page 17).

NO thus formed diffuses to neighbouring VSMC [130] to stimulate soluble guanylate cyclase (GC) by interacting with its haem group. GC then catalyzes the synthesis of cGMP from GTP [131]. It has been hypothesized that formation of cGMP leads to a fall in cytoplasmic calcium concentration, by decreasing the calcium influx (this can occur by inhibition of inositol 1,4,5 triphosphate formation, inhibition of protein kinase C activity, activation of myosin light chain phosphatase pathway [132]) or by stimulating protein kinase G which results in phosphorylation of the calcium channel shifting the calcium inside endoplasmic reticulum/sarcoplasmic reticulum or by eliciting membrane hyperpolarization. Some workers have suggested that cGMP may cause a substantial part

of its effect via mechanisms that are independent of changes in calcium concentrations, probably involving endothelium-derived hyperpolarizing factor [118]. How this leads to smooth muscle relaxation is not completely understood [133, 134]. Rybalkin et al [135] have proposed that reduced cytoplasmic calcium leads to dissociation of calcium from calmodulin which in turn dissociates from myosin light chain kinase (MLCK) thus inactivating it. With MLCK inactivated, de-phosphorylated myosin light chain inhibits binding of myosin to actin leading to smooth muscle relaxation [136-138].

cGMP thus formed is hydrolyzed by a specific class of enzymes called phosphodiesterases (PDE) [139].

NITRIC OXIDE –MEDIATED VASODILATION

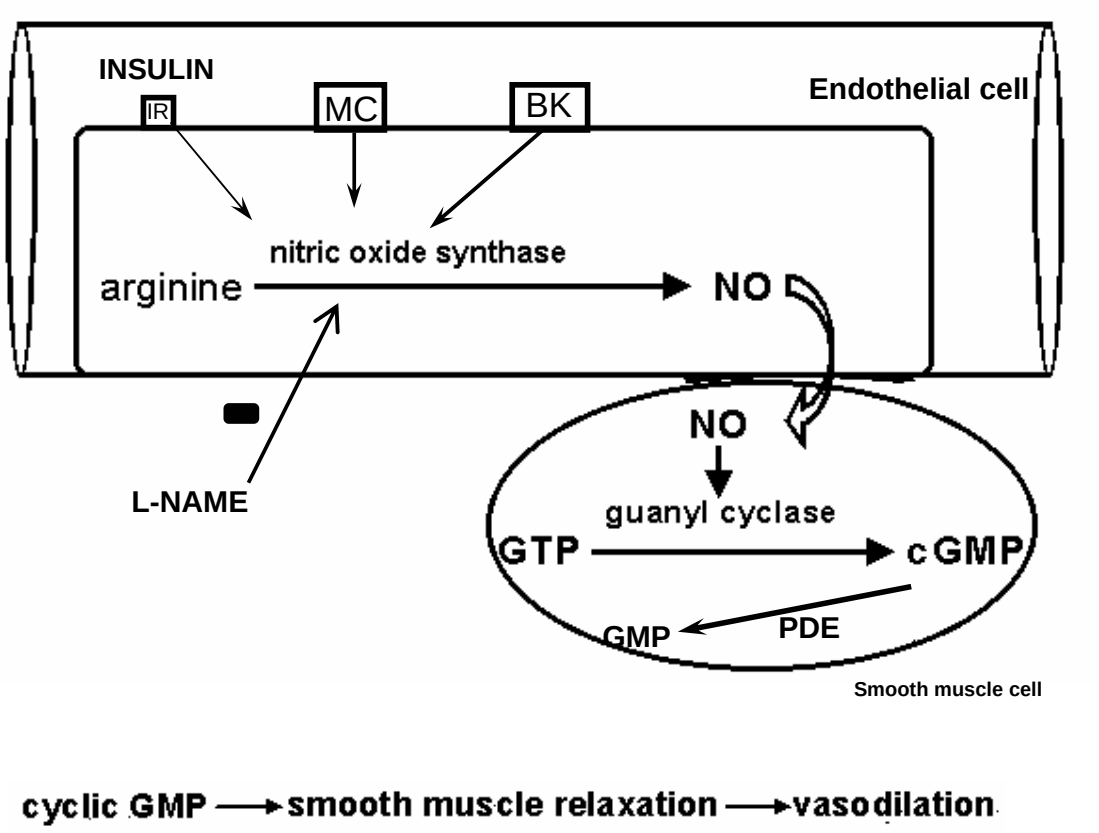


Fig. 1. Proposed mechanism of insulin-mediated vasodilatation.

Insulin binds to insulin receptors on endothelial cells and stimulates an intracellular cascade involving IRS1/2, PI3 kinase, PKB and phosphorylation of eNOS, which forms NO. NO thus formed permeates to neighboring VSMC to stimulate soluble guanylate cyclase (GC). GC then catalyzes the synthesis of cGMP from GTP. cGMP formation leads to a fall in intracellular calcium concentrations causing vasorelaxation and vasodilatation. Also shown is bradykinin (BK) which binds to B_2 receptors, and methacholine (MC) which binds to M_3 receptors [140] on the endothelial cell, both leading to activation of nitric oxide synthase.

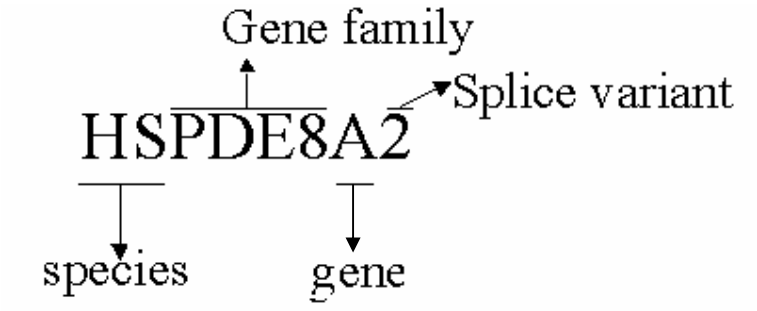
1.10 cGMP Phosphodiesterases

Phosphodiesterases (PDEs) are considered to be the negative regulators of cyclic nucleotide signaling cascades. The major function of PDEs in the cell is to act as homeostatic regulators. They terminate the cyclic nucleotide 2nd messenger signal and therefore regulate the steady state levels of cAMP and cGMP.

PDEs are metallo phospho-hydrolases which specifically cleave 3′, 5′-cyclic phosphate moiety of cAMP and/or cGMP to produce the corresponding nucleotide, AMP/GMP which is converted back to GTP by a kinase. There are ≥ 80 PDE isoforms discovered so far which complicates the issue as to which PDEs are involved in the breakdown of cGMP in smooth muscle of terminal arterioles of skeletal muscle [141]. Out of 11 gene families described, some are specific for cAMP, some for cGMP and some can hydrolyze both. Since only cGMP and not cAMP is the effector molecule in NO pathway and because it is thought that capillary recruitment is mediated by NO-cGMP pathway, an inhibitor which aims at the cGMP hydrolyzing PDEs is likely to enhance insulin action. One of my aims was to see whether the systemic administration of a selective cGMP phosphodiesterase 5 inhibitor, T-1032 enhances insulin action.

1.10a PDE Families

Sequence analysis and low stringency probing has led to the identification of 11 different families of PDE based on amino acid sequence, substrate specificity, endogenous and exogenous regulators and pharmacological properties. The nomenclature of various PDE family members is indicated as follows:



In this new, more systematic nomenclature, a PDE isoform is indicated by an Arabic numeral followed by a capital letter indicating the gene within the family and a 2nd Arabic numeral indicating the splice variant derived from a single gene [142, 143]. Out of the 11 classes of PDEs, PDE5 is specific for cGMP and is considered as the sole cGMP hydrolyzing PDE present in VSMC [144, 145].

1.10b PDE5 family

This family has 2 genes with 3 splice variants each. This family has been called the cGMP binding cGMP-specific family. Both the allosteric and catalytic sites are highly specific for cGMP (Km 1–5 μM). PDE5A1 is abundant in lung, platelets, VSMC, kidney and skeletal muscle. It has been suggested that PDE5 isozymes contain 2 or more tightly bound Zn^{2+} that are involved in catalysis [142]. PDE5 contains 2 homologous allosteric cGMP binding sites which are required for phosphorylation of a serine residue. This phosphorylation by cGMP-dependent protein kinase may cause an increase in catalytic activity of enzyme. PDE5 has been seen as a regulator of cGMP function. It has been suggested that it plays an important part in regulation of pulmonary vascular tone [143]. Dipyridamole and T-1032 (PDE5 antagonists) have been suggested as particularly efficient in decreasing pulmonary vascular resistance [143, 146, 147]. It was suggested by some workers [145] that PDE5 is almost exclusively responsible for hydrolyzing cGMP in rat VSMC and this is the only cGMP-selective PDE in VSMC, but this may be an overstatement. In another study by Mercapite et al, [144] selective inhibition of PDE5 by zaprinast in porcine aortic smooth muscle cells, did not potentiate sodium nitroprusside/atrial natriuretic peptide-induced rise of cGMP. This questioned the widespread opinion that PDE5 exclusively accounts for cGMP hydrolysis in VSMC. In contrast, IBMX at concentrations inhibiting both PDE1 and 5 isozymes, potentiated the rise in cGMP compared with zaprinast alone. The authors suggest that PDE5 is not responsible for all the cGMP hydrolysis in VSMC.

Tables I and II provide a description of all the PDE isoforms (pages 20, 21).

Family name	PDE1	PDE2	PDE3	PDE4	**PDE5**
Known genes	1A 1B 1C	2A	3A 3B	4A4B4C4D	**5A 5B**
Splice variants	9 1 5	3	3 1	2 2 2 2	**3 3**
Descriptive name	Ca/CAM-stimulated	cGMP-stimulated	cGMP-inhibited	cAMP-specific	**cGMP-binding**
Structural information	535aa PDE1A3	941aa PDE2A3	1141aa PDE3A1	647aa PDE4A1	**874aa PDE5A1**
Regulators	Ca/CAM Phosphorylation	cGMP	cGMP insulin	PKA	**PKG**
Substrate	cAMP & cGMP	cAMP & cGMP	cAMP & cGMP	cAMP-specific	**cGMP**
Tissue Expression					
SKM	Y	Y	Y, 3B	Y	**Y**
VSM	Y	Y	Y	Y	**Y**
Others	Brain, heart, olfactory cilia	Adrenal cortex CNS	Platelets, heart Adipose tissue Pancreas	Wide distribution	**Lungs, Platelets Corpus callosum**
Selective inhibitors **(non-selective, mixed, highly selective)**	Vinpocetin Phenothiazines MMPX	EHNA	Cilostamide Cilostazol Milrinone Siguazodan Zardaverine	Rolipram Ro-201724 Etazolate Zardaverine	**T-1032 Sildenafil Zaprinast Dipyridam-ole MBCQ MY5445**
Role of inhibitors	VSM relaxation central actions	Augment inhibition of platelet aggregation	Inotropic Smooth muscle relaxation Platelet aggregation	Airway SM relaxation Inhibition of inflammatory response	**Inhibition of platelet aggregation Viagra®**

Table I showing the tabulated summary of phosphodiesterase classes.

Family name **Known genes** **Splice variants**	PDE6 6A 6B 1 1	PDE7 7A 7B 1 1	PDE8 8A 8B 1 1	PDE9 9A 20	PDE10 10A 14	PDE11 11A 4
Descriptive name **Structural information**	Photoreceptor PDE 860aa PDE6A1	cAMP-specific 482aa PDE7A1	cAMP-specific 713aa PDE8A1	High affinity cGMP-specific 593aa PDE9A1	Dual specificity 779aa PDE10A1	Dual specificity 490aa PDE11A1
Regulators **Substrate**	Light cGMP	? cAMP	? cAMP	? cGMP	? both	? both
Tissue Expression **SKM** **VSM** **Others**	 N N Rod cone photoreceptors	 Y ? T-cells	 Y ? Testis, Liver, thyroid	 Y ? Kidney	 ? ? Testis, Brain	 Y ? Prostate
Selective inhibitors **(non-selective, mixed, highly selective)**	Zaprinast Dipyridamole	?	Zaprinast Dipyridamole	Zaprinast SCH151866	Zaprinast Dipyridamole SCH151866 Sildenafil	Zaprinast Dipyridamole
Role of inhibitors	Modulation of signal transduction	?	?	?	?	?

Table II showing the phosphodiesterase classes.

From tables I and II it is clear that there are more than 80 PDE isoforms described. If capillary recruitment is mediated by NO-cGMP pathway, only the specific inhibitors of cGMP hydrolyzing PDE isozymes will be of special interest. The cGMP hydrolyzing PDE families include 1,2,5,6,9,10 and 11. Out of these PDE6 is found exclusively in eye, which cuts down the PDE isoforms of interest to ~60. Interestingly, PDE families show tissue-specific distribution as reported by Wallis et al [148] with regards to cardiovascular system. The major PDE activity in human cardiac ventricle has been shown to be PDE1 with no detectable PDE5. In contrast human saphenous vein contains PDEs 1, 4 and 5 and human mesenteric artery contains PDEs 1–5. Human pulmonary artery has been shown to contain a high level of PDE5 activity compared to PDE1, while in canine aorta PDE1 activity is more abundant than PDE5. VSMC contains PDEs 1,3,4 and 5 and endothelial cells contain PDEs 2 and 4 [146, 149].

1.10c Vascular effects of cGMP PDE inhibitors

Non-selective PDE inhibitors include theophylline, papaverine, caffeine, IBMX and pentoxifylline that produce vasodilatation by increasing cAMP and cGMP levels. Selective PDE3 inhibitors which are being used are amrinone and milrinone. They are potent vasodilators, and good inotropes. Relatively selective PDE5 inhibitors are sildenafil, zaprinast, dipyridamole and a selective PDE5 inhibitor is T-1032. Sildenafil citrate (Viagra®) has certainly been useful in the treatment of erectile dysfunction. We know that nitrergic nerves innervating the corpus callosum mediate erection through increased cGMP levels and sildenafil elevates cGMP signal by inhibiting its degradation, thus increasing the erectile response to sexual stimulation. Another relatively selective PDE5 inhibitor is zaprinast. It has been used extensively by researchers, is not selective for any particular isoform but it most potently inhibits PDE5 and also 1,3,6,9,10 and 11 [150]. Zaprinast has been reported to promote vasorelaxation *in vitro* by increasing cGMP in VSMC and decrease peripheral vascular resistance and blood pressure *in vivo* [150, 151]. It causes pulmonary vasodilatation in rats and dogs and also has venodilator properties in anesthetized rats [152].

1.10d The PDE5 inhibitors

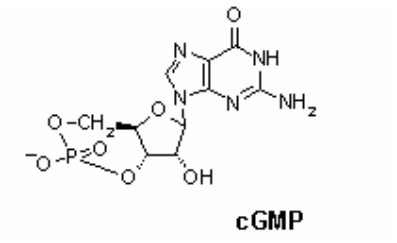
PDE5 has been implicated as the predominant cGMP-specific isozyme present in VSMC [144, 145] and its mRNA has been located in mRNA harvested from skeletal muscle which contains endothelial cells, VSMC, myocytes and neural cells [153]. Other PDEs associated with cGMP degradation in VSMC are 1,2,9,10 and 11 [146]. Zaprinast and sildenafil are relatively selective inhibitors of PDE5 while T-1032 is highly selective [147]. Table III (page 24) gives the IC-50 (μM) values for T-1032, sildenafil and zaprinast [147, 154-156].

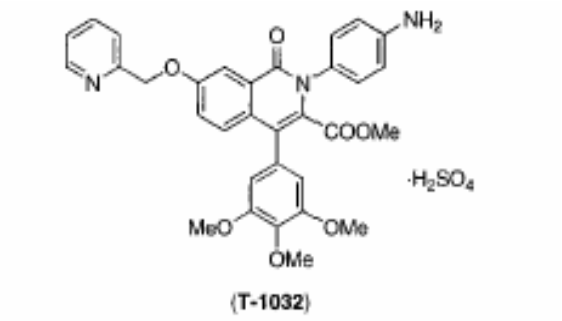
Fig. 2 (page 25) depicts the structure of T-1032 along with cGMP, zaprinast and sildenafil. It is not clear why T-1032 is more selective for PDE5 than sildenafil and zaprinast, considering that the structure of T-1032 is quite different from that of cGMP whereas the other two are structurally related to cGMP.

Table III

Compound	PDE5	5A1	5A2	PDE1	PDE2	PDE3	PDE4	PDE6
T-1032	0.001	0.003	0.002	3.0	9.7	7100	3.3	0.028
Sildenafil	0.0036	0.005	0.0045	0.27	43	7100	11	0.029
Zaprinast	0.76			45				0.15

Table III depicts the IC50 (μM) values. PDE1, 4, and 5 were isolated from canine lung, PDE3 from canine heart, PDE2 from canine adrenal gland and PDE6 was from canine retina [147, 154-156].





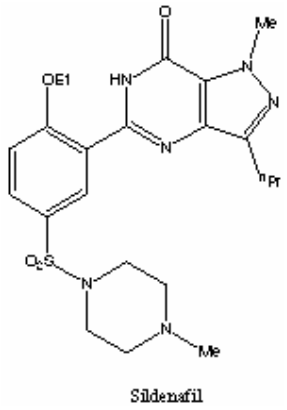


Fig. 2. This shows the structures of T-1032, cGMP, sildenafil and zaprinast. T-1032 is methyl-2-(4-aminophenyl)-1,2-dihydro-1-oxo-7-(2-pyridinylmethoxy)-4-(3,4,5-trimethoxyphenyl)-3-isoquinoline carboxylate sulphate [147].

1.11 Metabolic vasodilatation in capillary recruitment

It has been observed by Cleland et al [157] that local insulin-mediated vasodilatation is significantly augmented by co-infusion of D-glucose. They suggested that insulin-mediated glucose uptake may determine skeletal muscle blood flow via cross talk with NO pathway at an endothelial level. For example, glucose-induced changes in the intracellular environment (changes in pH by aerobic glycolysis) and increase in ATP production could activate ion pumps (eg, Na-K-ATPase) resulting in hyperpolarization and changes in fluxes in both endothelium and VSMC.

Interestingly, McKay et al [77] have demonstrated impaired insulin-mediated vasodilatation in hamster cremaster muscle, with adenosine receptor antagonism and blockade of ATP-sensitive potassium channels. They visualized the 1^{st}, 2^{nd}, 3^{rd}, and 4^{th} order arterioles under the microscope. In this preparation, NOS inhibition had no effect on insulin-mediated vasodilatation at the level of 3^{rd} and 4^{th} order arterioles, but had some inhibition on 1^{st} and 2^{nd} order arterioles. They have suggested that NO-like vasoactive molecules regulate vasodilatation at the level of larger 1^{st} and 2^{nd} order arterioles which control the blood flow to muscle, while smaller 3^{rd} and 4^{th} order arterioles which are more involved with the redistribution of blood flow within muscle are regulated by local tissue metabolism.

1.12 Role of hyperpolarizing phenomenon in insulin-mediated capillary recruitment

The existence of endothelium-dependent hyperpolarization factor (EDHF) has been presumed to play a role in vasodilatation by the apparent failure of NOS inhibitors to completely block endothelium-dependent vasodilation by acetylcholine, first described by Bolton and coworkers in 1984 [158]. EDHF is defined as that potassium channel opening factor which produces vascular smooth muscle hyperpolarization and which cannot be explained by NO or by a cyclooxygenase product such as prostacyclin [159]. Evidence for involvement of EDHF in insulin-mediated responses is discussed in section 1.12e.

The important thing is that hyperpolarization is a phenomenon that has been measured only in *in vitro* preparations. Both in human and animal arteries, the contribution of EDHF to endothelium-dependent relaxations elicited by acetylcholine [160, 161] or bradykinin [162, 163] appears to be significantly greater in small than in large arteries [77, 164-170]. The various candidates that have been suggested to mediate EDHF responses in animal and human blood vessels include: (a) potassium ions in rat mesenteric and femoral arteries and in human interlobar renal arteries [169, 171-175]; (b) epoxyeicosatrienoic acid derived from cytochrome P450 monooxygenases in coronary, internal mammary and subcutaneous arteries [176-181]; (c) myo-endothelial gap junction communication in animals and human subcutaneous arteries [182-185]; (d) hydrogen peroxide in coronary and mesenteric circulation [178, 186-191]; (e) anandamide (arachidonylethanolamide) an endogenous ligand at cannabinoid receptors in rat mesenteric arteries [192-194]. Not only are there species- [167] and vessel-related differences but also gender differences have been found in animals in EDHF response; the EDHF responses are greater in females [195].

1.12a Role of endothelial Ca^{2+}-dependent K^+ channels in insulin action

Insulin may stimulate hyperpolarization by stimulating transient increases in intracellular calcium in the endothelial cell via non-selective cation channels and via release of calcium from intracellular stores causing endothelial hyperpolarization [196-199]. The term "EDHF-mediated responses" reflects the mechanism by which this endothelial hyperpolarization is transferred to vascular smooth muscle cells [200, 201]. The endothelial hyperpolarization could then either spread to the adjacent smooth muscle cells through myo-endothelial gap junctions [184, 185] and/or the efflux of potassium through the endothelial SK_{Ca} [202] and IK_{Ca} [169, 203] channels (calcium-dependent small conductance potassium channels and calcium-dependent intermediate conductance potassium channels) [172, 200, 204, 205]. This elicits the hyperpolarization of surrounding vascular smooth muscle by activating the Na/K-ATPase, [1, 169, 172, 206] and/or increases the conductance of inward-rectifying potassium channels (K_{IR}) [169]. This leads to closure of voltage-dependent calcium channels, a decrease in intracellular

calcium and vessel dilatation. The reduction in intracellular sodium which results from an increase in the activity of the Na^+/K^+-ATPase may stimulate the Na^+/Ca^{2+} exchanger, leading to a further reduction in intracellular calcium [200, 207, 208].

1.12b Role of vascular smooth muscle Ca^{2+}-dependent K^+ channels in insulin action

Insulin may act directly on VSMC to initiate hyperpolarization phenomenon. It has been reported that insulin activates sodium potassium ATPase in VSMC by stimulating the translocation of this to the plasma membrane [209]. This leads to hyperpolarization of VSMC, blocking voltage-dependent calcium channels, and a decrease in the calcium influx resulting in relaxation [210]. It has been reported that insulin decreases calcium concentration directly by increasing calcium efflux through activation of calcium ATPase. Ouabain, a sodium potassium ATPase inhibitor, significantly inhibited the increase in forearm blood flow in humans [118].

On the other hand, insulin may activate calcium-dependent potassium channels in VSMC leading to potassium efflux, which hyperpolarizes VSMC (Fig. 4, page 35). This closes the voltage–dependent calcium channels reducing the calcium influx. A decrease in intracellular calcium may lead to vasorelaxation and capillary recruitment (as explained in section 1.9, page 15).

1.12c Inhibitors of Ca^{2+}-dependent K^+ channels

Not many pharmacological agents have been used to block these channels [211]. A non-specific inhibitor is tetrabutylammonium (TBA) [175, 212, 213] which blocks all types of potassium channels. More specific ones than TBA include, tetraethylammonium chloride (TEA), 1-EBIO, TRAM 34 and TRAM 39 as blockers of IK_{Ca} [169, 181, 214], toxins such as apamin to block SK_{Ca} [202], iberiotoxin, a blocker of large conductance calcium-dependent potassium channels (BK_{Ca}) [215], charybdotoxin which blocks both $IK_{Ca,}$ BK_{Ca} and also voltage-sensitive potassium channels [216], and scyllatoxin, a

structurally distinct SK_{Ca} inhibitor [217-220]. It was first observed by Garland's group that EDHF-mediated response can be abolished by combination of apamin plus charybdotoxin [221, 222].

1.12d Tetraethylammonium chloride (TEA)

Tetraethylammonium chloride (TEA) has been known to pharmacologists as an autonomic ganglion blocker (0.5 mg/min/kg) [223]. At doses of 10 mM it also acts as a weak nicotinic acetylcholine receptor agonist and a muscarinic acetylcholine receptor antagonist [224]. But the action which has gained much importance is the blockade of calcium-dependent large conductance potassium channels (BK_{Ca}) [225-227]. A concentration of TEA between 0.2 and 3 mM selectively blocks calcium-dependent potassium channels in smooth muscle cells *in vitro* [202, 228, 229] (Fig. 4, page 35) while higher concentrations (>5 mM) can inhibit both ATP and voltage-dependent K^+ channels [230].

1.12e Hyperpolarization-mediated insulin response in blood vessels

In vitro evidence

A number of *in vitro* studies point towards the role of EDHF in insulin-mediated hemodynamic effects. Insulin-induced relaxation of rat mesenteric artery was abolished by charybdotoxin and endothelial denudation but not by L-NAME suggesting a role of large-conductance Ca^{2+}-activated potassium channels and EDHF [231]. Iida and coworkers [231] also showed that clotrimazole, an inhibitor of cytochrome P450 inhibited insulin-induced vasodilatation as effectively as the blockers of Ca^{2+}-activated potassium channels, as it has been suggested that EDHF activity may reflect the action of cytochrome P450-derived arachidonic acid metabolites (EETs). However, an endothelium independent, nitric oxide independent vasorelaxation of rings from human internal mammary artery and saphenous vein in response to both insulin and IGF-I, through a mechanism involving activation of potassium channels has also been described [232].

Relaxation was not affected by the removal of the endothelium and by inhibition of the production of nitric oxide, but the vascular relaxation caused by insulin and IGF-I was completely abolished by KCl, and was attenuated by the potassium channel blocker tetraethylammonium (TEA) indicating that activation of potassium channels is involved in cellular action of insulin [232]. However the high dose of TEA (10 mM) used in this study reflected a non-specific antagonism of all potassium channels.

Five studies have been published using resistance arteries. An endothelium-dependent vasodilatation in small (≈112 μm) dog coronary arteries demonstrated that insulin-induced vasodilatation could be inhibited by KCl or tetrabutylammonium chloride (TBA) but not by L-NNA, indomethacin, TEA, glibenclamide or charybdotoxin plus apamin [168]. Inhibition by TBA, a non-specific potassium channel blocker but not by specific potassium channel blockers suggested that hyperpolarization via some other K_{Ca} channels is probably involved in insulin-induced vasodilatation. On the other hand, insulin-induced vasodilatation was inhibited by indomethacin, glibenclamide and potassium chloride but was resistant to L-NNA, charybdotoxin plus apamin in fourth order branches (≈211μm) of rat superior mesenteric artery [94]. Thus, the role of specific calcium-dependent potassium channels in the vascular response to insulin was not found. Conversely, Chen and Messina [116] showed that insulin-induced vasodilatation in isolated rat skeletal muscle arterioles (≈80μm) could be completely inhibited by the nitric oxide synthase inhibitor L-NNA. The differences in these studies could be either species- or vascular bed-dependent because similar concentrations of insulin and of inhibitors were used in all 3 experiments. Hyperpolarization in one way or another seems to play a role in insulin-mediated vasodilatation as McKay et al [77] have also shown that insulin-induced dilatation in hamster cremaster arterioles is NO-dependent in second order but not in third-or fourth-order arterioles while blockade of ATP-sensitive potassium channels by glibenclamide prevented insulin-induced dilatation in both second and fourth-order arterioles. Oliveira and coworkers [233] induced diabetes in rats and then looked at the potentiation of bradykinin relaxation by angiotensin-(1-7) in A_2 resistance arterioles (15-25μm). They demonstrated that the potentiating effect of angiotensin-(1-7) on bradykinin-induced vasodilatation restored in diabetic rats by chronic insulin,

disappeared in the presence of TEA while L-NAME did not interfere with the restoring effect of insulin on the potentiation. Their finding reinforced the contribution of hyperpolarization on the alteration of microvascular reactivity in diabetic rats.

Nitric oxide is also capable of hyperpolarizing smooth muscle. It has been suggested that activation of calcium-dependent potassium channels plays an important role in mediating the vasorelaxation caused by NO [81, 201, 234-236].

Attenuated EDHF-mediated responses have been noticed with no or minor alteration in NO-dependent responses in the fructose-fed rat, the leptin deficient, genetically obese and mildly hypertensive Zucker rat and the Otsuka Long-Evans Tokushima fatty rat [237-241].

***In vivo* evidence**

In the intact animal, the involvement of hyperpolarization in the vasodilator response to insulin is difficult to assess and very few studies have been designed specifically to address this issue. EDRF as well as the EDHF response induced by acetylcholine in the presence of indomethacin and L-NNA was significantly attenuated in the type II diabetic rats [237]. There is only one *in vivo* study to date in which the role of potassium (K_{Ca} and K_{ATP}) channels in insulin-induced increase in total flow and glucose uptake has been demonstrated using TEA and glibenclamide [242]. This study argues against a role for calcium- and ATP-dependent potassium channels in insulin action in humans *in vivo*. One of the aims of this thesis is to deduce the role of calcium-dependent potassium channels in insulin's action in muscle *in vivo* using TEA during euglycemic hyperinsulinemic clamp. The other potassium channel antagonists (the toxins) are probably too toxic for *in vivo* use [243].

1.13 The present study- summary of aims

The work presented in this thesis was designed to investigate the mechanisms operating behind hemodynamic (with special emphasis on capillary recruitment) and metabolic (glucose uptake) changes in muscle. Insulin has been shown to stimulate both the capillary recruitment and glucose uptake in muscle. Insulin has two hemodynamic actions in muscle: it increases total flow to the muscle, which was measured using Transonic® flow probes and, it also increases the capillary perfusion by causing flow distribution (capillary recruitment) which was measured using the 1-MX technique. To measure the metabolic effect of insulin in muscle, 2-deoxy glucose uptake was measured as an index of insulin-mediated glucose uptake. The technique used for this purpose was euglycemic hyperinsulinemic clamp.

Since systemic infusion of vasoactive agents can affect systemic hemodynamics, leading to activation of homeostatic mechanisms and compensatory changes which can mislead the interpretation of results, my aim was therefore to develop a technique so that the test agents could be given locally in only the regional circulation of hindleg.

The nitric oxide-cGMP pathway has been thought to play a role in insulin's action in muscle. Three different sets of experiments were designed to explore the role of such a pathway in insulin's action (Fig. 3, page 34).

First, since cGMP is thought to be the downstream effector molecule which mediates NO action in muscle, it was thought a phosphodiesterase inhibitor would elevate cGMP levels and potentiate insulin action.

Second, NO production was enhanced by using local infusion of methacholine, an endothelium-dependent nitro-vasodilator during an insulin clamp. Blood flow was increased similarly by using another endothelium-dependent nitro-vasodilator bradykinin, to detect whether the effects seen were methacholine-specific or related to increase in NO production. Possible correlation of glucose uptake with total flow and/or capillary recruitment was also investigated.

Third, the effect of NOS inhibition by L-NAME was observed by using both the systemic and local infusion of the inhibitor during an insulin clamp. Thus, the aim was both to perturb and amplify the NO-cGMP axis and to determine their effects on insulin-mediated capillary recruitment and glucose uptake (Fig. 3, page 34).

Alternatively, insulin action in muscle may not be controlled by a single mechanism. It might be the net result of the combination of effects with several agents involved each playing its own part. Some recent *in vitro* reports have suggested the role of calcium-dependent potassium channels in insulin action. The effect of blocking calcium-sensitive potassium channels in vascular smooth muscle cells by TEA was assessed during an insulin clamp (Fig. 4, page 35).

To summarize, the overall aim of this study was to elucidate the possible mechanisms in muscle microvasculature leading to capillary recruitment and glucose uptake. Since a reduction in insulin-mediated hemodynamic action in muscle might contribute to a decreased glucose uptake, it was important to explore the mechanisms. Mechanisms involved in capillary recruitment may thus constitute new targets for the treatment of insulin resistance.

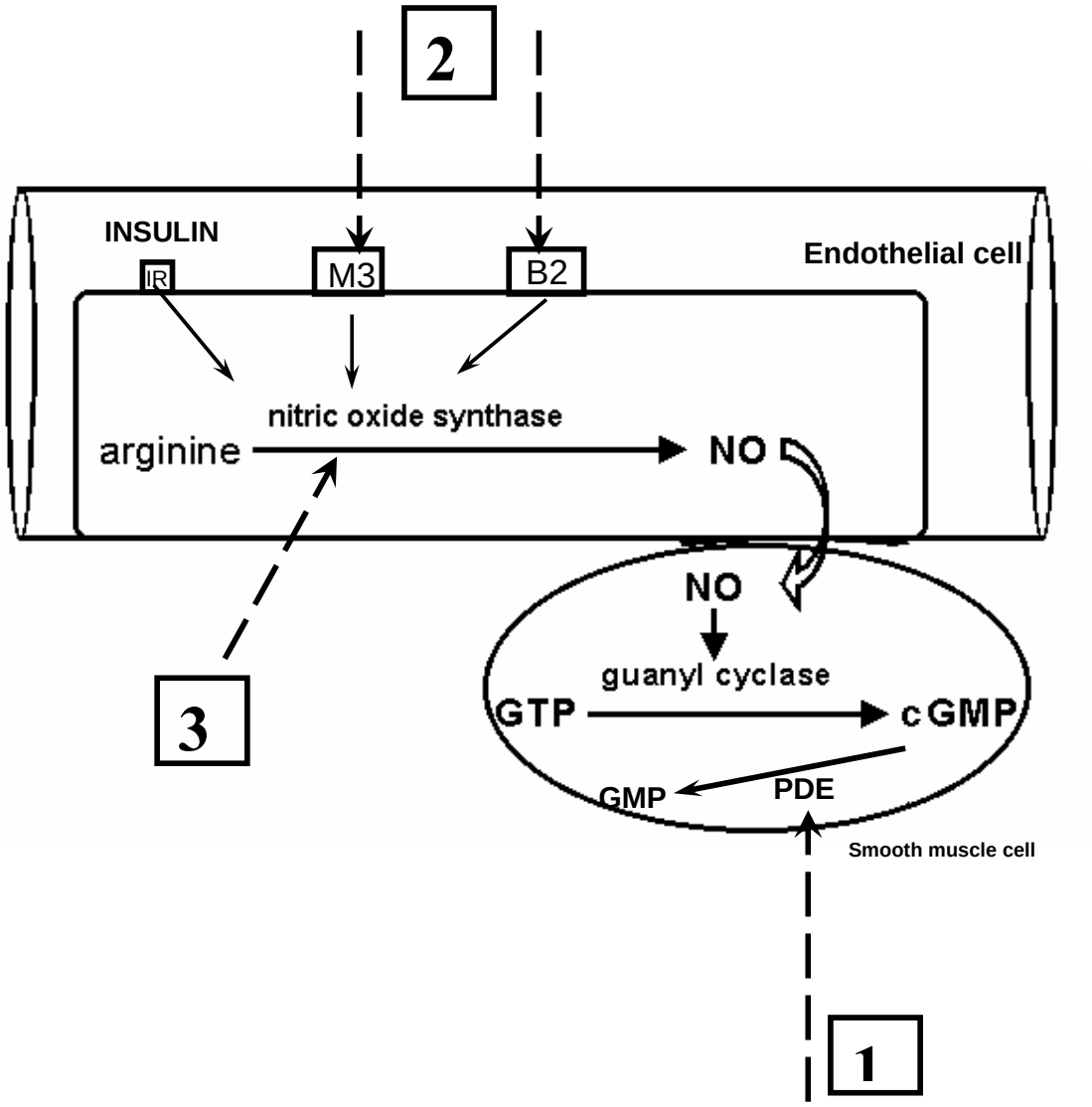


Fig. 3. Experimental strategies to investigate if insulin action in muscle is nitric oxide dependent.

1. Increase cGMP action with T-1032, a specific PDE5 inhibitor
2. Increase NO production using methacholine (MC) and bradykinin (BK)
3. Decrease NO synthesis using nitric oxide synthase inhibitor, L-NAME.

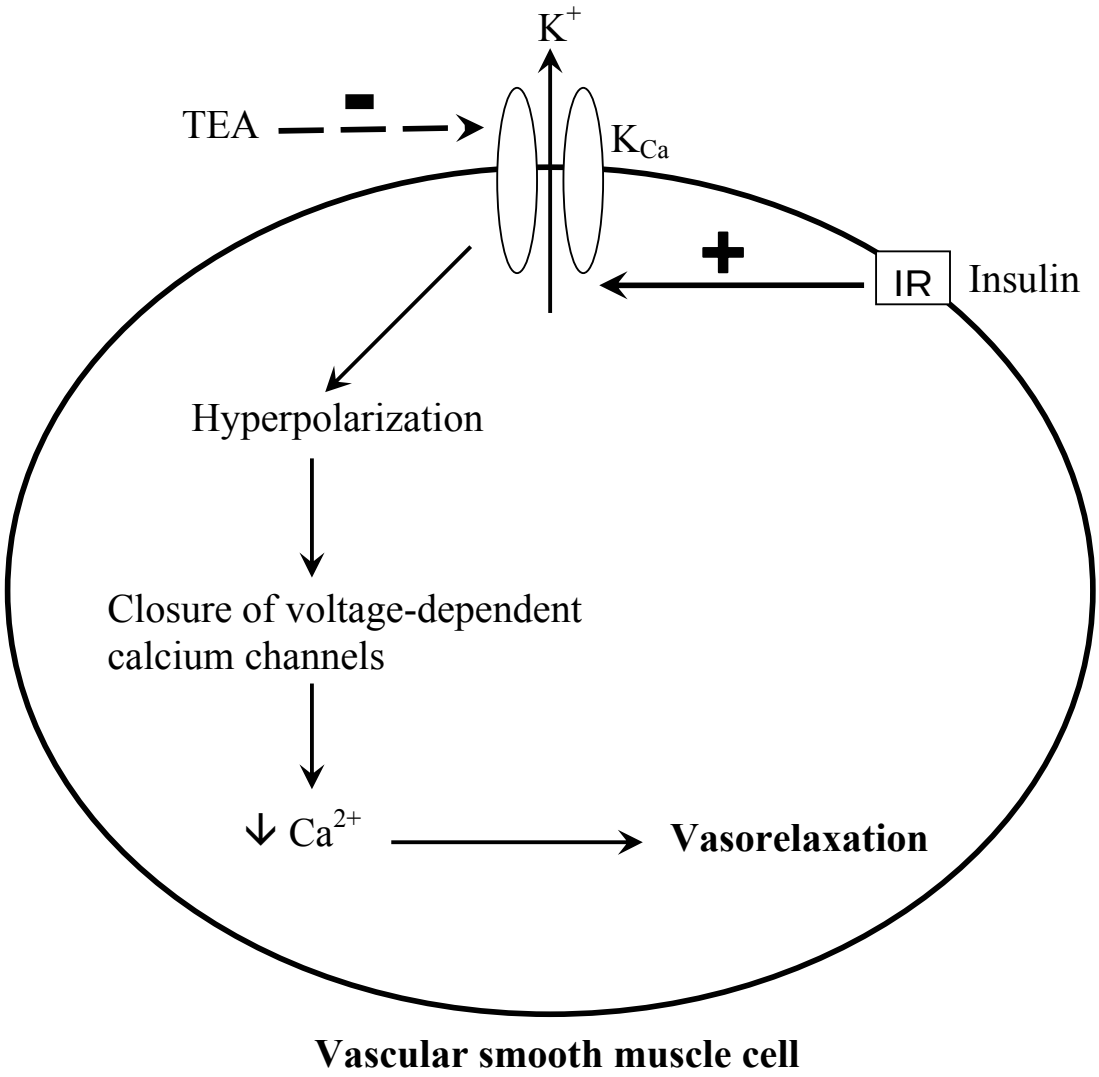


Fig. 4. Proposed mechanism of insulin-mediated vasodilatation by activation of calcium-dependent potassium channels [244]. K_{Ca} – calcium-dependent potassium channels and TEA – tetraethylammonium chloride.

CHAPTER 2

MATERIALS AND METHODS

2.1 Animal care

Male Hooded Wistar rats (240-350 grams) were raised on a commercial diet (Gibsons, Hobart) containing 21.4% protein, 4.6% lipid, 68% carbohydrate, and 6% crude fiber with added vitamins and minerals together with water *ad libitum*. Rats were housed at a constant temperature of 21 ± 1°C in a 12 h/12 h light-dark cycle. All procedures adopted and experiments undertaken were approved by the University of Tasmania, Animal Ethics Committee in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (1990).

2.2 Surgery

The rats were fasted overnight (12 h) and then anesthetized using pentobarbital sodium (50 mg/kg body weight). Polyethylene cannulas (PE-50, Intramedic®) were surgically implanted into the carotid artery, for arterial sampling and measurement of mean arterial pressure (pressure transducer Transpac IV, Abbott Critical Systems). Both jugular veins were cannulated for continuous infusion of anesthetic and other intravenous infusions. A tracheotomy tube was inserted, and the animal was allowed to breathe room air spontaneously throughout the course of the experiment. Small incisions (1.5 cm) were made in the skin overlaying the femoral vessels of each leg, and the femoral artery was separated from the femoral vein and saphenous nerve. The epigastric vessels were then ligated, and an ultrasonic flow probe (Transonic Systems, VB series 0.5 mm) was positioned around the femoral artery of the right leg just distal to the rectus abdominus muscle. The cavity in the leg surrounding the probe was filled with lubrication jelly (H-R, Mohawk Medical Supply, Utica, NY) to provide acoustic coupling to the probe. The probe was then connected to the flow meter (Model T106 ultrasonic volume flow meter,

Transonic Systems). This was in turn interfaced with an IBM compatible PC computer which acquired the data at a sampling frequency of 100 Hz for femoral blood flow (FBF), heart rate (HR) and mean arterial pressure (MAP) using WINDAQ data acquisition software (DATAQ Instruments). Most studies of the hemodynamic effects of insulin have been made in humans or large animals, where repetitive measurement of skeletal muscle blood flow is relatively easy. With the miniaturized flow probe used in this study, it is possible to make continual measurements in a small animal such as the rat, where until recently, flow measurement were limited to a few measurements using microspheres or indirect methods [245-248]. The surgical procedure generally lasted approximately 30 min and then the animals were maintained under anesthesia for the duration of the experiment using a continual infusion of pentobarbital sodium (0.6 mg/min/kg). The femoral vein of the left leg was used for venous sampling, using an insulin syringe with an attached 29G needle (Becton Dickinson). A duplicate venous sample (V) was taken only on completion of the experiment to prevent alteration of the blood flow from the hindlimb due to sampling, and to minimize the effects of blood loss. The total blood volume withdrawn from the animals before the final arterial and venous samples did not exceed 1.5 ml and was easily compensated by the volume of fluid infused. The body temperature was maintained at 37˚C using a water-jacketed platform and a heating lamp positioned above the rat. The rat was sacrificed using an intra-cardiac injection of Nembutal and both left and right hindleg muscles were collected as described later.

2.3 Cannulation of epigastric artery

A new technique was developed (figure 1, page 43) to infuse the test substances locally in one leg. If vasoactive substances are given systemically, they have a profound effect on the blood pressure which activates counter-regulatory reflex mechanisms. Some have generalized side effects, for example, methacholine. To avoid the systemic effects of a test substance, the epigastric artery was cannulated, which is a branch of the femoral artery, in the middle part of thigh. The cannula was used to infuse the test substances methacholine, bradykinin, L-NAME and tetraethylammonium. Another advantage of this technique was that the opposite leg served as a control. Only the substances which were

rapidly metabolized could be used by this technique. Since all the substances used were vasoactive, any effect on blood pressure and heart rate were taken as an indication that the substance had appeared in the systemic circulation. An effect on femoral blood flow in the test leg was taken as definitive indicator of the substance being infused and to calculate the optimum dose to be used. Transonic flow probes were placed on the femoral artery of both legs to measure the femoral blood flow simultaneously; the contralateral leg served as control. At the end of the experiment, after taking the arterial sample (200 μl), the venous sample (150 μl) was taken from the femoral veins of control and test legs.

2.4 Euglycemic hyperinsulinemic clamp

Once the surgery was completed, a 60-min equilibration period was allowed so that leg blood flow and blood pressure could become stable and constant. Details of experimental protocols are given in individual chapters. An arterial blood sample was taken at the end of equilibration for glucose analysis. During the hyperinsulinemic clamp blood glucose was maintained at this level with the infusion of a 30% w/v solution of glucose. In the control groups, saline infusion was matched to the volumes of insulin (Humilin®, Eli Lilly and Co.) and glucose administered.

2.5 1-MX infusion and analytical method

A previously established method utilizing the metabolism of exogenously added I-MX (Sigma-Aldrich Inc.) was used to assess the perfused capillary surface area. 1-MX infusion (0.5 mg/min/kg, dissolved in saline) was commenced at 60 min prior to the end of the experiment. Since 1-MX clearance was very rapid, it was necessary to partially inhibit the endogenous xanthine oxidase activity in non-muscle tissues [57, 249]. We have performed allopurinol dose-response curves in the rat *in vivo* (data not shown) and found that 10 μmol/kg partially inhibited the xanthine oxidase, lowered the Km for 1-MX, and allowed steady-state systemic levels of 1-MX to be obtained [249].

To do this, a bolus injection of a specific xanthine oxidase inhibitor, allopurinol [250] (10 μmol/kg) was administered 5 minutes prior to commencing the 1-MX infusion. This

allowed constant saturating arterial concentrations of 1-MX to be maintained throughout the experiment.

Duplicate arterial (A) and venous (V) samples (300µl) were taken at the end of the experiment and placed on ice. These blood samples were immediately centrifuged. 1-MX measurement involved protein precipitation of plasma using perchloric acid. The PCA treated samples were then stored at -20°C until assayed for 1-MX. When required, samples were thawed on ice, centrifuged for 10 min and the supernatant used to determine 1-MX, l-methylurate and oxypurinol concentrations by reverse-phase HPLC as described previously [251, 252]. The rest of the plasma was used for glucose, insulin and other analyses.

2.6 2-Deoxyglucose injection and analytical method

In experiments (insulin clamps and saline controls) measuring glucose uptake into individual muscles, a 100 µCi bolus of 2-deoxy-D-[2,6-^{14}C]glucose or 50 µCi of 2-deoxy-D-[2,6-^{3}H] glucose (2-DG; specific activity-44.0 Ci/mmol, Amersham Life Science) in saline was administrated at 45 min prior to the completion of the experiment. Plasma samples (25 µl) were collected at 5, 10, 15, 30 and 45 min after the 2-DG injection to determine the plasma radioactivity decay or time course.

A modified technique for 2-DG uptake

A new technique was developed where instead of measuring the plasma decay curve, the averaged plasma specific activity of [^{3}H]2-DG was obtained by continuous arterial sampling after giving 2-DG bolus. This enabled (i), to decrease the labeling period from 45 min to 10 min and (ii), to see the effect of vasoactive agents during insulin clamps over short periods (1 h). This technique has been used in local L-NAME and TEA studies (chapters 5 and 6). R'g assessed in insulin treated rats over 10 min with continuous blood sampling was approximately twice the value obtained from 45 min labeling with decay curve estimation (data not shown). However, insulin stimulated a 2-fold increase in R'g

as measured by either technique. Because continuous sampling also includes the initial 5 min period that decay curve sampling misses, the former is likely to more accurately represent the entire plasma glucose specific activity measured by the muscle. Plasma 2-DG specific activity is likely to be the highest in this initial 5 min period, when muscle is taking up most radioactivity.

At the conclusion of the experiment, the soleus, plantaris, red gastrocnemius (RG), white gastrocnemius (WG), extensor digitorum longus (EDL) and tibialis muscle were removed, freeze clamped in liquid nitrogen and stored at –20°C until assayed for 2-DG radioactivity. The frozen muscles were ground under liquid nitrogen and homogenized using an Ultra Turrax™. Free and phosphorylated 2-DG were separated by ion exchange chromatography using an anion exchange resin (AG1-X8) [253, 254]. Scintillant (16ml; Biodegradable Counting Scintillant-BCA, Amersham USA) was added to each radioactive sample and radioactive counts (disintegrations per minute, dpm) were determined using a scintillation counter (Beckman LS3810, USA). From this measurement and a knowledge of plasma glucose and the time course of plasma 2-DG disappearance, R'g, which reflects glucose uptake into the muscle, was calculated as previously described in detail by others [253, 254] and is expressed as μg/min/g wet weight of muscle [253].

2.7 Glucose assay

A glucose analyzer (Model 2300 Stat plus, Yellow Springs Instruments, Yellow Springs OH) was used to determine whole blood glucose and plasma glucose (by the glucose oxidase method) during and at the conclusion of the insulin clamp. A sample volume of 25 μl was required for each determination. Insulin levels at the beginning and the end of the experiment were determined from arterial plasma samples by ELISA assay (Mercodia AB, Sweden).

2.8 Reproducibility of techniques

The hyperinsulinemic euglycemic clamp, along with the infusion of 2-DG is a widely used technique which gives a reliable and accurate measure of insulin sensitivity. Whilst the methods used to assess both total flow (transonic flow probe) and capillary flow (l-MX metabolism) were only recently established, they have now been utilized in a number of studies and have yielded consistent results. Unfortunately, the nature of experiments does not allow comparison of day-to-day variation using the same animal. Nevertheless, measurements of total flow are continuous and show very little drift over a 2-hour saline infusion. Furthermore, the values of both basal and insulin-stimulated blood flow have been comparable in previous studies and in the work presented herein. In addition, when flow is measured simultaneously on both legs, the values show little difference.

2.9 Data analysis

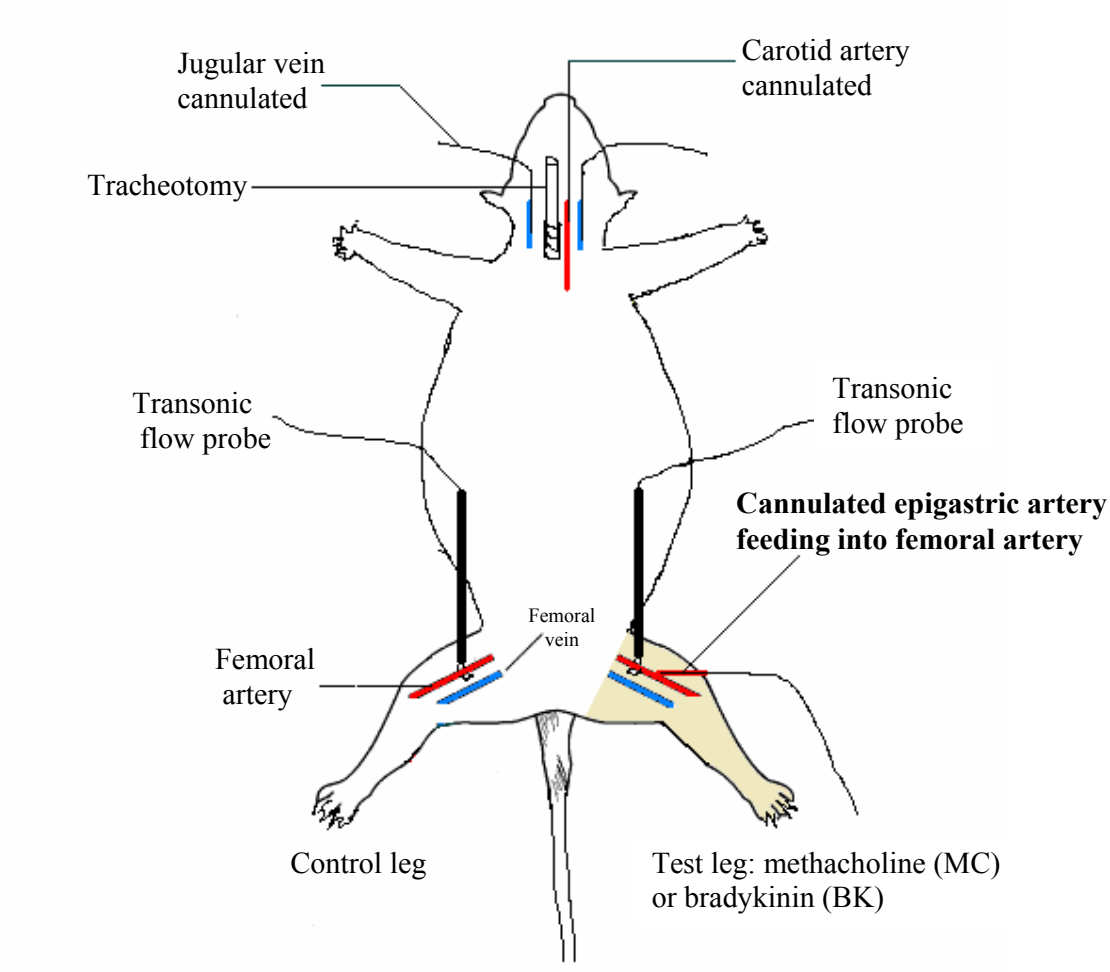
All data are expressed as means ± SEM. Mean femoral blood flow, mean heart rate and mean arterial pressure were calculated from 5 second subsamples of the data, representing approximately 500 flow and pressure measurements every 15 min. Vascular resistance in the hindleg was calculated as mean arterial pressure in millimeters of mercury divided by femoral blood flow and expressed as resistance units (R.U.). Glucose uptake in the hindlimb was calculated (chapter 3) from A-V glucose difference and multiplied by femoral blood flow and expressed as μmol/min. R'g for the combined muscle was calculated from the sum of R'g of each individual muscle times the dry weight of the individual muscle and divided by the sum of each individual muscle dry weight. The 1-MX metabolism was calculated from A-V plasma 1-MX difference and multiplied by femoral blood flow (corrected for the volume accessible to 1-MX, 0.871, determined from plasma concentrations obtained after additions of standard 1-MX to whole blood) and expressed as nmol/min.

2.10 Statistical analysis

Repeated measures two-way analysis of variance was used to test the hypothesis that there was no difference among treatment groups for femoral blood flow, blood pressure, heart rate, vascular resistance, 1-MX, and oxypurinol concentrations throughout the time course. When a significant difference ($P < 0.05$) was found, pair wise comparisons by the Student-Newman-Keuls test were used to determine at which individual times the differences were significant. Statistical differences between the treatments for arterial glucose and 1-MX, hindleg glucose extraction and uptake, and hindleg 1-MX extraction and disappearance were determined by one way ANOVA. These tests were performed using the SigmaStat^{TM} statistical program (Jandel Software, version 2.03).

Figure 1: A schematic diagram of Epigastric technique for local infusion of test agents in the rat.

The agent is confined to the test leg (no systemic effects). The contralateral leg serves as the control.



CHAPTER 3

EFFECT OF CYCLIC GMP PHOSPHODIESTERASE-5 INHIBITOR T-1032 ON INSULIN-MEDIATED MUSCLE HEMODYNAMIC EFFECTS AND GLUCOSE UPTAKE *IN VIVO*

3.1 INTRODUCTION

It has been proposed by Baron et al that insulin increases glucose uptake by muscle causing vasodilatation, described as functional capillary recruitment within the muscle, and in doing so, increases its own access and that of glucose by increasing blood distribution within the muscle [57]. This process of insulin-mediated vasodilatation has been proposed [39, 115] to involve production of nitric oxide which then migrates to neighbouring vascular smooth cells activating guanylate cyclase to produce cyclic GMP which thereby leads to a decrease of intracellular calcium with vasodilatation.

cGMP is degraded to GMP by class of enzymes called phosphodiesterases. Agents which prolong the survival of cGMP by inhibiting the PDEs in vascular smooth muscle in terminal arterioles controlling the entry to nutritive bed have the potential for increasing capillary recruitment and enhancing insulin action. PDEs are substrate- and tissue-specific enzymes and are divided in 11 classes; each class has a number of isoenzymes (Tables I and II, pages 20, 21) [141]. The property of PDE inhibitors to increase cGMP has been exploited before. The PDE5 inhibitor sildenafil (Viagra®) is one such example which has dominant effects on the vasculature of the corpus cavernosum and has been used to increase penile tumescence. Since NO signaling involves activation of the soluble form of guanylate cyclase to produce cGMP, the relationship between NO, cGMP, and muscle glucose uptake has been explored.

3.1.1 Recent reports on T-1032

Shortly after its discovery by Kotera et al in April 2000 [156], it was reported that the PDE inhibitor, T-1032 dose dependently enhanced the penile tumescence induced by pelvic nerve stimulation with the same potency as sildenafil in the corpus cavernosum of anesthetised dogs [255]. In an *in vitro* study, Takagi et al [256] compared the effects of T-1032 in isolated rabbit corpus cavernosum and rat aorta. They concluded that the influence of T-1032 was more profound on cardiovascular than on penile tissue in anesthetised rats, since T-1032 produced more marked relaxation in the rat aorta than in the rabbit corpus cavernosum.

So far it has been believed that T-1032 and sildenafil have similar vasorelaxant properties. But Mochida et al [257] demonstrated that sildenafil produced a more potent vasorelaxation at higher concentration than T-1032 in endothelial denuded aortic rings and in the presence of L-NAME. They concluded that sildenafil at high concentration has an additional vasorelaxant property, other than PDE5 inhibition in isolated rat aorta, probably the calcium channel antagonism. Since T-1032 did not show an additional vasorelaxant property, T-1032 should be considered to be the better tool as a selective PDE5 inhibitor.

Studies by Yano et al [244] and Inoue et al [258] in pulmonary hypertensive dogs and rats respectively, suggested T-1032 as a useful drug for the treatment of pulmonary hypertension as it potently and selectively dilated pulmonary vessels. Another study by Inoue and coworkers [259, 260] indicated that T-1032 may have therapeutic potential for the treatment of chronic heart failure.

3.1.2 Aim of the study

As discussed in the first chapter, cGMP hydrolyzing PDEs are involved in regulation of vascular tone with type 5 likely to be the most important cGMP hydrolyzing enzyme present in VSMC [144, 145]. Thus it was hypothesized that a specific inhibition of cGMP

PDE specifically expressed in the terminal arteriolar VSMC controlling blood flow entry to the capillaries would improve insulin action to recruit capillary flow and skeletal muscle glucose uptake and may therefore help in ameliorating insulin resistance. The aim of this study was to investigate the effect of the highly selective cGMP hydrolyzing PDE inhibitor T-1032 on insulin-mediated capillary recruitment and skeletal muscle glucose uptake, to see if T-1032 amplifies the effects of insulin *in vivo*.

3.2 RESEARCH DESIGN AND METHODS

3.2.1 Animals

Rats were raised as described in section 2.1.

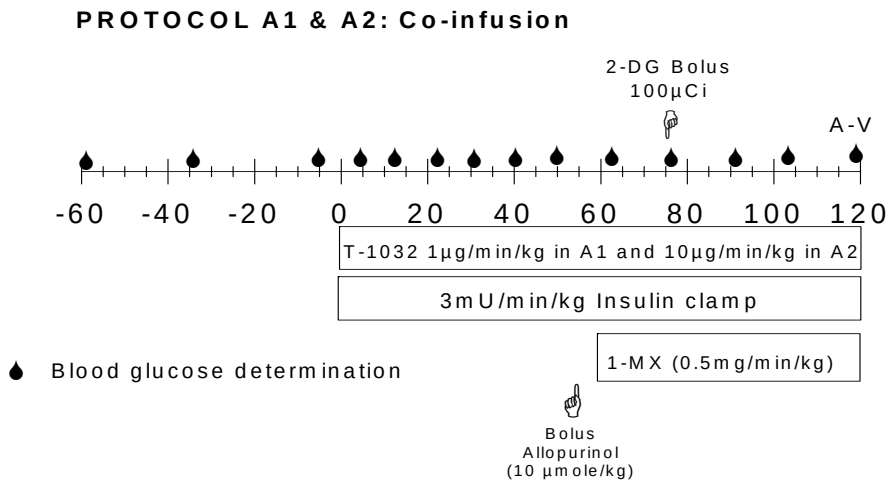
3.2.2 *In vivo* experiments

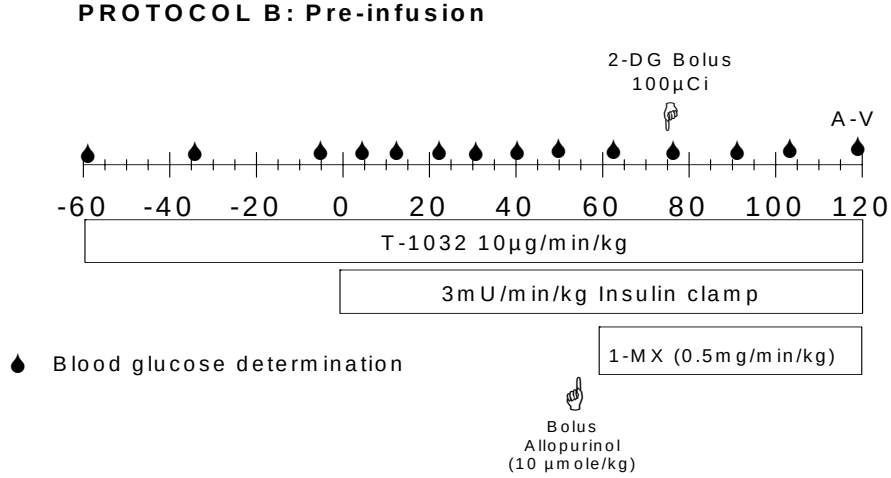
Hyperinsulinemic euglycemic clamps were performed in fasted anesthetized rats as described in section 2.2. Once the surgery was completed, a 60-min equilibration period was allowed so that leg blood flow and blood pressure could become stable and constant. Femoral blood flow in one leg was continuously measured from a Transonic® flow probe positioned around the femoral artery. Rats were then allocated into either control (saline), T-1032 or euglycemic insulin clamp (insulin alone or T-1032 + insulin) group (n = 5-7 in each group). Glucose (30% w/v solution) was infused to maintain blood glucose levels at or above basal whilst infusing insulin for a period of 120 min. T-1032 was dissolved in 1 mM HCl in saline. At the end of the experiment samples were taken from the femoral artery and vein. Hindleg glucose uptake and 1-MX metabolism were calculated from the arterio-venous difference multiplied by the flow. 1-MX metabolism was an indicator of perfused capillary surface area. At 45 min prior to the completion of the experiment, a 100 μCi bolus of [^{14}C]2-DG was administrated. Plasma samples (25μl) were collected at 5, 10, 15, 30 and 45 min after the 2-DG injection to determine the time course for plasma radioactivity decay. At the conclusion of the experiment, the muscles were removed,

freeze clamped in liquid nitrogen and stored at –20°C until assayed for 2-DG radioactivity as described in section 2.6.

3.2.3 Experimental protocols

Completion of the surgical procedures was followed by a 60-min equilibration period to allow leg blood flow and blood pressure to become constant. Rats were then subjected to Protocol A or B (Fig. 1), where they were infused with saline or T-1032 for 3 h (some received T-1032 for only 2 h starting at 0) and underwent euglycemic insulin clamp (3 mU/kg/min, Humulin R, Eli Lilly and Co., Indianapolis), or saline alone for the final 2 h. T-1032 was infused at 1 μg/min/kg in protocol A1 and at 10 μg/min/kg in protocol A2 and B. T-1032 was also infused 1h prior to insulin clamp (protocol B).





Study design. Fig. 1. Arterial and venous samples were collected at times indicated as A-V. for HPLC analysis and plasma glucose determination. Arterial blood glucose were determined at time ♦. Venous infusion periods are indicated by bars. Bolus infusion periods are indicated by ☞. T-1032 was infused at a dose of 1 µg/min/kg in protocol A1 and at a dose of 10 µg/min/kg in protocol A2 and B. n = 5-7.

3.2.4 Plasma T-1032 assay

A novel assay was developed for the measurement of plasma T-1032 by reverse-phase HPLC using 5 µm C-18 reverse-phase column (Luna) with 64% methanol in 50 mM $NH_4H_2PO_4$ buffer as the mobile phase at a flow rate of 1.2 ml/min. 1 ml of plasma was mixed with 3 ml of ethanol to precipitate the proteins and centrifuged at 3500 rpm for 15 minutes. To the 3.4 ml of supernatant 10.2 ml chloroform was added, mixed and briefly centrifuged (30 sec). The lower chloroform layer was then air dried at 60°C, the residue re-dissolved in HPLC buffer (100 µl) and an aliquot (50 µl) injected into the HPLC system to measure the plasma concentration of T-1032. A standard curve was constructed using different concentrations of spiked plasma samples that were treated in the same manner as above.

3.2.5 Plasma insulin assay

Rat insulin levels at the end of the euglycemic insulin clamp (and other groups) were determined from arterial plasma samples by ELISA assay (Mercodia rat insulin ELISA) using rat insulin standards.

3.2.6 Muscle cGMP assay

Muscle cGMP levels (soleus) were determined using a Biotrak cGMP enzyme immunoassay kit (Amersham Pharmacia Biochem., UK) on trichloroacetic acid extracts of muscle according to the instructions provided.

3.2.7 Plasma free fatty acid assay

Plasma free fatty acid were determined using an enzymatic colorimetric assay kit (Wako Pure Chemical Industries Ltd).

3.2.8 Muscle glucose-6-phosphate

Muscle glucose-6-phosphate was determined enzymatically in the neutralized perchlorate extracts of muscle. Extracted muscle glucose-6-phosphate was treated with glucose-6-phosphate dehydrogenase in the presence of $NADP^+$. Change in absorbance at 340 nM after formation of $NADPH^+$ was proportional to the levels of glucose-6-phosphate.

3.2.9 Data analysis

All data are expressed as means ± SEM. Data-analysis was done as described in section 2.9.

3.2.10 Statistical analysis

Repeated measures two-way analysis of variance was used to test the hypothesis that there was no difference among treatment groups for femoral blood flow, blood pressure, heart rate, vascular resistance, 1-MX, and oxypurinol concentrations throughout the time course. When a significant difference ($P < 0.05$) was found, pair wise comparisons by the Student-Newman-Keuls test were used to determine at which individual times the differences were significant. Statistical differences between the treatments for arterial glucose and 1-MX, hindleg glucose extraction and uptake, and hindleg 1-MX extraction and disappearance were determined by one way ANOVA. These tests were performed using the SigmaStat™ statistical program (Jandel Software, version 2.03).

3.3 RESULTS

3.3.1 EFFECT OF LOW DOSE T-1032 (1 μg/min/kg) ON PHYSIOLOGIC INSULIN (3 mU/min/kg) (Protocol A1)

3.3.1a Hemodynamic effects

Fig. 2 A and B show the MAP and heart rate during saline control, T-1032 1 μg/min/kg (hereafter referred as 1 μg), euglycemic 3 mU/min/kg (hereafter referred to as 3mU insulin) insulin clamp and T-1032 1 μg + 3 mU insulin clamps. There were no significant differences observed in MAP and heart rate among the 4 groups.

Fig. 2 C and D show the changes in FBF and vascular resistance. Saline and 1 μg T-1032 (2 h) infusion alone had no effect on either FBF or vascular resistance. Insulin infusion alone caused a significant increase in FBF when compared with saline towards the end of the clamp (from 0.7 ± 0.1 to 1.0 ± 0.1 ml/min, an increase of 33%). Co-infusion of 1 μg T-1032 did not have any significant effect on the insulin-mediated increase in FBF (0.8 ± 0.1 to 1.0 ± 0.1 ml/min). The vascular resistance did not significantly decrease at the 120min time point in the insulin group nor did the co-infusion of T-1032 affected vascular resistance (from 155.6 ± 18.3 to 120.0 ± 20.0 R.U.).

3.3.1b Glucose metabolism

There were no differences in arterial glucose or lactate concentrations between any of the groups during the course of the experiments (Fig. 3 A and B). During the insulin clamp, blood glucose was maintained at or above this level. Fig. 3C shows the GIR during the insulin clamp with and without T-1032 infusion. In order to maintain this basal blood glucose level, glucose was infused at a significantly higher rate in the insulin group. In contrast, co-infusion of T-1032 with insulin led to a significant decrease in GIR (42%) in the last 30 minutes (insulin 12.8 ± 0.5, insulin + T-1032 7.4 ± 0.8 mg/min/kg); after an

initial rise, GIR started to decrease after 40 minutes and showed a steady decline until the end of the experiment.

Hindleg glucose uptake (Fig. 4A) calculated from the A-V difference (glucose extraction) multiplied by the FBF showed a significant elevation in insulin group (saline 0.1 ± 0.01 to insulin 0.4 ± 0.05 μmol/min) while the insulin + T-1032 group showed a significant 39% decrease in insulin-mediated glucose uptake (0.25 ± 0.04 μmol/min). Glucose extraction showed the same changes as hindleg glucose uptake (Fig. 4B).

3.3.1c [^{14}C] 2-DG uptake

R'g or 2-deoxyglucose uptake for combined muscles (Fig. 4C) showed a significant 66% increase with insulin infusion (saline 3.6 ± 0.4, insulin 10.7 ± 1.1 μg/g/min). Insulin infusion alone increased the R'g for soleus (2.3 fold), plantaris (2.1 fold), red gastrocnemius (3.1 fold), white gastrocnemius (1.5 fold), EDL (4.9 fold) and tibialis (5.3 fold) when compared with saline controls (Fig. 4D). When combined, the increase represented 2.9-fold basal. T-1032 infusion did not show a significant difference from saline. The co-infusion of T-1032 with insulin partly inhibited (~20%) the insulin-mediated increase in 2-DG uptake (8.5 ± 0.9 μg/g/min).

3.3.1d 1-MX metabolism

1-MX infusion was started at 60 min before the end of the experiment. A bolus injection of allopurinol was given via the carotid artery 5 min before 1-MX infusion. Allopurinol is converted to oxypurinol, the major inhibitor of the enzyme xanthine oxidase. The arterial levels of oxypurinol did not show significant differences between the various treatment groups, indicating that xanthine oxidase was inhibited to the same extent in all the groups (saline 6.8 ± 0.8, insulin 5.6 ± 0.8, T-1032 7.3 ± 0.7, ins + T-1032 6.6 ± 1.2 μM). No significant difference was found between the treatment groups in arterial 1-MX concentrations (saline 26.2 ± 2.5, insulin 44.2 ± 10.0, T-1032 32.6 ± 2.9, T-

1032 + insulin 25.4 ± 1.7 μM). 1-MX metabolism (Fig. 5) was significantly elevated by insulin, with a 1.6 fold increase in 1-MX metabolism compared to saline. In the insulin + T-1032 group, however, the 1-MX metabolism was not significantly different from insulin alone although it did show a 15% decrease in 1-MX metabolism (saline 6.2 ± 0.5, insulin 9.9 ± 1.4 and insulin + T-1032 8.5 ± 1.0 nmol/min).

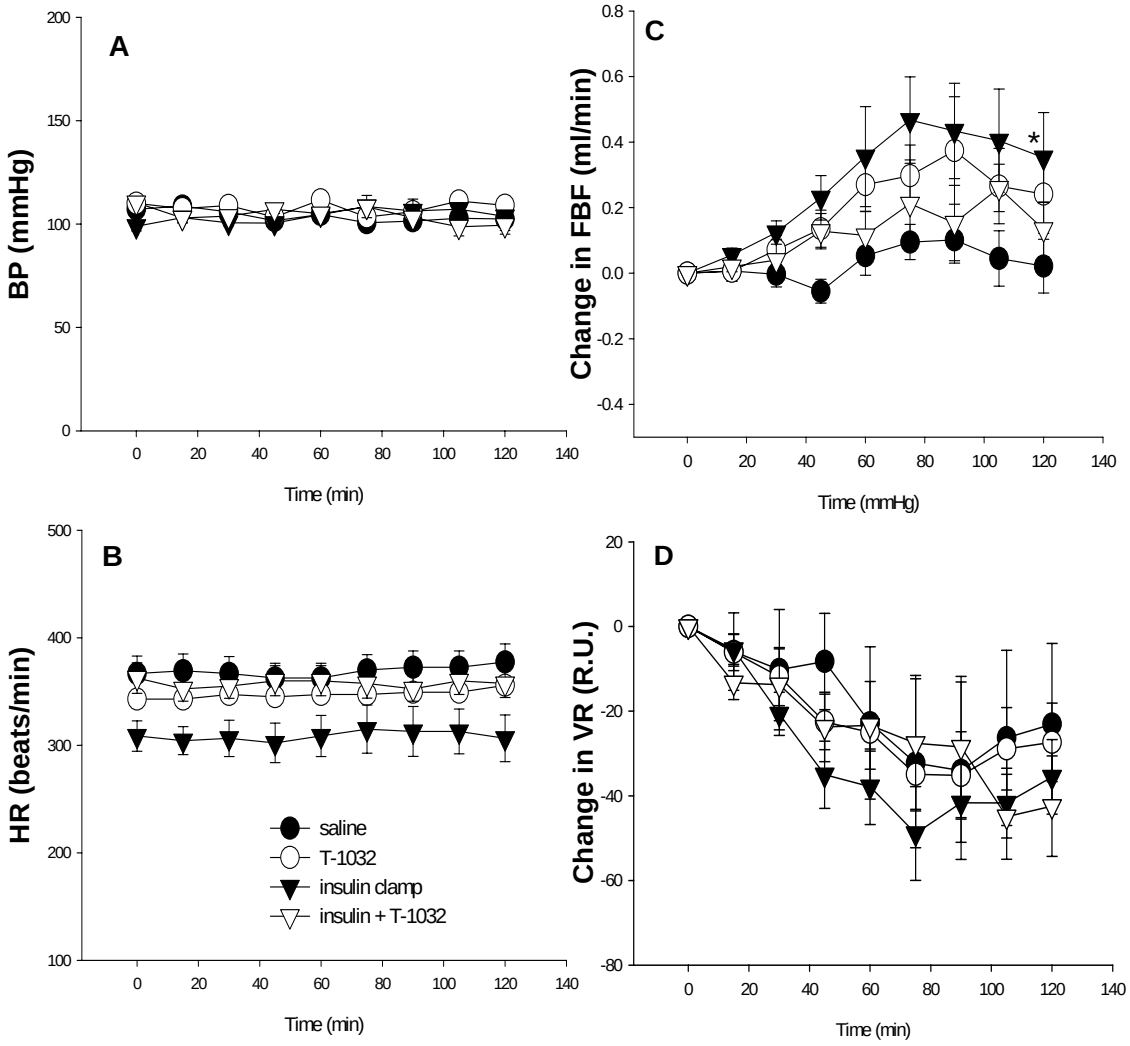


Fig. 2. Mean arterial pressure (A), heart rate (B), changes in femoral blood flow (C) and vascular resistance (D) for saline, 1µg T-1032, 3mU insulin, insulin + 1µg T-1032 treated rats (Protocol A1). Data were collected from 5s sub-samples each 15 minutes. Values are means ± SEM. Significant values from saline are indicated by *, $P < 0.05$.

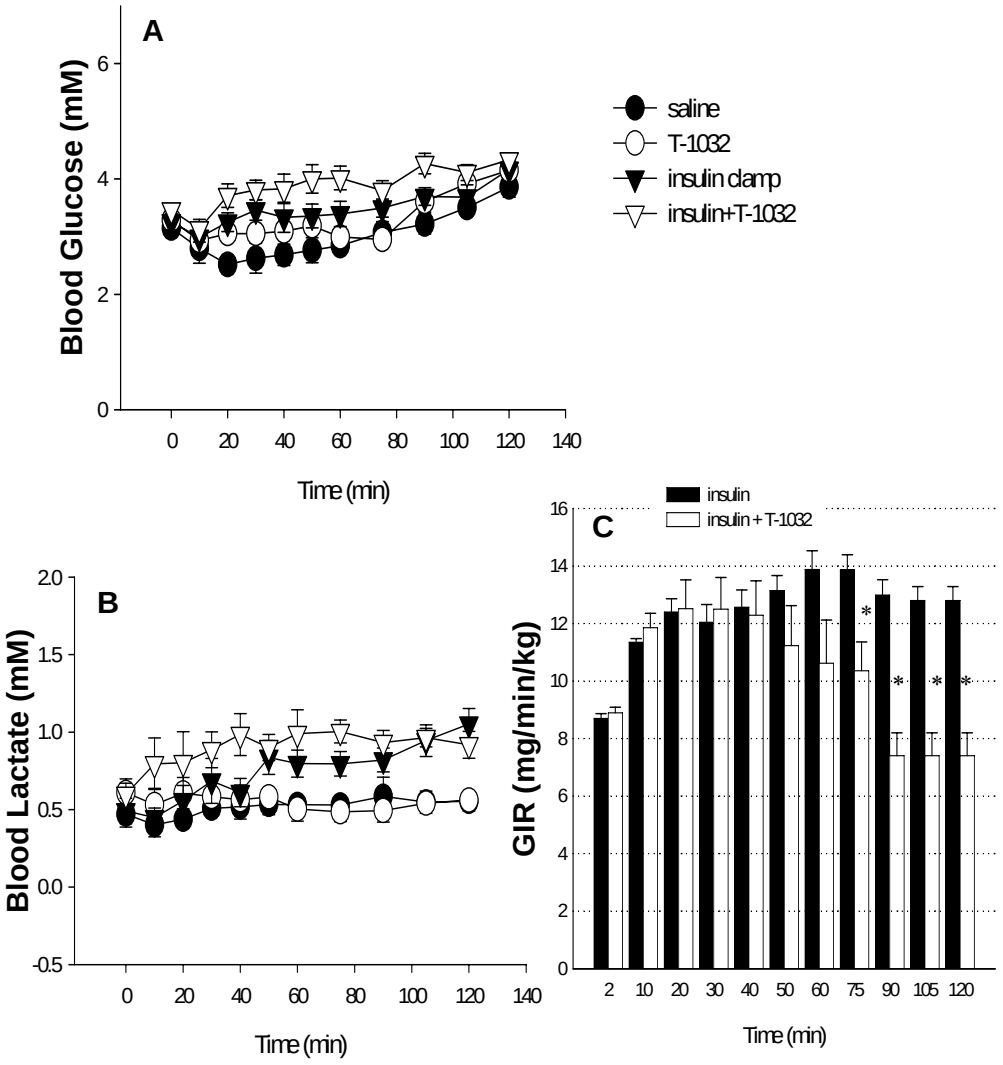


Fig. 3. Blood glucose (A) and blood lactate (B) concentration for saline, 1 μg T-1032, 3mU insulin, insulin + 1 μg T-1032 treated rats (Protocol A1). It also shows glucose infusion rate (C) to maintain blood glucose level at or above basal level during insulin and insulin + 1 μg T-1032 infusions. Values are means ± SEM. Significant values from insulin are indicated by *, $P < 0.001$.

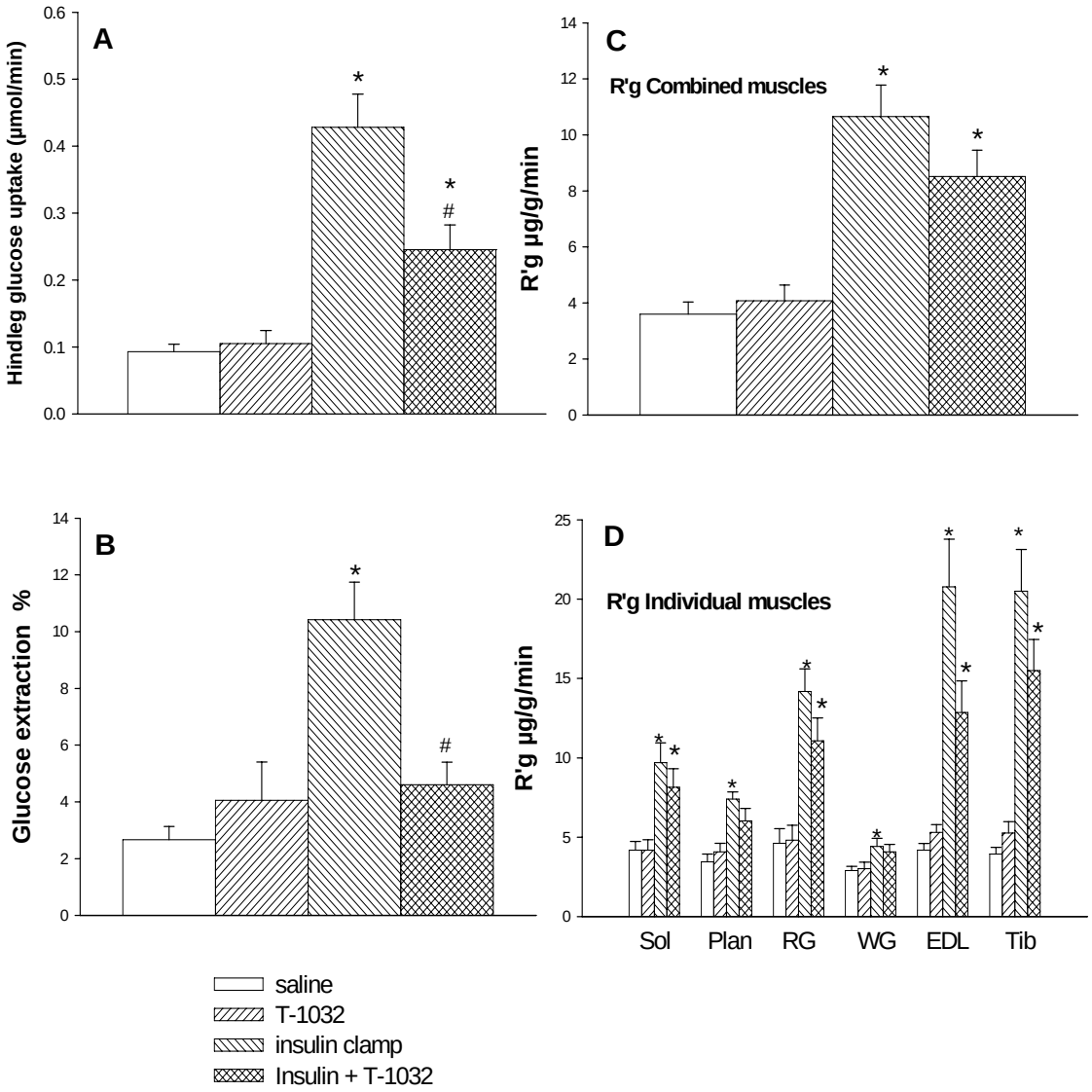


Fig. 4. Hindleg glucose uptake (A), glucose extraction (A-V difference) (B) and R'g calculated from [^{14}C]2-DG uptake for the combination of 6 muscles (C) and for individual muscles (D) (Protocol A1). Different treatment groups were saline, 1 µg T-1032, 3 mU insulin, insulin + 1 µg T-1032. Values are means ± SEM. Significant values from saline are indicated by *, $P < 0.001$. # Indicates that insulin + T-1032 is significantly different from insulin, $P < 0.001$.

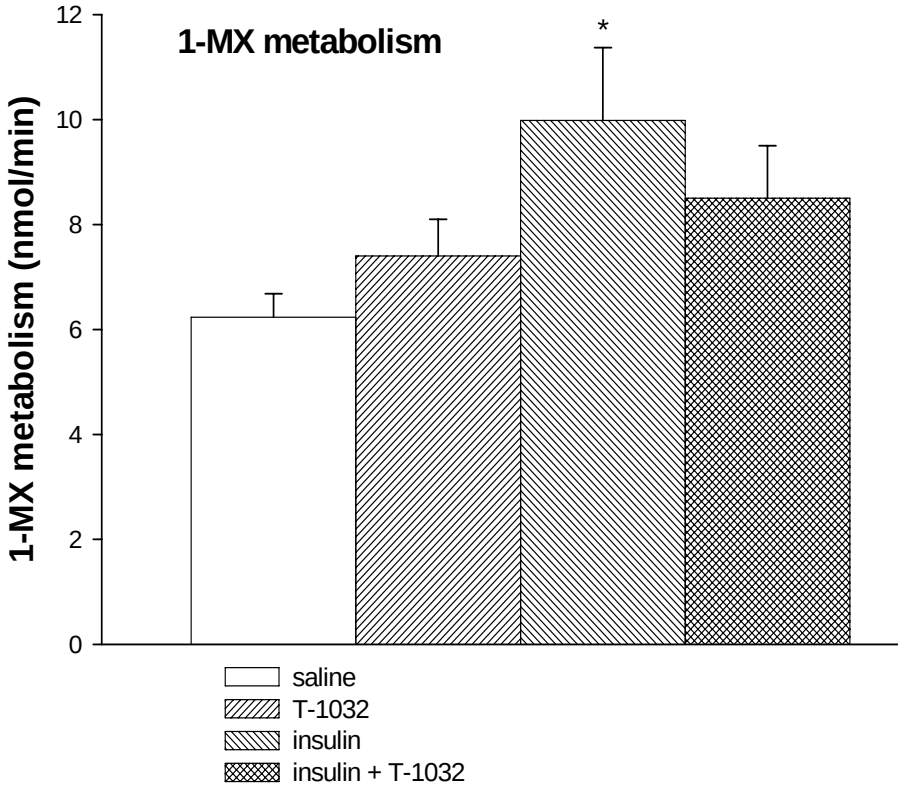


Fig. 5. Hindleg 1-MX metabolism values for saline, 1 µg T-1032, 3mU insulin, insulin + 1 µg T-1032 treated rats (Protocol A1). Values are mean ± SEM. Significant differences from saline are indicated by *, $P < 0.001$.

3.3.2 EFFECTS OF T-1032 (10 µg/min/kg) ON PHYSIOLOGIC INSULIN (Protocol A2)

3.3.2a Hemodynamic effects

There were no significant differences observed in MAP and heart rate between the 4 groups (Fig. 6 A and B) of saline control, T-1032 10 µg/min/kg (hereafter referred as 10µg), euglycemic 3 mU/min/kg insulin clamp and T-1032 10 µg + 3 mU insulin clamps**.** Saline and 10 µg T-1032 2 h infusion alone had no effect on either FBF or vascular resistance (Fig. 6 C and D). Insulin infusion alone caused a significant increase in FBF when compared with saline towards the end of the clamp (from 0.7 ± 0.1 to 1.0 ± 0.1 ml/min), an increase of 33%. Co-infusion of 10 µg T-1032 did not have any significant effect on insulin-mediated increase in FBF but the magnitude of insulin-mediated increase in FBF was decreased from 33 to 20% (0.85 ±. 0.05 at basal to 1.1 ± 0.1 ml/min at 120min). The vascular resistance did not significantly decrease at the end of the experiment in the insulin group and nor did the co-infusion of T-1032 have any effect (from 134.5 ± 7.5 to 103.4 ± 10.7 R.U., co-infusion group).

3.3.2b Glucose metabolism

There were no differences in arterial glucose concentrations between any of the groups during the course of the experiments (Fig. 7A). During the insulin clamp, blood glucose was maintained at or above this level. Fig. 7B also shows the GIR during insulin clamp with and without T-1032 infusion. In order to maintain this basal blood glucose level, glucose was infused at a significantly higher rate in the insulin group. In contrast, co-infusion of T-1032 with insulin led to a significant (64%) decrease in GIR at 120 min (insulin 12.6 ± 0.5, insulin + T-1032 4.6 ± 0.9 mg/min/kg). GIR did not rise as in the insulin group and showed a steady decline. The insulin + T-1032 1 µg group has been included for comparison.

Hindleg glucose uptake, (Fig. 8A) calculated from the extraction multiplied by the FBF, showed a significant elevation in the insulin group (saline 0.1 ± 0.01 to insulin 0.4 ± 0.05 μmol/min). The insulin + T-1032 group showed a significant 70% decrease in hindleg glucose uptake (0.1 ± 0.04 μmol/min). Glucose extraction showed similar changes as hindleg glucose uptake (Fig. 8B).

3.3.2c [^{14}C] 2-DG uptake

R'g or 2-deoxyglucose uptake (Fig. 8 C and D) showed a significant increase with insulin infusion (saline 3.6 ± 0.4, insulin 10.7 ± 1.1 μg/g/min). 10 μg (2 h) T-1032 infusion did not show a significant difference from saline. The co-infusion of 10 μg T-1032 with insulin resulted in a trend indicative of a 20% inhibition (8.5 ± 1.0 μg/g/min, but this was not significant, P = 0.101) of insulin-mediated 2-DG uptake in combined muscles. Insulin + 1 μg T-1032 group showed a similar trend.

3.3.2d Capillary recruitment

1-MX metabolism, indicative of capillary recruitment (Fig. 9) was significantly elevated by insulin, with 1.6 fold significant increase in 1-MX metabolism when compared with saline. In the insulin + 10 μg T-1032 group, however, the 1-MX metabolism was not significantly different from saline, i.e., insulin-mediated capillary recruitment was completely inhibited by T-1032 (saline, 6.2 ± 0.5; insulin, 10.0 ± 1.4; and insulin + 10 μg T-1032, 6.1 ± 1.1 nmol/min). In contrast, 1-MX metabolism in insulin + 1 μg T-1032 group did not differ significantly from insulin.

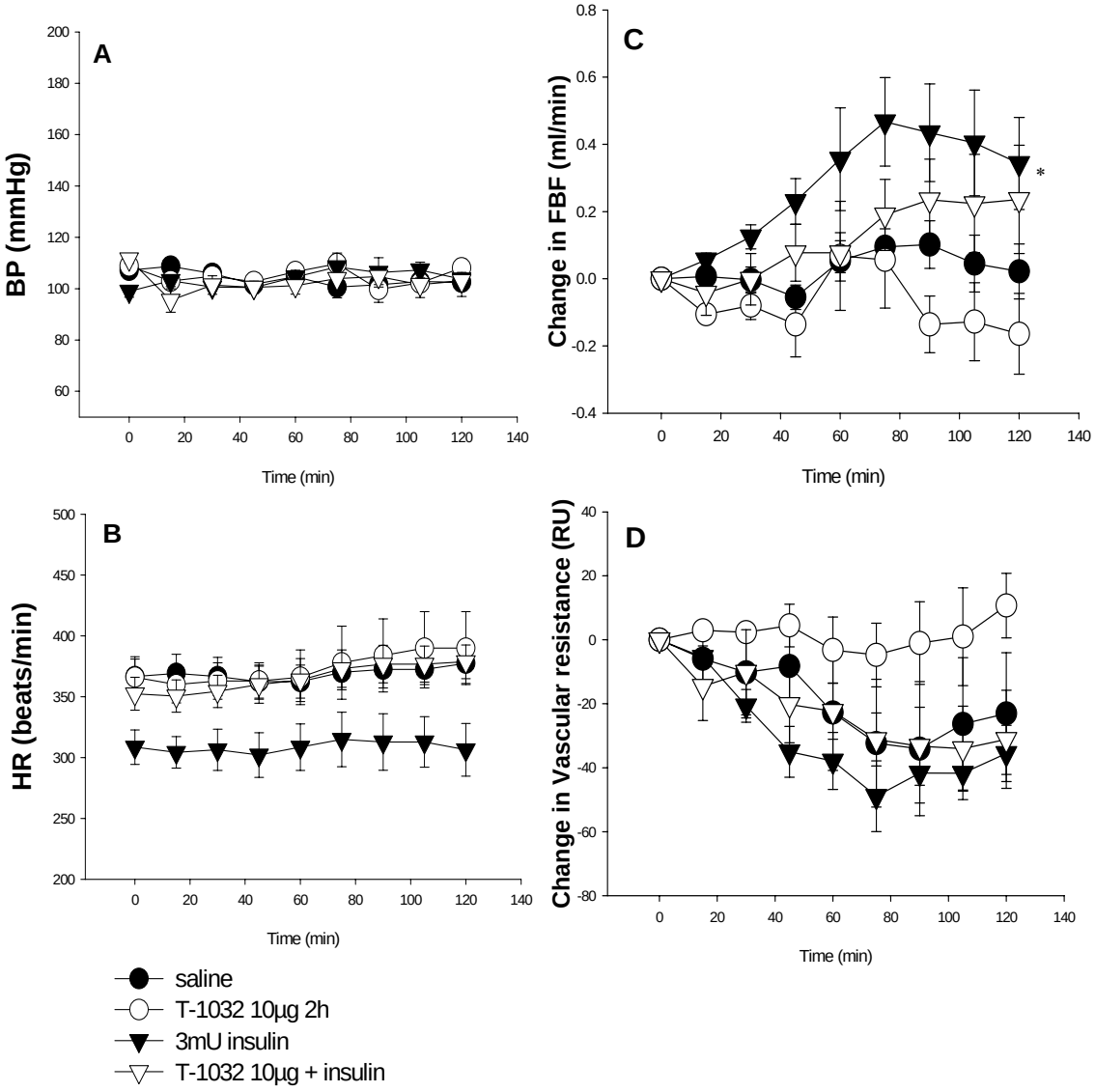


Fig. 6. Mean arterial pressure (A), heart rate (B) and changes in femoral blood flow (C) and vascular resistance (D) for saline, 10 µg/min/kg T-1032, 3 mU/min/kg insulin, and insulin + 10 µg T-1032 treated rats (Protocol A2). Data were collected from 5s sub-samples each 15 minutes. Values are means ± SEM. Significant values from saline are indicated by *, $P < 0.001$.

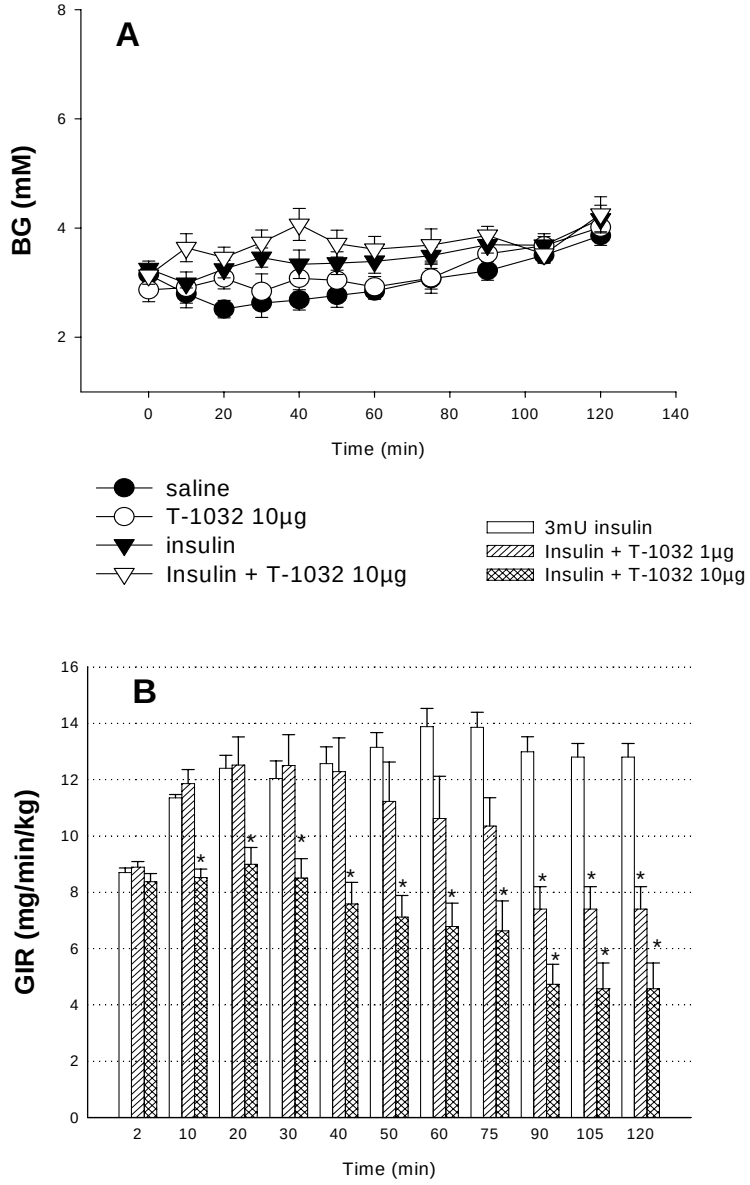


Fig. 7. Arterial blood glucose (A) and glucose infusion rate (B) for saline, 10 µg T-1032, 3 mU insulin, and insulin + 10 µg T-1032 treated rats (Protocol A2). Data from 1 µg T-1032 (protocol A1) is added for comparison. Values are mean ± SEM. Significant values from insulin are indicated by *, $P < 0.001$.

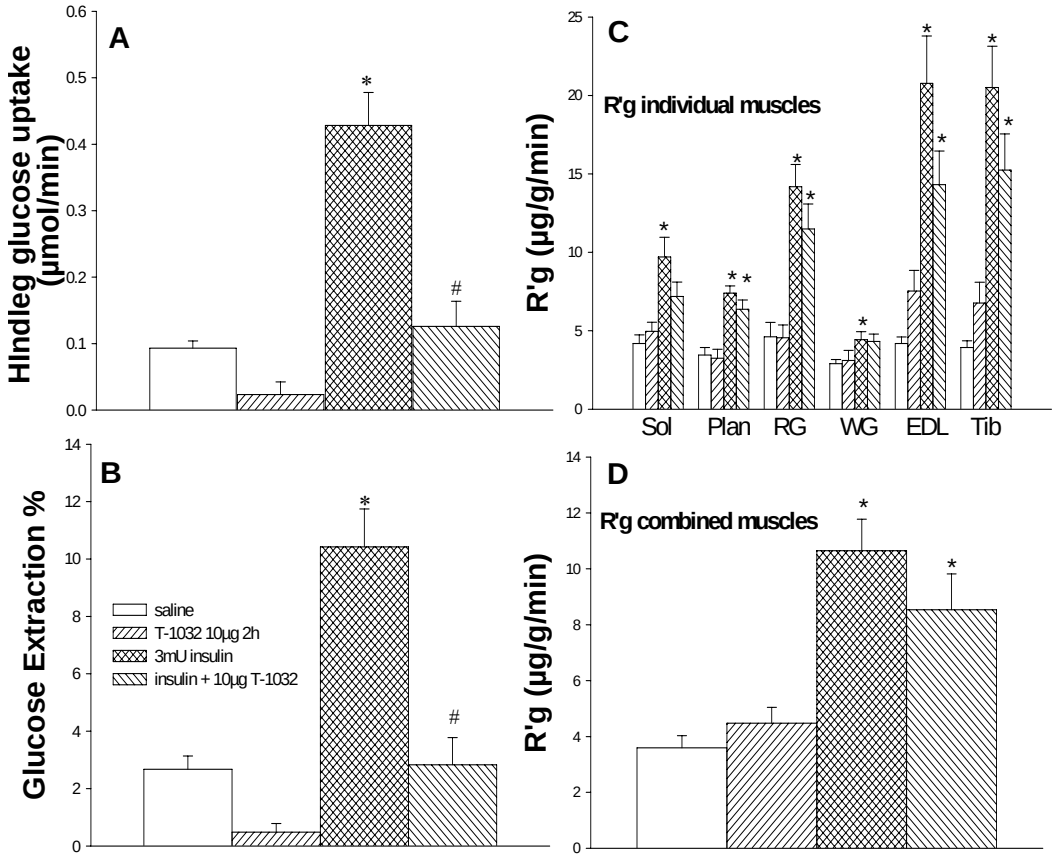


Fig. 8. Hindleg glucose uptake (A), glucose extraction (B) and [^{14}C]2-DG uptake values for combination of 6 muscles (D) and for individual muscles (C). Different treatment groups were for saline, 10 µg T-1032, 3 mU insulin, and insulin + 10 µg T-1032 (Protocol A2). Values are mean ± SEM. Significant values from saline are indicated by *, $P < 0.001$. # indicates that insulin + T-1032 is significantly different from insulin, $P < 0.001$.

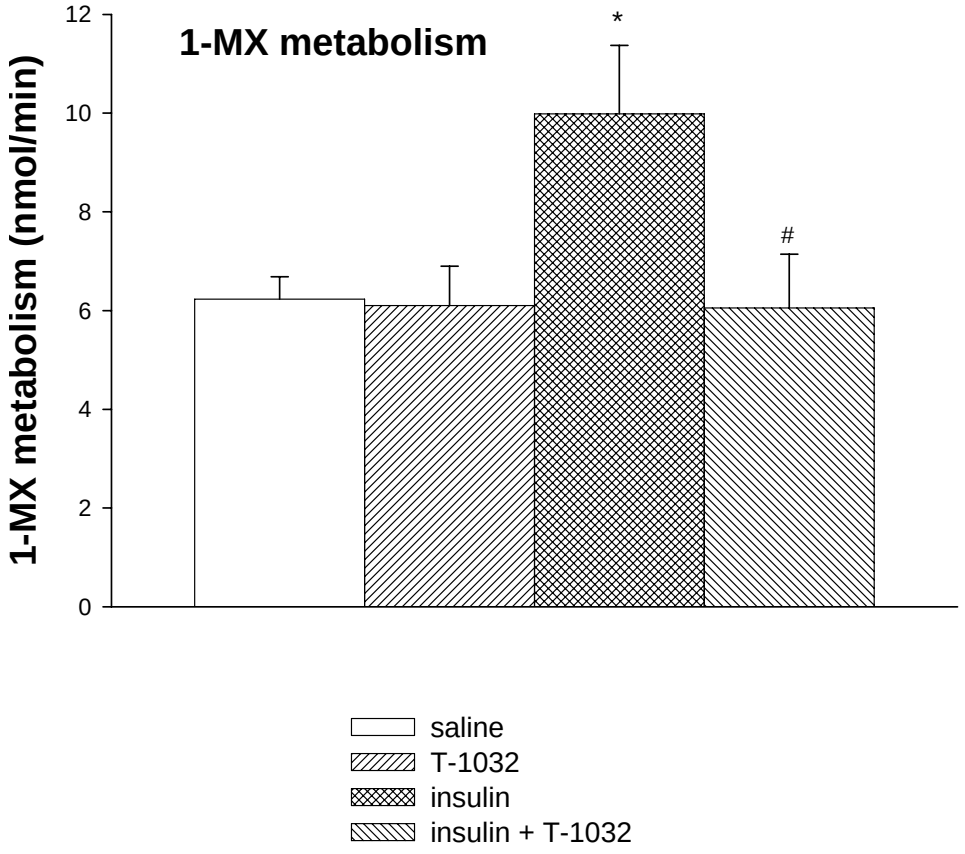


Fig. 9. Hindleg 1-MX metabolism values for saline, 10 µg T-1032, 3 mU insulin, and insulin + 10 µg T-1032 treated rats (Protocol A2). Values are mean ± SEM. Significant values from saline are indicated by *, $P < 0.001$, and from insulin by #, $P < 0.001$.

3.3.3 EFFECTS OF HIGH DOSE OF T-1032 10 µg/min/kg STARTED 1 h BEFORE AND CONTINUED THROUGHOUT THE PHYSIOLOGIC INSULIN CLAMP (Protocol B)

In this group T-1032 10 µg/min/kg was commenced 1 h before 3 mU insulin clamp and then continued with insulin. Thus T-1032 was given for the duration of 3 h. It is obvious from the results obtained so far that co-infusion of T-1032 with insulin did not lead to a significant inhibition of 2-DG uptake, while hindleg glucose uptake was almost completely inhibited. The 2-DG uptake method is more precise since it is measured in 6 individual hindleg muscles, and it indicates an average glucose uptake over a period of 45 minutes, while hindleg glucose uptake is based on single time point measurement at the end of the experiment. Individual GIR values indicated that rats showed variable levels of inhibition, with maximum inhibition at the completion of the experiment, since the decrease in GIR occurred at different time points in different rats, and did not correlate perfectly with 2-DG uptake. One explanation is that inhibition of insulin-mediated glucose uptake by T-1032 is cumulative and exerts its maximum effect at the completion of the experiment when it is indicated by hindleg glucose uptake.

To confirm whether T-1032 results in complete inhibition of insulin-mediated glucose uptake, infusion of T-1032 was commenced 1 h before and during insulin clamp.

3.3.3a Hemodynamic effects

Fig. 10 A and B show the MAP and heart rate during saline control, T-1032 10 µg/min/kg (hereafter referred as 10 µg), euglycemic 3 mU/min/kg (hereafter referred to as 3 mU insulin) insulin clamp and T-1032 10 µg + 3 mU insulin clamps. There were no significant differences observed in MAP and heart rate between the 4 groups.

Saline and 10 µg T-1032 3 h infusion alone had no effect on either FBF or vascular resistance (Fig. 11 A and B). Insulin infusion alone caused a significant increase in FBF over saline towards the end of the clamp (from 0.7 ± 0.1 to 1.0 ± 0.1 ml/min). Infusion of 10 µg T-1032 for 1 h before commencing insulin did not have any effect on FBF and it

completely abrogated the insulin-mediated increase in FBF (1.0 ± 0.1 to 0.9 ± 0.1 ml/min). The vascular resistance did not show any significant change in T-1032 + insulin group (from 112.4 ± 11.6 R.U. basal to 118.1 ± 10.2 R.U. at the end of the experiment).

3.3.3b Glucose metabolism

There were no differences in arterial glucose concentrations between any of the groups during the course of the experiment (Fig. 12A). During the insulin clamp, blood glucose was maintained at or above this level. Fig. 12B shows the GIR during insulin clamp with and without T-1032 infusion. In order to maintain this basal blood glucose, glucose was infused at a significantly higher rate in insulin group. In contrast, pre-infusion and co-infusion of T-1032 with insulin led to significant decreases in GIR from the beginning and glucose infusion was completely stopped by ~30min after starting the insulin in all animals (insulin 12.6 ± 0.5 at 120 min) in the pre-infusion group.

Hindleg glucose uptake, (Fig. 13A) exhibited a significant elevation in the insulin group (saline 0.1 ± 0.01 to insulin 0.4 ± 0.05 μmol/min). T-1032 completely inhibited the insulin effect reducing the hindleg glucose uptake to 0.1 ± 0.02 μmol/min. Glucose extraction showed the same changes as hindleg glucose uptake (Fig. 13B).

3.3.3c [^{14}C] 2-DG uptake

R'g or 2-deoxyglucose uptake (Fig. 13 C and D) showed a significant 66% increase with insulin infusion (saline 3.6 ± 0.4, insulin 10.7 ± 1.1 μg/g/min). 10 μg T-1032 (3 h) infusion did not show a significant difference from saline. The 1 h pre-infusion and co-infusion of T-1032 with insulin resulted in a significant 40% inhibition (6.4 ± 0.6 μg/g/min, $P < 0.05$) of insulin-mediated 2-DG uptake in combined muscles. Insulin-mediated 2-DG uptake was inhibited in soleus by 34%, plantaris 20%, red gastrocnemius 47%, white gastrocnemius 7%, EDL 49% and tibialis by 54%.

3.3.3d 1-MX metabolism

The 1-MX metabolism (Fig. 14) was significantly elevated by insulin. Pre-infusion of T-1032 in the insulin + T-1032 group, however, completely inhibited the insulin-mediated increase in 1-MX metabolism (saline 6.2 ± 0.5, insulin 10.0 ± 1.4 and insulin + T-1032 5.7 ± 0.7 nmol/min).

For the purpose of comparison, 10 μg T-1032 + insulin group has been included for GIR, hindleg glucose uptake, R'g and 1-MX metabolism.

3.3.3e Plasma T-1032 assay

To determine whether T-1032 achieved significant, pharmacologically active concentration in plasma, HPLC analysis of extracted plasma was carried out. Plasma T-1032 levels were measured in the pre-infusion 3 h 10 μg/min/kg T-1032 group by HPLC. The plasma concentration of T-1032 was 0.22 ± 0.003 μM (n = 3, separate animals) at the end of the experiment.

3.3.3f Muscle cGMP assay

A cGMP assay was performed on soleus muscle extracts from saline, insulin, T-1032 10 μg 3 h and T-1032 10 μg 1 h before and during insulin (pre-infusion) groups to see if T-1032 infusion resulted in significant PDE inhibition. Muscle cGMP levels of various treatment groups were (fmol/mg wet wt), saline 38 ± 8.0, T-1032 90 ± 18, insulin 31 ± 4.9 and insulin + T-1032 90 ± 11 (Fig. 15A). T-1032 significantly increased the muscle cGMP levels compared to saline or insulin alone, and was unaffected by insulin co-infusion.

3.3.3g Plasma insulin assay

It is possible that the inhibitory effect of T-1032 on insulin action is due to the suppression of endogenous insulin production. Therefore plasma insulin levels were monitored. Plasma insulin levels (pM) were, saline 143 ± 11, T-1032 10 μg/min/kg × 3 h 159 ± 22, 3 mU insulin 592 ± 91, T-1032 10 μg before and during insulin 518 ± 58 (Fig. 15B). The insulin levels did not differ between insulin and insulin + T-1032 groups.

3.3.3h Plasma FFA assay

To see if T-1032 resulted in a Randle type defect (in which excess FFA would be expected to increase glucose-6-phosphate levels secondary to inhibition of pyruvate oxidation), both plasma FFA and muscle glucose-6-phosphate assay were done. The colorimetric plasma FFA assay did not differ significantly between the various treatment groups. The FFA levels were (meq/L), saline 1.0 ± 0.12, T-1032 10 μg 3 h 1.2 ± 0.16, 3 mU insulin 1.0 ± 0.1 and T-1032 10 μg 1 h before and during insulin 1.0 ± 0.2 (Fig. 16B).

3.3.3i Muscle glucose-6-phosphate assay

Muscle (tibialis) glucose-6-phosphate levels are shown in figure 16A. Muscle content of glucose 6-phosphate was 0.95 ± 0.10 (saline), 0.6 ± 0.09 (insulin), 0.10 ± 0.04 (T-1032) and 0.1 ± 0.05 for T-1032 + insulin (pre-infusion) μmol/g wet weight. Thus T-1032 significantly decreased ($P < 0.05$) glucose 6-phosphate levels with or without insulin.

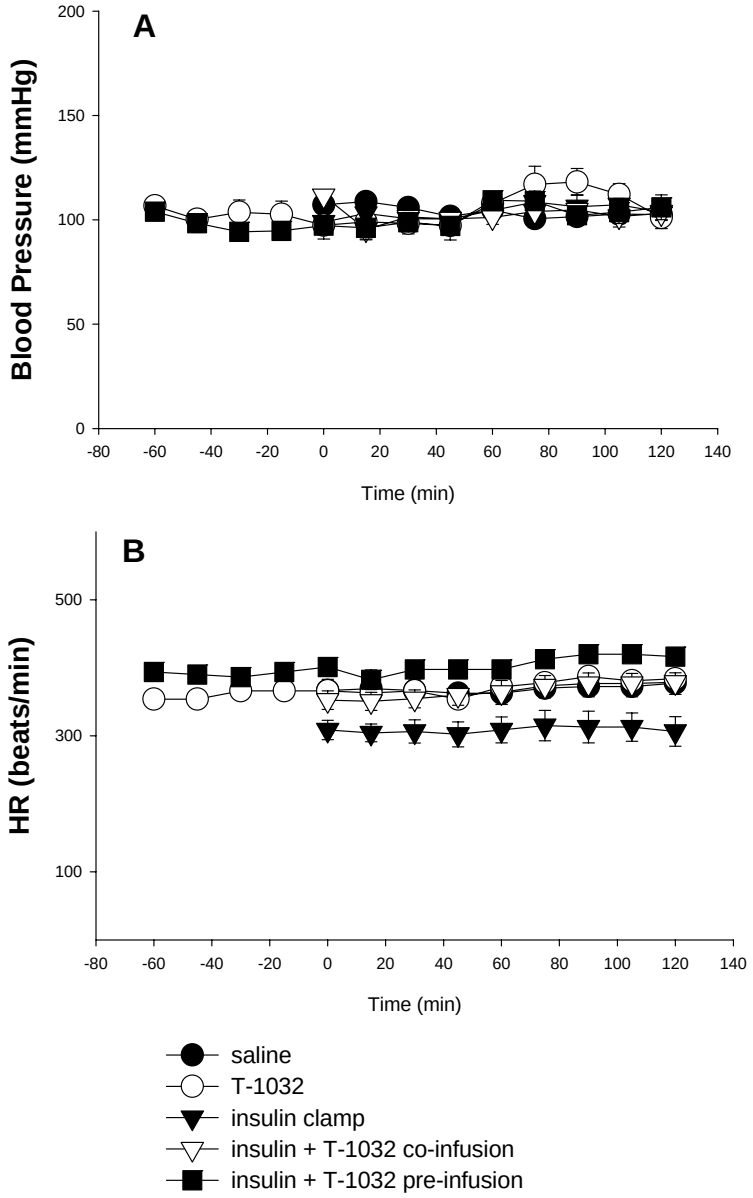


Fig. 10. Mean arterial pressure (A) and heart rate (B) for saline, 10 μg T-1032 × 3 h, 3 mU insulin, insulin + 10 μg T-1032 and (10 μg T-1032 1 h before and during insulin) treated rats (Protocol B). Data were collected from 5s sub-samples each 15 minutes. Values are means ± SEM. Co-infusion values (Protocol A2) are shown for comparison.

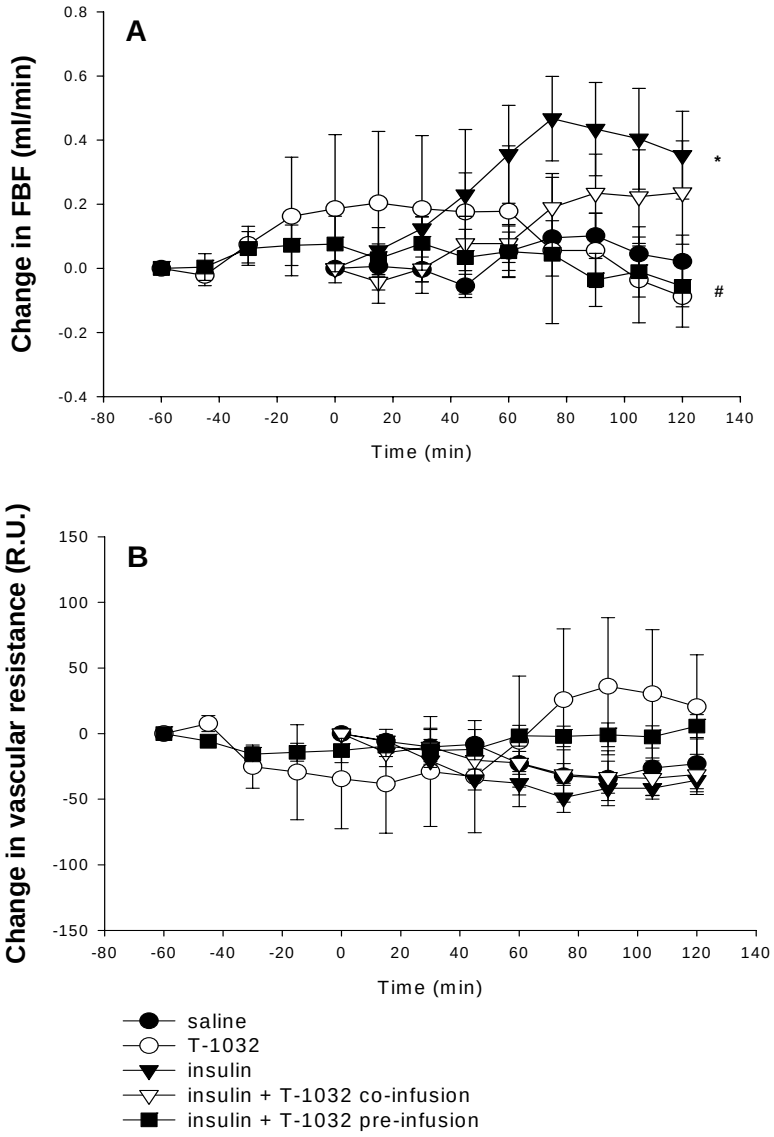


Fig. 11. Changes in femoral blood flow (A) and vascular resistance (B) for saline, 10 µg T-1032 × 3 h, 3 mU insulin, insulin + 10 µg T-1032 and (10 µg T-1032 1 h before and during insulin) treated rats (Protocol B). Co-infusion values (Protocol A2) are shown for comparison. Values are means ± SEM. Significant values from saline are indicated by *, $P < 0.001$. # indicates that insulin + T-1032 pre-infusion is significantly different from insulin, $P < 0.001$.

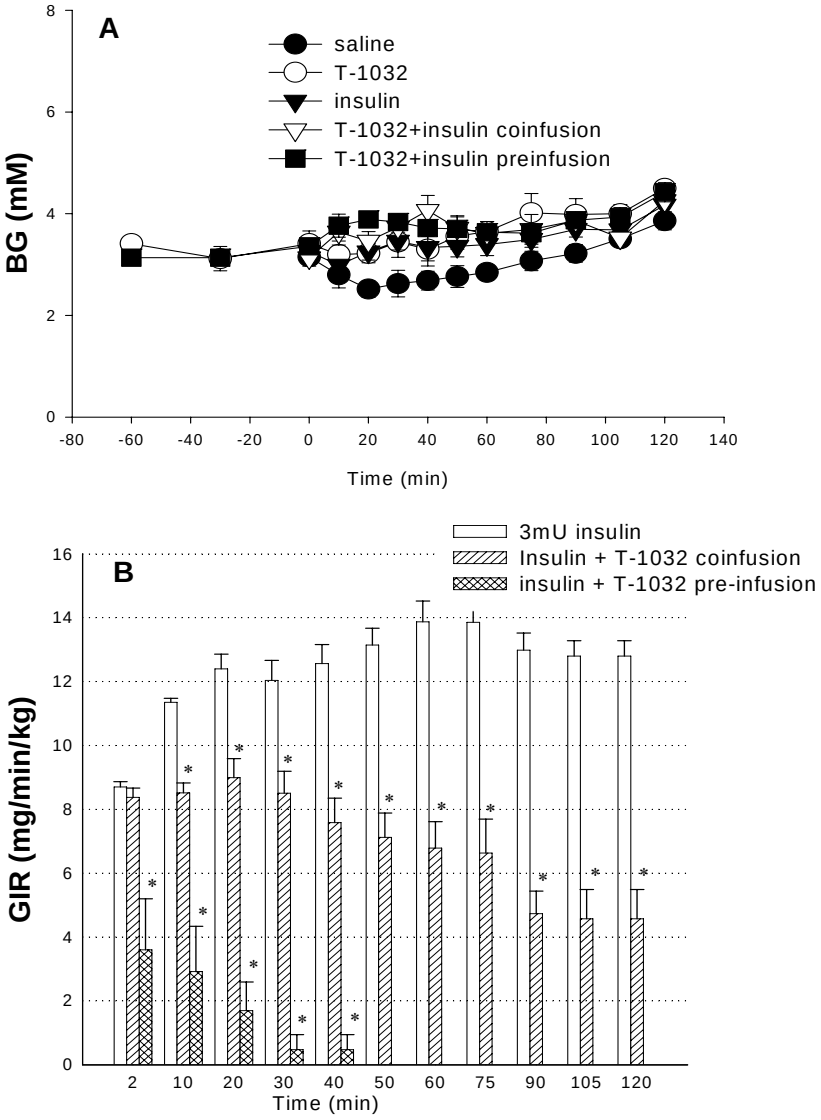


Fig. 12. Arterial blood glucose (A) and glucose infusion rate (B) for saline, 10 μg T-1032 × 3 h, 3 mU insulin, insulin + 10 μg T-1032 and (10 μg T-1032 1 h before and during insulin) treated rats (Protocol B). Co-infusion values (Protocol A2) are shown for comparison. Values are mean ± SEM. Significant values from insulin are indicated by *, $P < 0.001$.

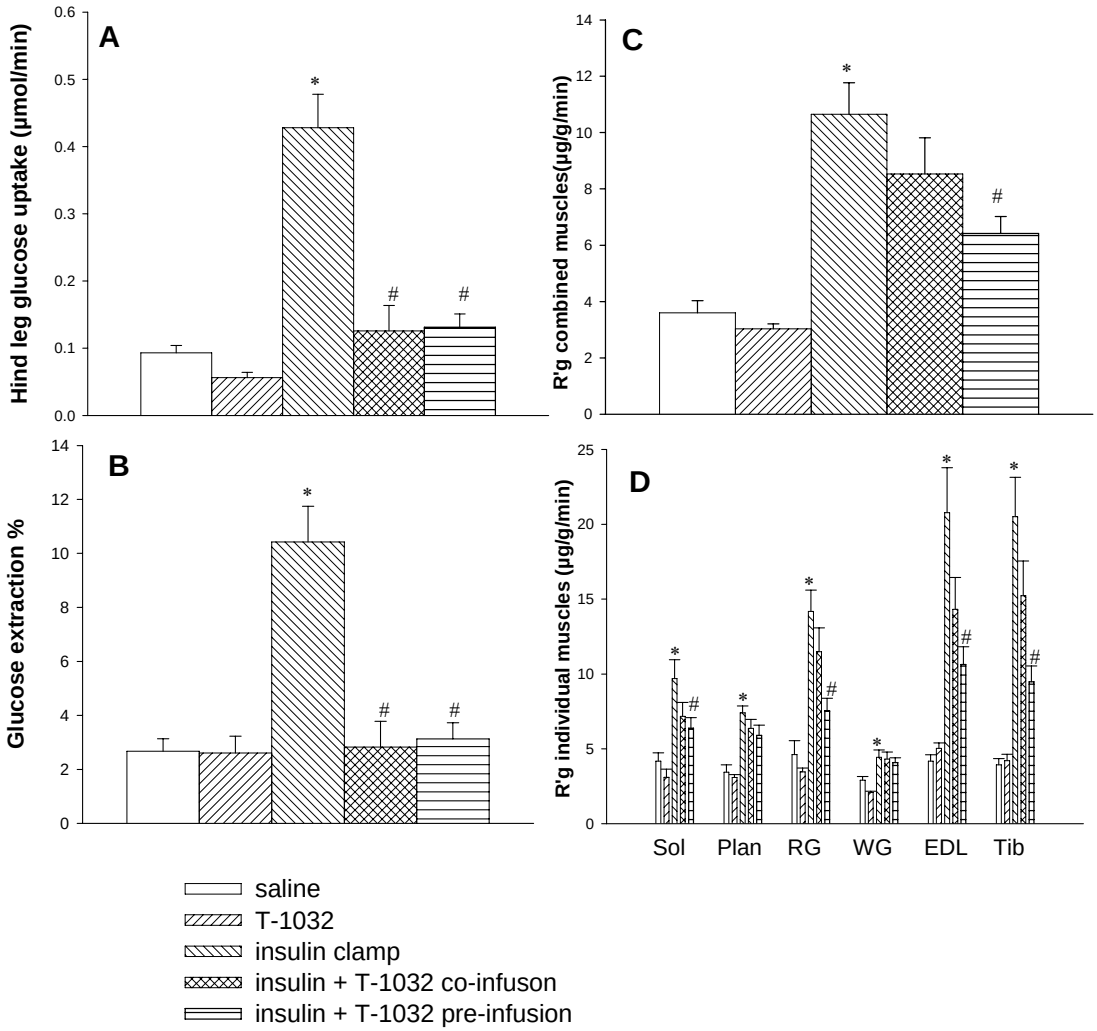


Fig. 13. Hindleg glucose uptake (A), glucose extraction (B) and [^{14}C]2-DG uptake values for combination of 6 muscles (C) and for individual muscles (D). Different treatment groups were saline, 10 µg T-1032 × 3 h, 3 mU insulin, insulin + 10 µg T-1032 and (10 µg T-1032 1 h before and during insulin) treated rats (Protocol B). Co-infusion values (Protocol A2) are shown for comparison. Significant differences from saline are indicated by *, $P < 0.001$. # indicates that insulin + T-1032 is significantly different from insulin, $P < 0.001$.

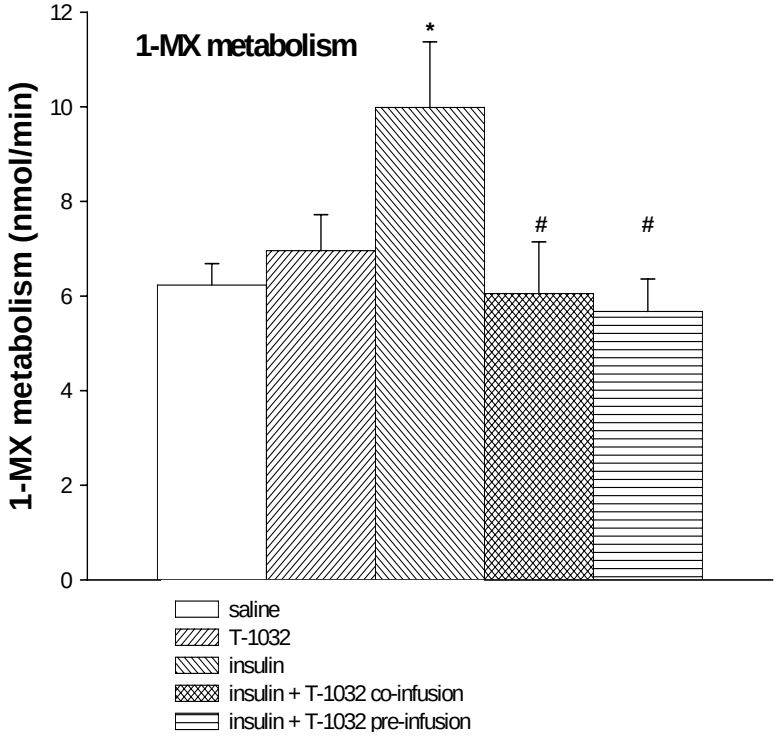


Fig. 14. Hindleg 1-MX metabolism values for saline, 10 µg T-1032 × 3 h, 3 mU insulin, insulin + 10 µg T-1032 and (10 µg T-1032 1 h before and during insulin) treated rats (Protocol B). Co-infusion values (Protocol A2) are shown for comparison. Values are mean ± SEM. Significant differences from saline are indicated by *, $P < 0.001$, and from insulin by #, $P < 0.001$.

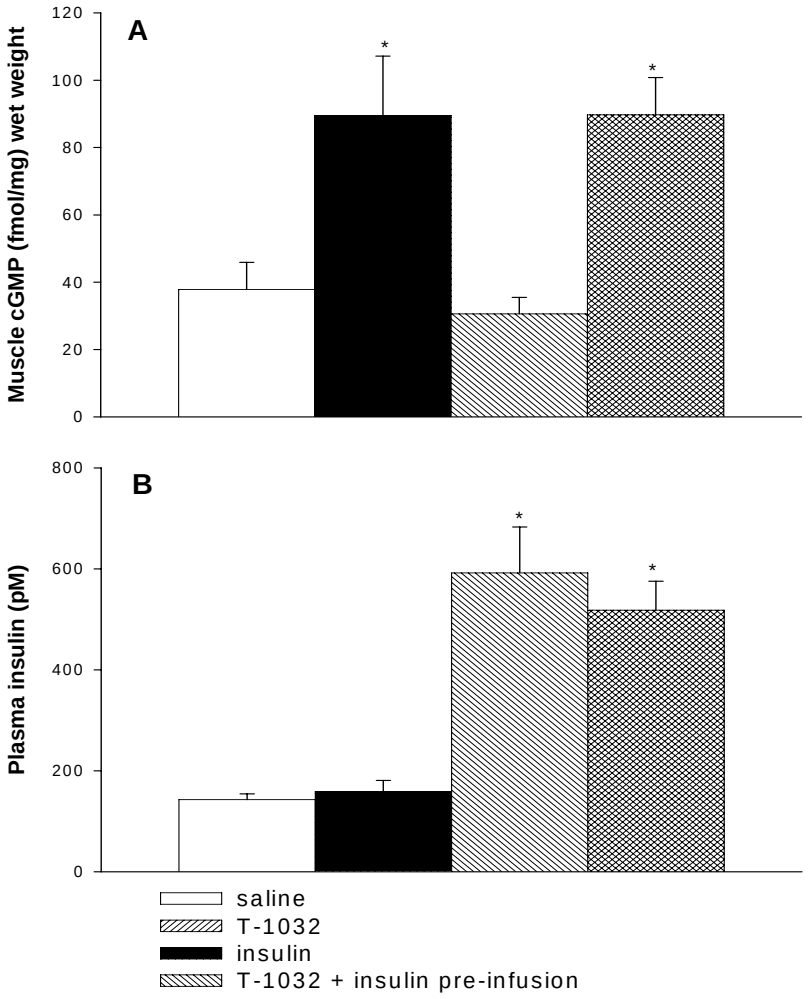


Fig. 15. Muscle cGMP (A) and plasma insulin (B) concentrations for saline, T-1032 10 μg × 3 h, 3 mU insulin, and (T-1032 10 μg 1 h before and during insulin) treated rats (Protocol B). Values are mean ± SEM. Significant differences from saline are indicated by *, $P < 0.001$.

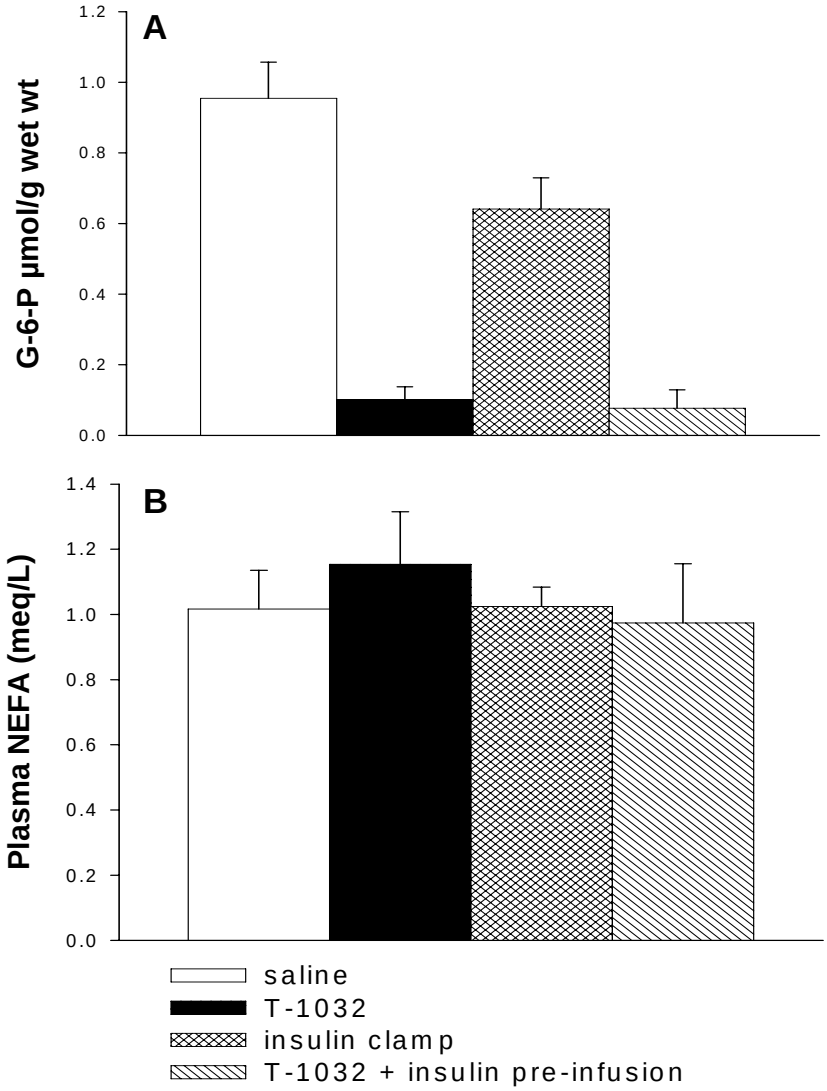


Fig. 16. Muscle glucose-6-phosphate (A) and plasma FFA (B) values for saline, T-1032 10 µg × 3 h, 3 mU insulin, and (T-1032 10 µg 1 h before and during insulin) treated rats (Protocol B). Values are mean ± SEM. Significant differences from saline and insulin are indicated by *, $P < 0.001$.

3.4 DISCUSSION

This study was undertaken to see if the cGMP-PDE5 inhibitor T-1032, potentiates insulin-mediated capillary recruitment and skeletal muscle glucose uptake. The main finding emerging from this study was that T-1032, a specific PDE-5 inhibitor, was antagonistic of insulin's vascular and metabolic actions. The current data presented here indicate that acute infusion of T-1032 induced a state of insulin resistance *in vivo.*

The study examined four treatment groups, (i), low dose T-1032 (1 μg/min/kg) with or without 3 mU/min/kg (hereafter referred as 3 mU) hyperinsulinemic euglycemic clamp; (ii), high dose T-1032, (10 μg/min/kg) with or without 3mU insulin; (iii), high dose T-1032 (10 μg/min/kg) commenced 1 hour before and during 3 mU insulin clamp and 3 h T-1032 infusion with saline; (iv), saline control.

An insulin clamp (3 mU) alone did not affect blood pressure/heart rate, but a significant increase in femoral blood flow was apparent from 60 minutes onwards when compared with saline. Whole body GIR, hindleg glucose uptake, R'g and 1-MX metabolism (an index of capillary recruitment) were significantly elevated in the insulin group. T-1032 alone did not show any significant effects on any of measured parameters. High dose T-1032 for 1 h preceding and during insulin almost completely inhibited insulin action. It was noted that T-1032 completely blocked GIR by 1 h, hindleg glucose uptake and capillary recruitment at the end of experiment and R'g at the end by 50%. In contrast, both the low and high doses of T-1032 co-infused with insulin showed only partial blocking activity against insulin with almost complete inhibition of insulin-mediated increases in FBF, capillary recruitment and hindleg glucose uptake, while the insulin-mediated stimulation of 2-deoxyglucose uptake was blocked by 25%.

The doses used in this study were based on previous studies by other investigators [259]. Nevertheless, preliminary studies showed that infusion of T-1032 resulted in a temporary fall in mean arterial pressure by 6-8 mmHg for 5-10 minutes. Also the co-infusion of T-1032 1 μg/min/kg with 3 mU/min/kg insulin significantly decreased the

GIR. It is difficult to compare the hemodynamic response to T-1032 observed in this study with other studies, since this is the first time T-1032 has been infused for 3 hours in an *in vivo* setting in Hooded Wistar rats. However, T-1032 infusion alone did not affect the heart rate during the 2-3 h infusion.

The results obtained in this study were unexpected because it was speculated that a cGMP-PDE5 inhibitor would cause vasorelaxation by increasing cGMP levels in terminal arterioles of skeletal muscle thereby potentiating insulin-mediated capillary recruitment and skeletal muscle glucose uptake. NO of endothelial origin is thought to act as a paracrine signal [103, 130] and after entering nearby vascular smooth muscle cells of the terminal arterioles that control blood flow entry to further capillary networks, activates guanylate cyclase to produce cGMP. Cyclic GMP so formed activates a phosphorylation cascade to lower intracellular calcium ions and relax the smooth muscle. Cyclic GMP is destroyed by a number of isoforms of PDE that can be expressed in a tissue-specific manner. NO could also be formed in the muscle under the impetus of insulin. NO donors and cGMP analogues have been demonstrated to stimulate glucose transport and the rates of lactate release and glucose oxidation in isolated incubated rat skeletal muscle preparations [111]. Whereas the effector mechanism used by NO to stimulate glucose metabolism in this tissue is yet to be defined, the neuronal form of nitric oxide synthase is expressed in the myocytes. Zaprinast has been reported to have such activity *in vitro*. Thus, it has been demonstrated by Young et al that zaprinast stimulated cGMP formation and indices of increased glucose metabolism by isolated incubated muscle of lean Zucker rats [261].

Also, PDE5 has been shown as the most important cGMP hydrolyzing isoform in VSMC [144, 145]. Vasorelaxation by PDE5 inhibition has previously been demonstrated using other PDE5 inhibitors such as zaprinast [151, 152], sildenafil [144] and dipyridamole [143] in a number of studies. Moreover, T-1032 has been shown to produce vasorelaxation by several groups of workers [244, 257-260]. Taken together, there are enough data available to suggest that PDE5 inhibitors act as vasorelaxants and it could reasonably be expected that T-1032 administered to anaesthetized rats as in the present

study would have vascular effects in the hindlimb consistent with vasodilation. In fact, apart from a transient lowering of pressure that quickly reversed, this did not occur either for T-1032 administered alone or when administered with insulin.

There are no previous studies reporting a possible direct effect of T-1032 on insulin action or muscle metabolism. It is clear from the present study that T-1032 adversely affected the insulin-mediated increase in capillary recruitment and skeletal muscle glucose uptake. T-1032 infusion alone had no effect on hindlimb glucose uptake but markedly inhibited the insulin stimulation of glucose uptake and was associated with decreased glucose infusion rates to maintain euglycemia. In conjunction with this, the insulin-mediated increase in FBF and decrease in vascular resistance was prevented. In addition, the effects of this agent were time- and dose-dependent. Exposure for 2 h to T-1032 infused at 10 μg/min/kg produced inhibitory effects that were considerably less than exposure for 3 h and a dose of 1 μg/min/kg for 2 h had an even milder effect.

This study has been the first of its kind, looking at the effect of a specific PDE5 inhibitor, T-1032 on insulin action *in vivo*. The question arises as to what could be the probable causes of T-1032-induced inhibition of insulin-mediated increase in GIR and glucose uptake.

The main cause of inhibition of insulin-mediated 2-DG uptake induced by acute *in-vivo* infusion of T-1032 appears to be hemodynamic i.e., complete inhibition of insulin-mediated capillary recruitment leads to partial attenuation of insulin-mediated glucose uptake. It is probable that T-1032 has negatively affected insulin's action at the endothelium. Thus the complete blockade of capillary recruitment in the present study may account for the partial inhibition of lower leg R'g and some of the inhibition of hindleg glucose uptake. In addition, since T-1032 also inhibited the insulin-mediated increase in FBF, the effect of the PDE inhibitor was not restricted to the microvasculature. Thus a general inhibitory effect of T-1032 targeted at insulin-mediated signaling in endothelial cells of both small vessels (affecting capillary recruitment) and large vessels (affecting bulk blood flow) is likely.

T-1032 might also be redistributing the blood flow to the non-nutritive capillaries, in effect denying access of glucose and insulin to metabolically active tissue, (nutritive route), and thus might be responsible for the inhibition of insulin action. However, a direct cellular action of T-1032 to inhibit insulin signaling on glucose uptake, analogous to TNF-α, cannot be ruled out. It is possible that an inhibitory effect of T-1032 has developed over time to reduce insulin-mediated glucose uptake within the skeletal muscle cells. Inhibition of insulin-mediated capillary recruitment very likely would only account for about 50% of the hindleg glucose uptake and R'g. Thus complete inhibition of hindleg glucose uptake assessed by AV glucose difference at the end of the clamp would indicate that the inhibitory effects of T-1032 have become manifest in the myocytes. This time-dependent effect of T-1032, evident from the GIR results, might also explain why R'g values only reflected partial inhibition, as R'g values represent the average over the last 45 min of the clamp, whereas hindleg glucose uptake is determined from blood samples at the end of the clamp.

Another possibility is that T-1032 may inhibit metabolic vasodilatation, i.e., it may inhibit the effect of a vasodilator substance produced as a result of insulin stimulation of glucose uptake since T-1032 infusion prevented insulin-mediated increase in FBF. Thus a general inhibitory effect of T-1032 targeted at insulin-mediated signaling in endothelial cells of both small vessels (affecting capillary recruitment) and large vessels (affecting bulk blood flow) is likely.

This lab was the first to report and measure insulin's direct effect on capillary recruitment within skeletal muscle in rat hindleg after 2 h of hyperinsulinemic euglycemic clamp [57]. The technique uses 1-MX metabolism as a marker for capillary recruitment (nutritive flow) in muscle. 1-MX is metabolized by a capillary-endothelial enzyme, xanthine oxidase (XO) to 1-methylurate. The infusion of physiological levels of insulin, by recruiting nutritive capillaries, increases the skeletal muscle capillary endothelial XO and leads to enhanced metabolism of 1-MX. Inhibitory changes in capillary flow modulates 1-MX metabolism, and this has been demonstrated *in vivo* in studies with

alpha methyl serotonin, TNF-α..and Intralipid® heparin infusion [65-67]. It has been reported that hindleg glucose uptake and capillary recruitment are functionally coupled and show parallel adjustments. In fact, the T-1032 results from this study are similar to those obtained with TNF-α and to some extent with α-methyl serotonin. It has been suggested that inhibition of 1-MX metabolism by α-methyl serotonin involves redistribution of blood flow preferentially to the non-nutritive route, preventing insulin-mediated capillary recruitment. Acute infusion of α-methyl serotonin, results in inhibition of insulin-mediated capillary recruitment and glucose uptake, probably by constricting arterioles supplying nutritive capillaries. Thus, this study along with TNF-α and α-methyl serotonin studies, indicates that when capillary recruitment by insulin is blocked *in vivo*, an acute state of insulin resistance is induced.

It is possible that the PDE5 isoform inhibited by T-1032 is not present in terminal arterioles regulating the blood flow to nutritive capillaries in skeletal muscles or is not the one involved in insulin-mediated capillary recruitment and glucose uptake. It is also possible that PDE5 is not present in the terminal arterioles of skeletal muscle. Previous studies done in our lab using zaprinast, which is an inhibitor of multiple PDEs including PDEs 2, 5, 7, 9, 10 and 11 [148, 262] showed that zaprinast augmented the insulin-mediated glucose uptake and capillary recruitment but this elevation did not reach statistically significant levels. Keeping this in mind and the finding that T-1032 has been reported to be highly selective for PDE5 [156], it is possible that PDE isoforms other than PDE5 are involved in the NO-cGMP pathway in terminal arterioles of skeletal muscle. In support of this notion, it has been demonstrated that PDE families show tissue-specific distribution in cardiovascular system. PDE 1, 4 and 5 have been detected in saphenous vein while human mesenteric artery showed the presence of PDEs 1-5 [148]. Human pulmonary artery showed a high level of PDE5 activity; canine aorta contained higher activity of PDE1 than of PDE5 [146, 149].

Muscle cGMP levels were significantly elevated in response to T-1032 (10 μg/min/kg) with or without insulin. It does not, however, indicate the source of cGMP, which could be in the myocytes, smooth muscle cells, or both. However, it is likely that

the increase in cGMP is largely attributable to an inhibition of cGMP PDE in the myocytes. A build up of cGMP in vascular smooth muscle would be expected to cause vasodilatation and in this respect others have reported that T-1032 at 10 μg/min/kg lowered MAP by 16.8% in mecamylamine- and noradrenaline-treated anaesthetized rats [259]. In the present study, although a similar dose of T-1032 transiently lowered the MAP during the first 5 min, the effect was not sustained. Insulin infusion alone did not exhibit a detectable increase in cGMP levels.

Attempts were made to investigate other possible causes of T-1032-induced insulin resistance. It was thought that it could be a direct or indirect cellular effect of T-1032 to inhibit glucose uptake, a Randle type defect (in which excess FFA would be expected to increase glucose-6-phosphate levels secondary to inhibition of pyruvate oxidation). Increased triglyceride content in muscle increases the long chain acyl-CoA species which disrupts the insulin signaling cascade leading to attenuated GLUT4 transport [263, 264]. Artificial elevation of plasma FFA levels has been demonstrated in healthy humans to reduce insulin-mediated glucose uptake starting at 1.5 h [265]. It has been suggested that complete attenuation of insulin-mediated capillary recruitment will impair the clearance of triglyceride rich particles, thus creating a state of fat-induced insulin resistance [99].

It is also possible that T-1032 raises the levels of muscle cAMP inducing glycogenolysis thus increasing glucose-6-phosphate levels. Therefore, plasma FFA and muscle glucose-6-phosphate assays were done. Plasma FFA levels did not show any significant differences between the various treatment groups, thus ruling out the involvement of elevated FFA levels. This could happen if T-1032 elevated the cAMP levels in tissues such as adipocytes. T-1032 also significantly decreased muscle glucose-6-phosphate levels with or without insulin.

In the present study, no attempt was made to assess the relative contribution of the effects of T-1032 to muscle and liver glucose metabolism. Yet, since GIR was completely inhibited by T-1032 after 3 h and the combined muscle R'g was only inhibited by 50%, an effect of the PDE5 inhibitor to block insulin-mediated inhibition of hepatic glucose

output would seem likely. Clearly, this warrants further investigation. Finally, the possibility that T-1032 may have interacted with other pathways unrelated to cGMP cannot be ruled out. Such 'non-specific' interaction could account for the present findings and result from the particular chemistry of T-1032. For example, in preliminary studies (unpublished) using another PDE5 inhibitor, zaprinast, which differs in structure from T-1032, we found insulin-mediated glucose uptake by muscle *in vivo* not to be inhibited, even though it increased lactate release, as reported by others [261]. Clearly, additional members of this class of PDE5 inhibitors will need to be tested before a general conclusion can be made regarding the potential diabetogenic nature of these substances. However, HPLC analysis of the T-1032 revealed only one component, and thus the inhibitory effects reported herein are unlikely to be attributable to a contaminant.

It has been reported that PDEs play an important role in regulating the pool of pancreatic β-cell cyclic AMP and in the modulation of glucose-induced insulin secretion [266]. PDE inhibitors which elevate pancreatic β-cell cAMP potentiate glucose-stimulated insulin release, with no effect on basal insulin release. Although PDE5 has not been reported in pancreatic beta cells and T-1032 has not been found to raise cAMP levels, plasma insulin assay was done to rule out this possibility. Plasma insulin levels were not significantly affected by T-1032.

Thus, this study clearly indicates the unfavorable effects of the PDE5 inhibitor T-1032 on insulin sensitivity. In the lieu of studies being carried out by other groups with T-1032, emphasizing its role as a potential tool for pulmonary hypertension (T-1032 has been reported to specifically and potently dilate pulmonary vessels [244, 258]), this study clearly suggests that acute infusion of T-1032 induces insulin resistance in rats, thus highlighting the potential danger of using T-1032 in patients with diabetes mellitus.

In conclusion, the results show that the acute infusion of T-1032 in anesthetized rats *in vivo* led to insulin resistance in rats, uncovering the diabetogenic effect of T-1032. Administration of T-1032 before and during hyperinsulinemic euglycemic clamp prevented insulin action to increase skeletal muscle blood flow, capillary recruitment and

glucose uptake (50%). Although a direct or indirect effect of T-1032 on glucose uptake in muscle cannot be ruled out, the data presented in this study suggest that the complete inhibition of insulin-mediated capillary recruitment by T-1032 may account for the 50% inhibition of insulin-mediated glucose uptake, thus reinforcing the view that insulin-mediated capillary recruitment makes a hemodynamic contribution to glucose uptake. However, the mechanism by which T-1032 appears to block insulin action remains elusive.

CHAPTER 4

DIFFERENTIAL EFFECT OF VASODILATORS ON INSULIN-MEDIATED GLUCOSE UPTAKE AND CAPILLARY RECRUITMENT IN MUSCLE USING THE TECHNIQUE OF LOCAL INFUSION

4.1 INTRODUCTION

Recent evidence suggests that insulin-mediated increases in bulk blood flow [32] and capillary recruitment [57] are NO-dependent [52, 63]. When nitro-vasodilators such as bradykinin [44], nitroprusside [41] are infused, bulk flow is markedly augmented without an effect on insulin action. These results suggest that bulk blood flow changes are not necessary for insulin's metabolic actions in muscle. One exception is the vasodilator methacholine (MC), which increased total flow, and has been reported by two groups to enhance insulin action. In 1994 Baron et al [32] reported that methacholine augmented insulin-mediated glucose uptake across the leg of young healthy subjects. Similarly, Sarabi et al [55] reported that methacholine but not sodium nitroprusside increased forearm glucose uptake of hypertensive insulin resistant subjects. Thus it is puzzling why various vasoactive agents that act via NO-dependent vasodilatation have such different metabolic outcomes when insulin's hemodynamic actions are also NO-dependent. Baron et al [32] have proposed that the difference in vasodilator metabolic action may relate to the specific sites within the vasculature that are affected.

4.1.1 Aim of the study

In the present study using a novel approach of local infusion in the rat leg *in vivo,* the effects of enhancing NO production by using endothelium-dependent nitro-vasodilators methacholine and bradykinin on physiologic insulin in terms of macro- and microvascular

hemodynamic changes were compared. Methacholine was also infused systemically in a separate group of rats with or without insulin to see its effect on insulin-mediated capillary recruitment and glucose uptake. The systemic infusion had a profound effect on systemic hemodynamics, which led to the development of a local technique in which the epigastric artery, a branch of femoral artery, was cannulated to infuse the vasodilators methacholine and bradykinin. The data show that while both endothelium-dependent nitro-vasodilators infused locally increase leg blood flow to similar levels, only the one that augments capillary recruitment is able to enhance insulin's stimulation of glucose uptake.

4.2 RESEARCH DESIGN AND METHODS

4.2.1 Animals

Male Hooded Wistar rats weighing 286 ± 3 were raised on a commercial diet as described in chapter 2.1.

4.2.2 Surgical preparation

The intention was to conduct a hyperinsulinemic euglycemic clamp in rats, so that the effects of a local infusion of the vasodilators methacholine and bradykinin could be assessed in the absence of systemic perturbations. Particular focus was on effects imposed on the hemodynamic effects of insulin. Details were as essentially described previously in chapter 2.2. In these rats epigastric cannulation was done as described in section 2.3. A schematic drawing showing the positioning of cannulae and flow probes is given in chapter 2 (Fig. 1, page 43). Once the surgery was completed, a 45-60 min equilibration period was allowed so that leg blood flow and mean arterial pressure could become stable and constant. Rats were then subjected to the protocol (Fig. 1) where they were infused systemically with saline or insulin (as a euglycemic insulin clamp with 3 mU/kg/min) from t = 0 for 2 h, and locally (epigastric artery) into the test leg with methacholine or bradykinin for the last 45 min. Preliminary experiments were conducted to determine the

dose of methacholine or bradykinin that would produce the maximum increase in FBF without effects on MAP or heart rate. Some initial experiments involved systemic infusions of methacholine intravenously for the final 20 min of either saline (n = 5) or insulin clamp (3 mU/kg/min; n = 5).

4.2.3 Blood samples

Arterial samples were taken at the times indicated (Fig. 1) for blood glucose measurements. The femoral vein of each leg was used for venous sampling, using a 29G insulin syringe (Becton Dickinson). Duplicate venous samples (300µl) were taken only on completion of the experiment (total time 120 min) to prevent alteration of the blood flow from the hindlimb due to sampling, and to minimize the effects of blood loss.

4.2.4 Capillary recruitment

Plasma (20µl) from arterial and leg venous blood samples taken at the end of the experiment was mixed with 80µl of 0.42 M perchloric acid and centrifuged for 10 min. The supernatant was used to determine 1-MX, allopurinol and oxypurinol concentrations by reverse-phase HPLC as previously described in section 2.4. Capillary recruitment, expressed as 1-MX metabolism was calculated from arterio-venous plasma 1-MX difference and multiplied by femoral blood flow.

At 45 min prior to the completion of each experiment (Fig. 1), a 50 µCi bolus of [^{3}H]2-DG was administered. At the conclusion of the experiment, the soleus, plantaris, gastrocnemius white, gastrocnemius red, EDL and tibialis muscles were removed, clamp frozen in liquid nitrogen and stored at -20°C to be assayed for 2-DG uptake as described in section 2.6. A glucose analyzer was used to determine whole blood glucose (by the glucose oxidase method) during the insulin clamp.

4.2.5 Plasma insulin assay

Rat insulin levels at the end of the euglycemic insulin clamp (and other groups) were determined from arterial plasma samples by ELISA assay (Mercodia rat insulin ELISA) using rat insulin standards.

4.2.6 Expression of results

All data are expressed as means ± SEM. Mean femoral blood flow, mean heart rate and mean arterial pressure were calculated from 5 second sub-samples of the data, representing approximately 500 flow and pressure measurements every 15 minutes. Vascular resistance in the hindleg was calculated as mean arterial pressure in millimetres of mercury divided by femoral blood flow in millilitres per minute and expressed as resistance units (RUs).

4.2.7 Statistical analysis

Repeated measures two-way analysis of variance was used to test the hypothesis that there was no difference among treatment groups for femoral blood flow, blood pressure, heart rate, vascular resistance, 1-MX, R'g and GIR concentrations throughout the time course. When a significant difference ($P < 0.05$) was found, pair wise comparisons by the Student-Newman-Keuls test were used to determine at which individual times the differences were significant. All tests were performed using the SigmaStat™ statistical program (Jandel Software Corp.).

4.3 RESULTS

4.3.1 RESULTS: SYSTEMIC INFUSION OF METHACHOLINE

Systemic infusion of methacholine (Fig. 1) caused MAP to fall and blood glucose to rise, resulting in a nil GIR at a dose that achieved a small increase in FBF.

4.3.1a Hemodynamic effects

Methacholine infusion was given intravenously with saline or superimposed on the insulin clamp for the last 20 minutes (protocol, Fig. 1A). The beginning of infusion caused an immediate fall in mean arterial pressure in both methacholine and insulin + methacholine groups (MC 106 ± 4 to 83 ± 7 and insulin + MC 108 ± 2 to 84 ± 2 mmHg, $P < 0.05$, Fig. 1B). This decrease in MAP was accompanied by a significant decrease in heart rate (MC 378 ± 7 to 355 ± 4 and insulin + MC 360 ± 0.0 to 348 ± 2 mmHg, $P < 0.05$ Fig. 1C). The systemic infusion of methacholine also led to an increase in FBF, but even a modest increase of 0.42 ± 0.09 ml/min for the methacholine group and 0.4 ± 0.12 in insulin + MC group, (Fig. 2C) was accompanied by a decrease in MAP. Vascular resistance decreased as a result of infusion of methacholine (a decrease of 32 ± 7 in MC and 33 ± 8 R.U. in insulin + MC group, Fig. 2D).

4.3.1b Glucose and 1-MX metabolism

The decrease in MAP led to a counter-regulatory response with an increase in blood glucose (MC 4.5 ± 0.2 to 6.6 ± 0.3 and insulin + MC 4.1 ± 0.05 to 6.9 ± 0.3 mM, Fig. 2A) as well as a decrease in GIR (insulin 12.6 ± 1.8 at end while insulin + MC nil at end, Fig. 2B). 1-MX metabolism (Fig. 3) was increased in methacholine group as compared with saline (saline 5.1 ± 0.6, MC 9.8 ± 0.9 nmol/min).

Although methacholine infusion potentiated the insulin-mediated effects on 1-MX metabolism, (insulin 8.3 ± 1.2, ins + MC 10.7 ± 0.97 nmol/min) it was thought that the

interpretation of a study of this kind was complicated as the drug was likely to have secondary effects on glucose uptake such as those resulting from changes in systemic hemodynamics and accompanying counter-regulatory responses.

4.3.2 RESULTS: LOCAL EPIGASTRIC INFUSION OF METHACHOLINE AND BRADYKININ

The systemic infusion of methacholine affected the whole body causing a significant fall in blood pressure and rise in blood glucose. To avoid this, a novel technique was developed where the vasodilator was infused only (Fig. 4) in the regional milieu of one leg. This had the advantage that the systemic effects of each vasodilator were kept to a minimum, due to the infusion being local, and the short biological half-lives of the agents. The fact that the systemic hemodynamics did not change after the infusion of vasodilators and that the femoral blood flow did not change in the contralateral leg reflected that there was little overflow into the systemic circulation.

4.3.2a Hemodynamic effects

The dose of methacholine infused was determined from preliminary experiments (data not shown), to produce maximal increases in FBF in the test leg without changes in FBF in the contralateral control leg or systemic changes in heart rate or MAP. Fig. 5 shows the mean arterial pressure and heart rate. Fig. 6 shows the change in FBF and vascular resistance for two different combinations based on details of Fig. 4, where methacholine or bradykinin was infused locally via the epigastric artery of the test leg and measurements were made in both legs, while animals were receiving saline infusion systemically or were under hyperinsulinemic euglycemic clamps at 3 mU/kg/min. Methacholine increased FBF with or without insulin only in the test leg (Fig. 6A) and the dose used, as estimated from femoral arterial flow and infusion rate, was 0.31 ± 0.03 μM. The values for MAP before commencement of methacholine infusion were 110 ± 3 (saline) and 112 ± 2 mmHg (insulin). At 45 min after methacholine infusion there were no significant changes and the values were 104 ± 3 (saline) and 109 ± 2 mmHg (insulin).

Consequently, the calculated vascular resistance in the leg receiving methacholine was found to decrease whether or not insulin was infused (Fig. 6B). Bradykinin infusion, like that for methacholine was also adjusted in preliminary experiments to produce maximal increase in FBF without systemic effects of changes in MAP or heart rate. The dose used was estimated to be 0.07 ± 0.01 μM and the maximal increase in FBF was slightly lower than that produced by methacholine; higher doses had systemic effects and thus were unsuitable. At this dose, bradykinin increased FBF only in the test leg (Fig. 6C). This occurred whether or not insulin was infused systemically. The values for MAP before commencement of bradykinin infusion were 106 ± 4 (saline) and 109 ± 3 mmHg (insulin). At 45 min after bradykinin infusion these were 117 ± 4 (saline) and 116 ± 4 mmHg (insulin). Thus, the calculated vascular resistance in the leg receiving bradykinin was found to decrease whether or not insulin was infused (Fig. 6D).

4.3.2b Glucose metabolism

Blood glucose levels for the four groups involving local methacholine or bradykinin infusion with saline or insulin infused systemically were constant. For methacholine the blood glucose values (Fig. 7A) at 0 min were 4.5 ± 0.2 (saline) and 4.4 ± 0.1 mM (insulin) and at 120 min, 5.3 ± 0.2 (saline), 5.6 ± 0.3 mM (insulin). The glucose infusion rate (Fig. 7B) required to maintain euglycemia during insulin with local methacholine infusions reached a plateau at 8.7 ± 0.9 mg/kg/min. For bradykinin the blood glucose values (Fig. 7C) at 0 min were 4.4 ± 0.2 (saline), 4.0 ± 0.2 mM (insulin) and at 120 min, 4.7 ± 0.2 (saline), 4.8 ± 0.3 mM (insulin). Glucose infusion rate to maintain euglycemia when clamps were conducted during local bradykinin infusions reached a plateau at 9.4 ± 0.6 mg/kg/min (Fig. 7D).

Fig. 8 shows data for R'g of individual muscles of the lower leg. Methacholine had no effect on R'g of any of the muscles from the leg into which it was infused. However, methacholine when infused locally on a background of systemically infused insulin augmented the insulin-mediated increase in R'g for soleus, plantaris, red gastrocnemius, extensor digitorum longus and tibialis muscles (Fig. 8A). For the muscle combination,

insulin increased R'g ~3-fold from 3.0 ± 0.2 to 9.8 ± 0.8 μg/g/min and this was further increased to 12.5 ± 0.8 μg/g/min by methacholine ($P < 0.001$) (Fig. 8B). Bradykinin alone (saline background) had no effect on R'g of any of the muscles from the leg into which it was infused (Fig. 8C). Bradykinin (insulin clamp background), unlike methacholine, did not affect the insulin-mediated increase in R'g of any of the individual muscles (Fig. 8C) or of the combined group (Fig. 8D), of the leg into which it was infused.

4.3.2c Capillary recruitment

No significant differences were found between the two experimental groups in arterial plasma concentrations of 1-MX (saline ± local MC, 16 ± 1.4 μmol/l; insulin ± local MC, 20 ± 3 μmol/l) or oxypurinol, the metabolite of allopurinol and inhibitor of xanthine oxidase (saline ± local MC, 3.5 ± 0.5 μmol/l; insulin ± MC, 4.0 ± 1.2 μmol/l). Local infusion of methacholine increased capillary recruitment from 5.3 ± 0.7 to 8.4 ± 0.8 nmol/min ($P < 0.05$) in the test leg as judged from 1-MX metabolism. This increase was significant when compared to the contralateral control leg ($P < 0.05$). Systemic insulin infusion also increased 1-MX metabolism. This was significant when control legs were compared ($P < 0.05$; Fig. 9A). 1-MX was further increased from 8.8 ± 0.9 to 15.5 ± 1.2 nmol/min ($P < 0.008$) in the test leg when methacholine was infused locally on a background of insulin clamp (Fig. 9A). Bradykinin alone (saline background) did not increase 1-MX metabolism and did not further modify the stimulation due to systemic insulin infusion (Fig. 9B).

4.3.2d Plasma insulin assay

Plasma insulin levels were measured to see if the effects of methacholine or bradykinin on insulin action were due to the alteration of endogenous insulin production. Plasma insulin concentrations (pM) at –5 and 120 min were 382 ± 117 and 428 ± 37 (methacholine), 325 ± 134 and 819 ± 104 (insulin + methacholine), 352 ± 59 and 393 ± 87 (bradykinin), and 312 ± 51 and 756 ± 69 (insulin + bradykinin) (Fig. 10).

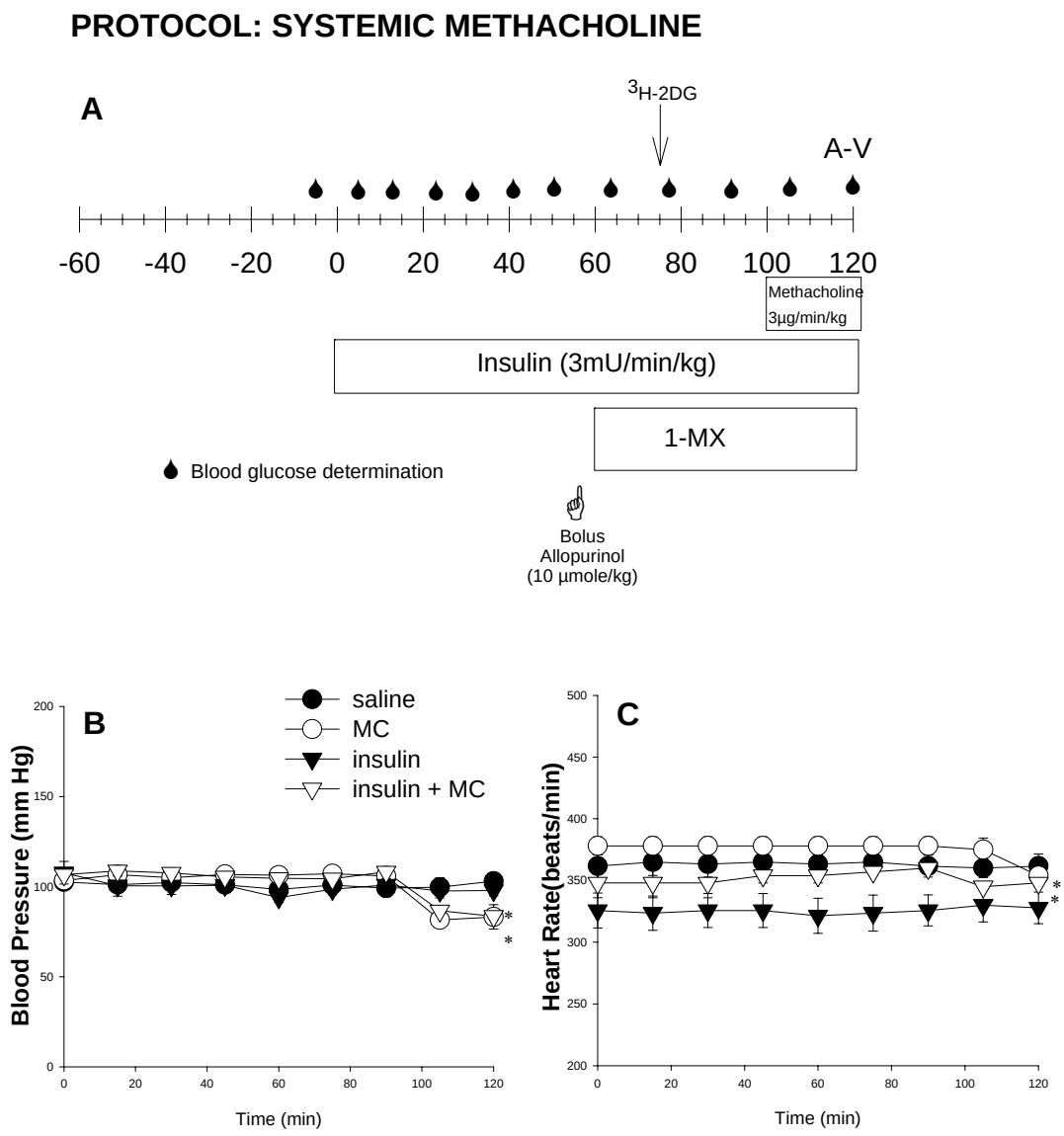


Fig. 1. This figure shows the protocol (A) used for systemic infusion of methacholine (MC) with or without hyperinsulinemic euglycemic clamp. It also depicts the effect of systemic infusion of MC on mean arterial pressure (B) and heart rate (B). * indicates a significant difference from the zero time point ($P < 0.05$). Saline n=9, 3 mU/min/kg insulin n=7, MC n=5, ins + MC n=5.

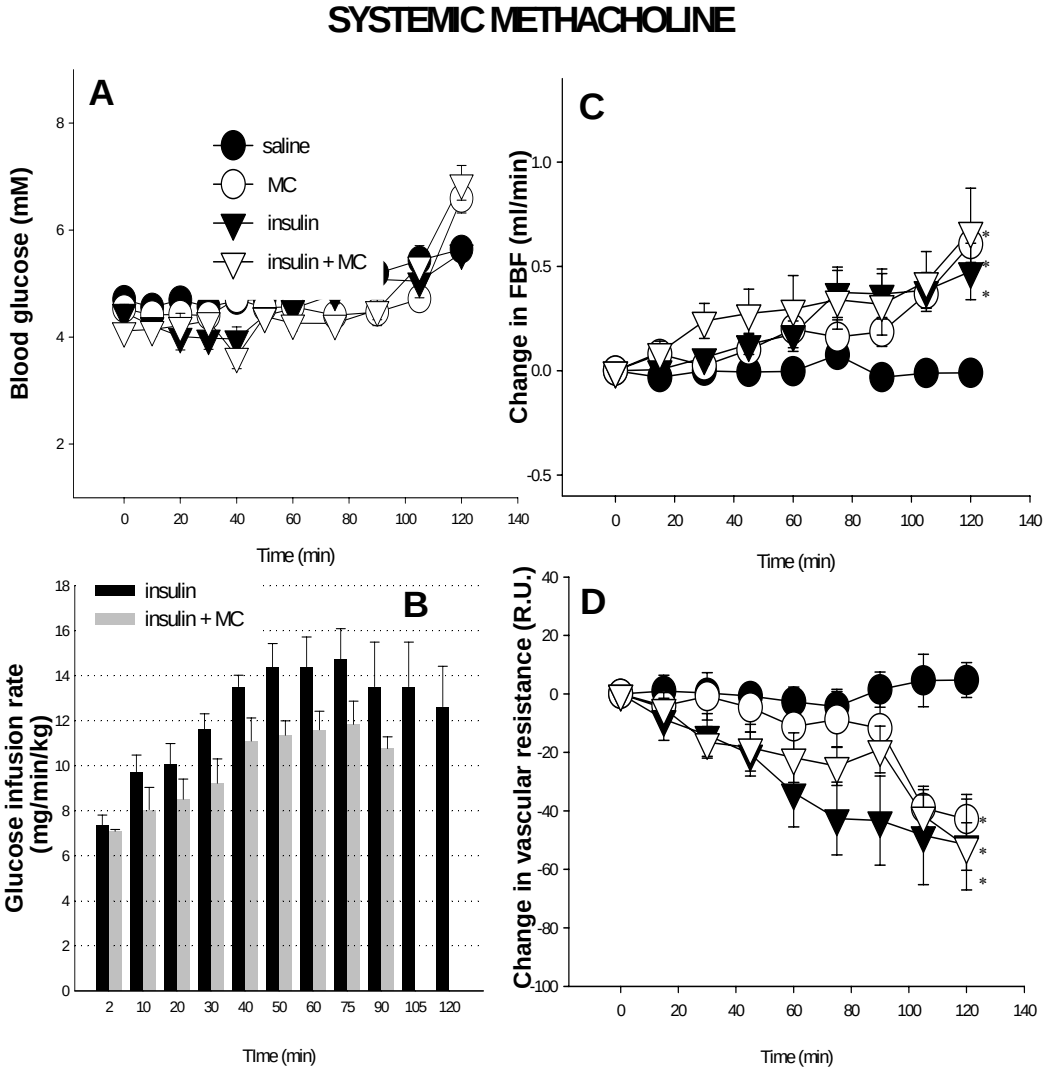


Fig. 2. Blood glucose (A), glucose infusion rate (B), change in FBF (C) and vascular resistance (D) after systemic infusion of methacholine (MC, last 20 minutes), saline, insulin and MC + insulin. NB: GIR was zero at 105 and 120 min for insulin + MC. *, indicates a significant difference from the saline ($P < 0.05$).

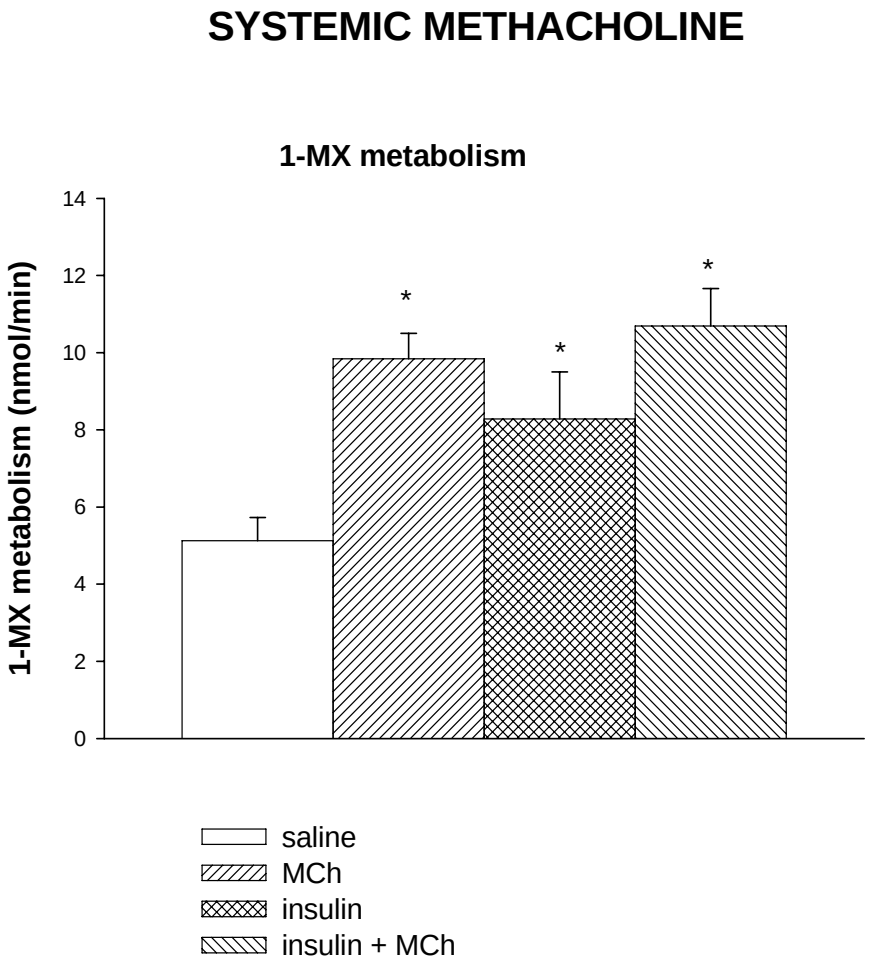


Fig. 3. 1-MX metabolism at the end of systemic infusion of methacholine (MC), saline, insulin and MC + insulin. *, Significantly different ($P < 0.05$) from the saline.

LOCAL INFUSION OF METHACHOLINE AND BRADYKININ IN ONE LEG

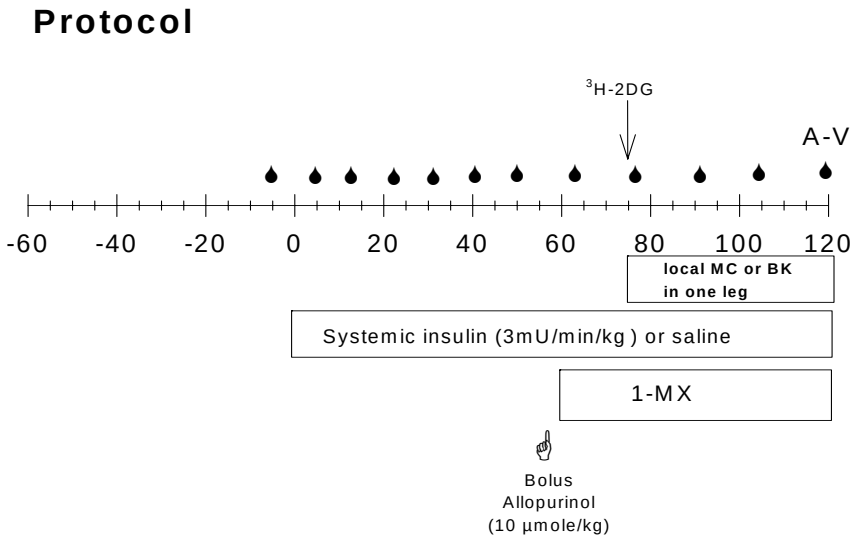


Fig. 4. Study design. The protocol involved the euglycemic clamp at 3 mU/kg/min insulin commencing at time = 0 min and either saline, MC (1 µg/kg/min) or BK (2 µg/kg/min) infused into the epigastric artery of the test leg for the last 45 min. Duplicate arterial and femoral venous plasma samples from each hindleg (test and contralateral control) were collected at 120 min, for HPLC analysis, and plasma glucose determinations. Systemic venous infusions are indicated by the bars. Bolus systemic injections of allopurinol or 2-DG were made as indicated. Arterial samples for glucose determinations are indicated by ♦. Muscle samples were taken at 120 min for 2-DG. The numbers of animals in each group were saline ± MC, n = 5; insulin ± MC, n = 6; and, saline ± BK, n = 6; insulin ± BK, n = 6.

LOCAL INFUSION OF METHACHOLINE AND BRADYKININ IN ONE LEG

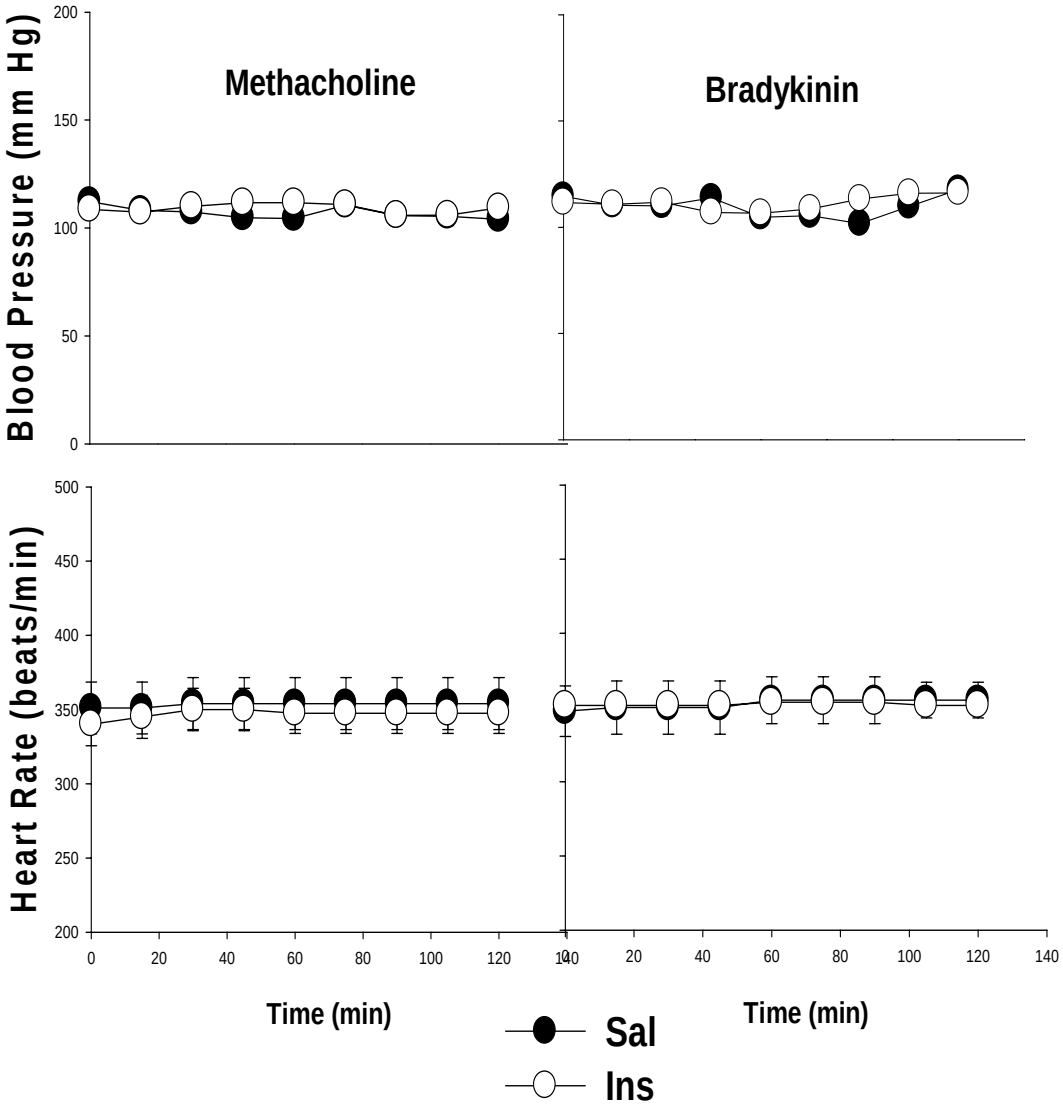


Fig. 5. MAP and HR as a result of local infusion of MC or BK via the epigastric artery into one leg with or without systemic infusion of insulin. Details are given in Fig. 4. Symbols are: ○, insulin treated; ●, saline treated.

LOCAL INFUSION OF METHACHOLINE AND BRADYKININ IN ONE LEG

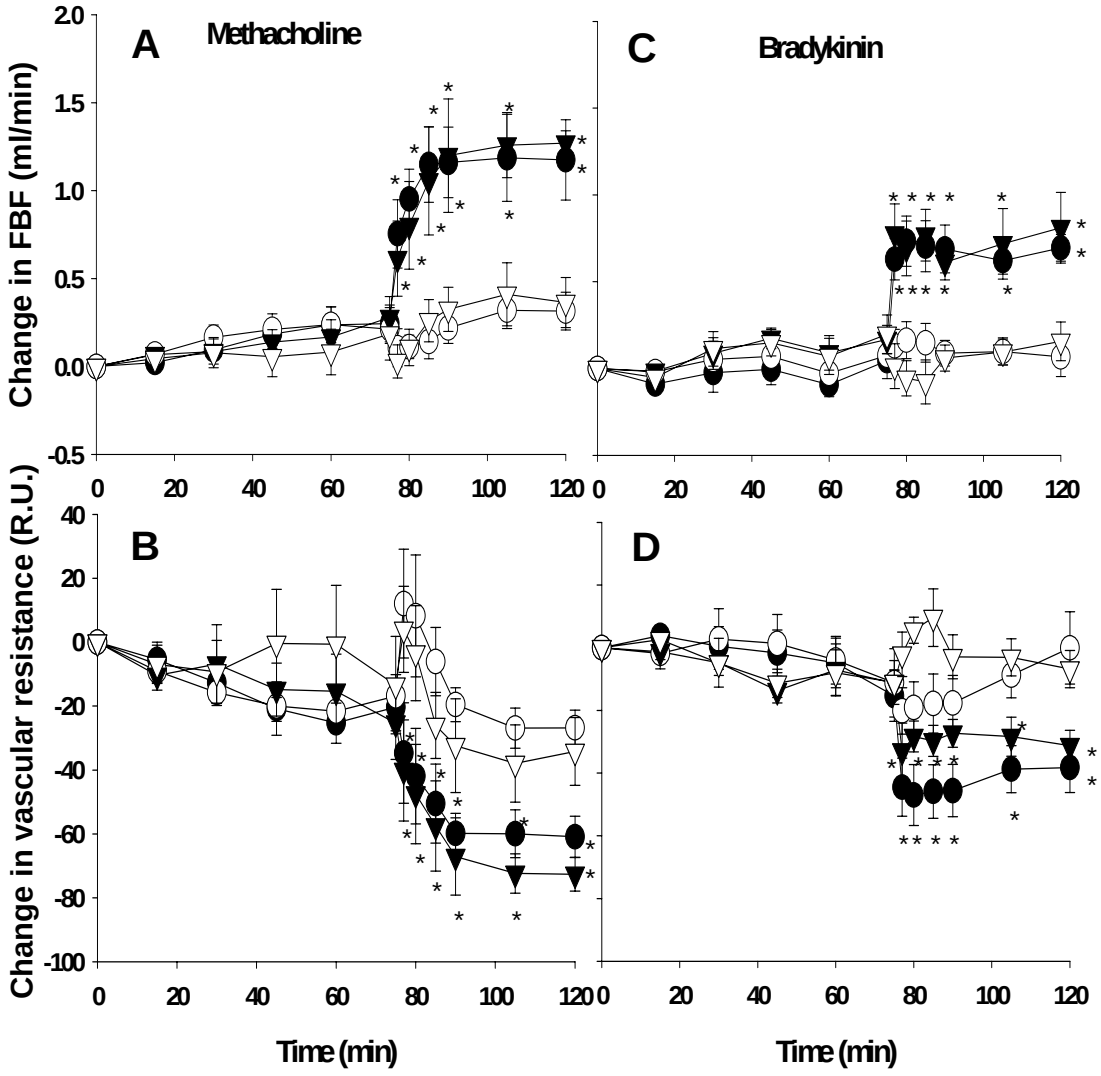


Fig. 6. Change in FBF and VR as a result of local infusion of MC (A, B) or BK (C, D) via the epigastric artery into one leg with or without systemic infusion of insulin. Details are given in Fig. 4. Symbols are:○, control leg saline treated; ●, test leg saline treated; ▽, control leg insulin treated; ▼, test leg insulin treated. *, Significantly different from control leg ($P < 0.05$).

LOCAL INFUSION OF METHACHOLINE AND BRADYKININ IN ONE LEG

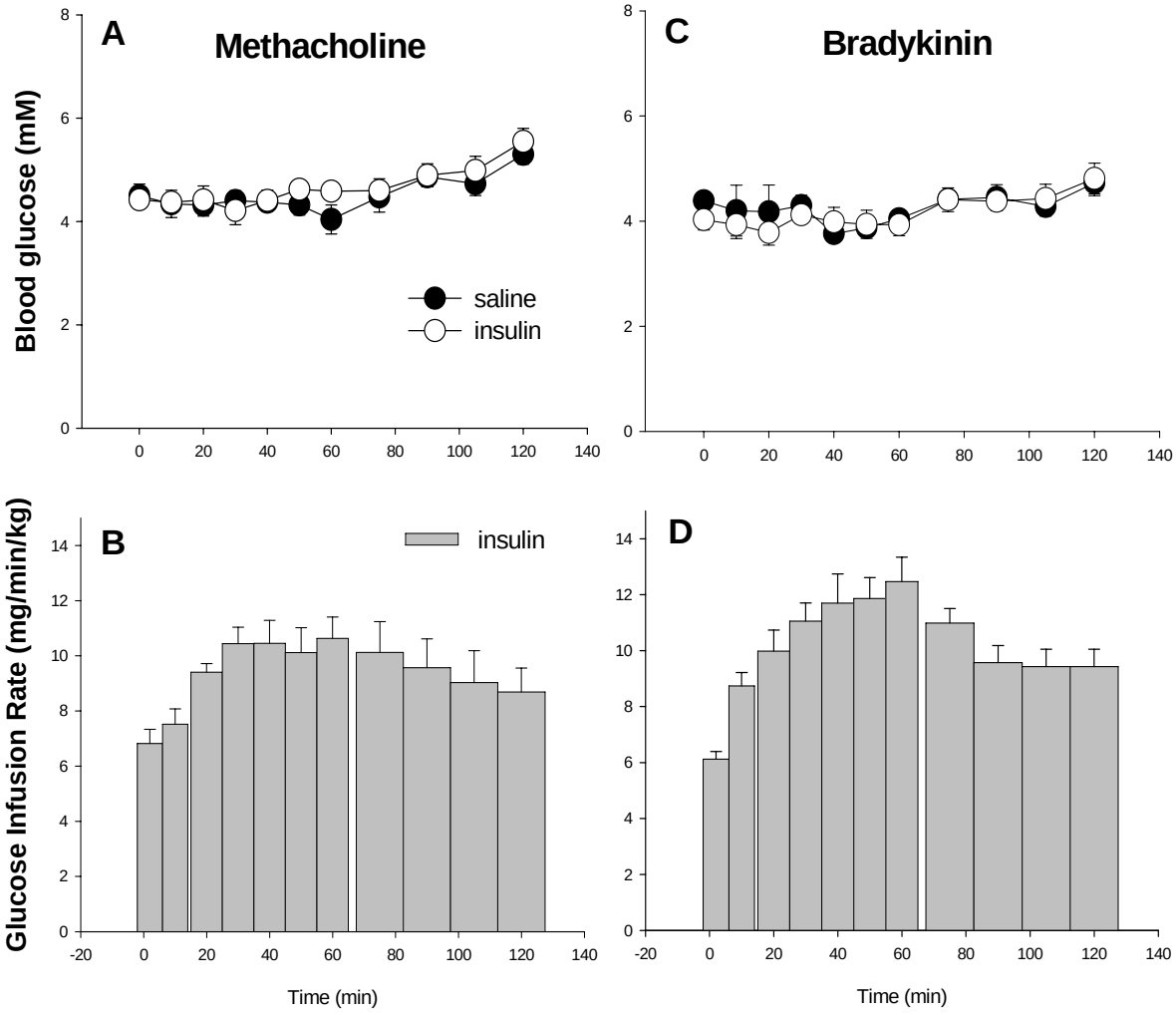


Fig. 7. Blood glucose and glucose infusion rate as a result of local infusion of MC (A, B) or BK (C, D) via the epigastric artery into one leg with systemic infusion of insulin or without (saline group).

LOCAL INFUSION OF METHACHOLINE AND BRADYKININ IN ONE LEG

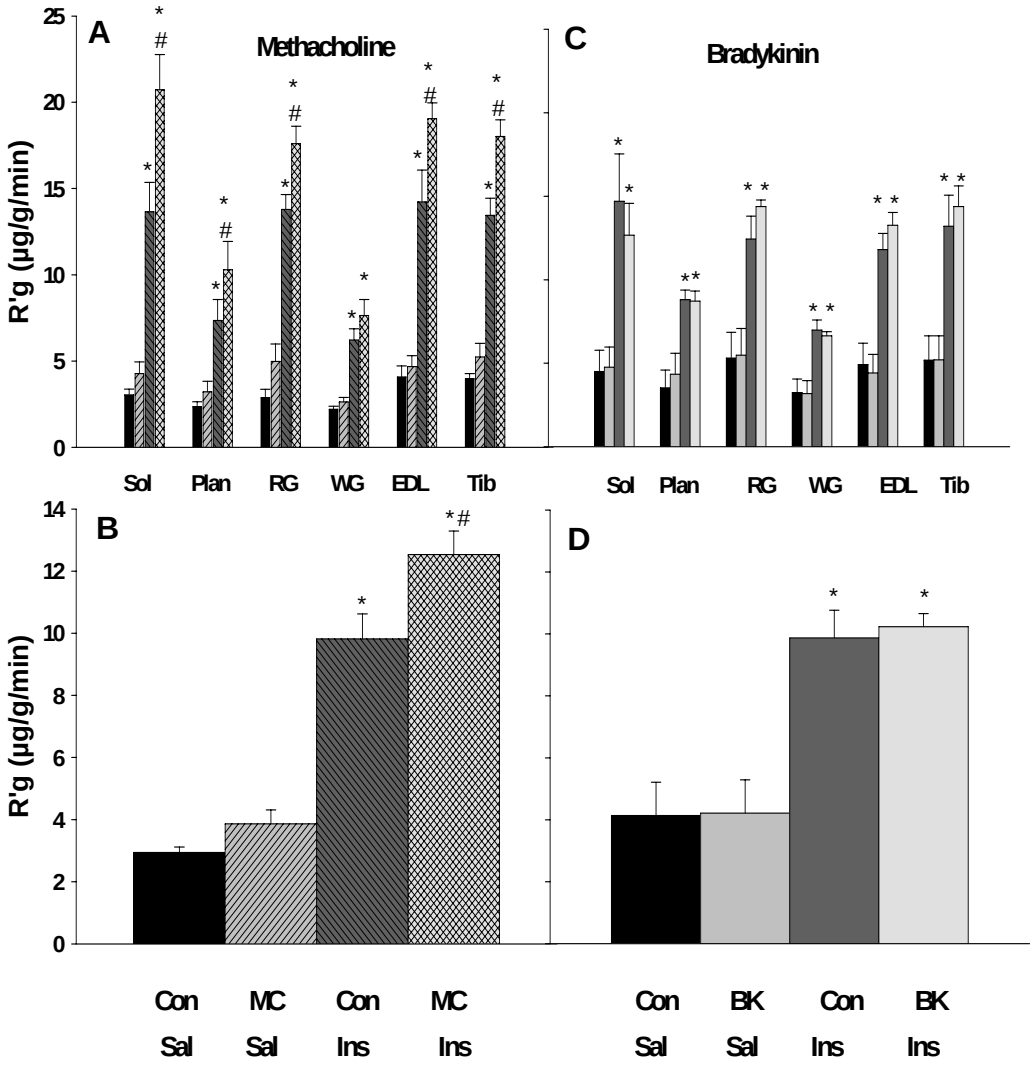


Fig. 8. Effect of local one-leg infusion of MC or BK on 2-deoxyglucose uptake (R'g) by lower leg muscles. MC or BK was infused only in the test leg; saline or insulin was infused systemically. From left to right: control leg saline treated; test leg saline treated; control leg insulin treated; test leg insulin treated. Other details are given in Fig. 4. *, Significantly different ($P < 0.05$) from the corresponding control leg (saline); #, significantly different ($P < 0.001$) from the corresponding control leg (insulin).

LOCAL INFUSION OF METHACHOLINE AND BRADYKININ IN ONE LEG

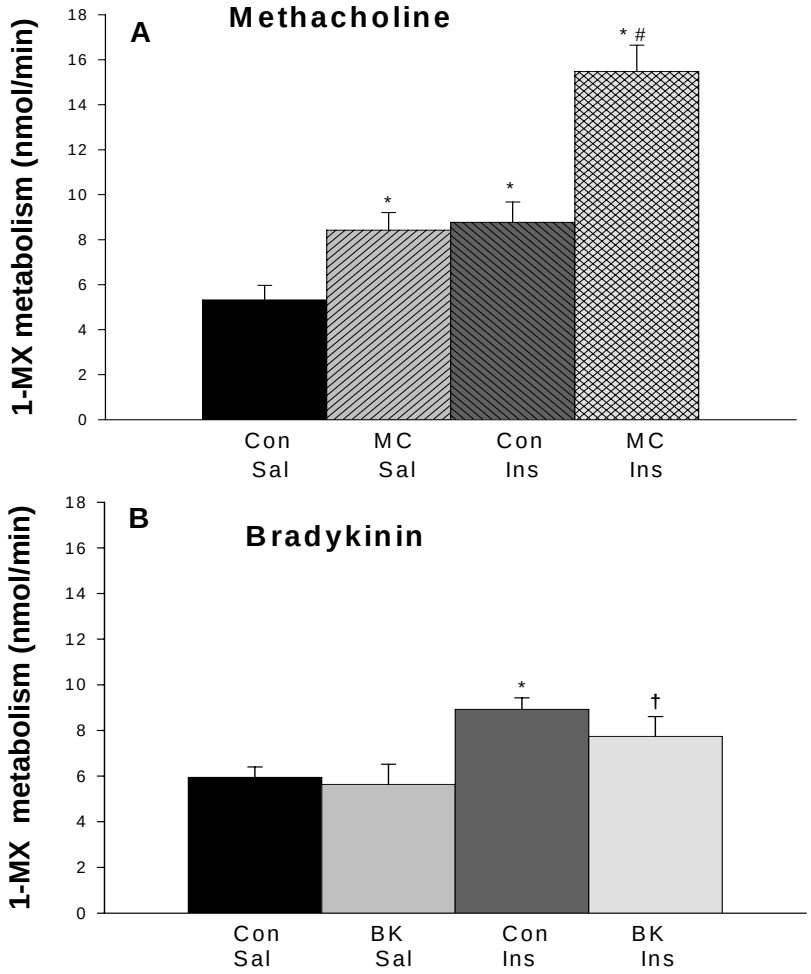


Fig. 9. Effect of local one-leg infusion of MC (A) or BK (B) on 1-MX metabolism. MC or BK was infused only in the test leg; saline or insulin was infused systemically. From left to right: control leg saline treated; test leg saline treated; control leg insulin treated; test leg insulin treated. Other details are given in Fig. 4. *, Significantly different ($P < 0.05$) from control leg (saline); #, significantly different ($P < 0.01$) from the corresponding control leg (insulin); †, significantly different from test leg without insulin.

LOCAL INFUSION OF METHACHOLINE AND BRADYKININ IN ONE LEG

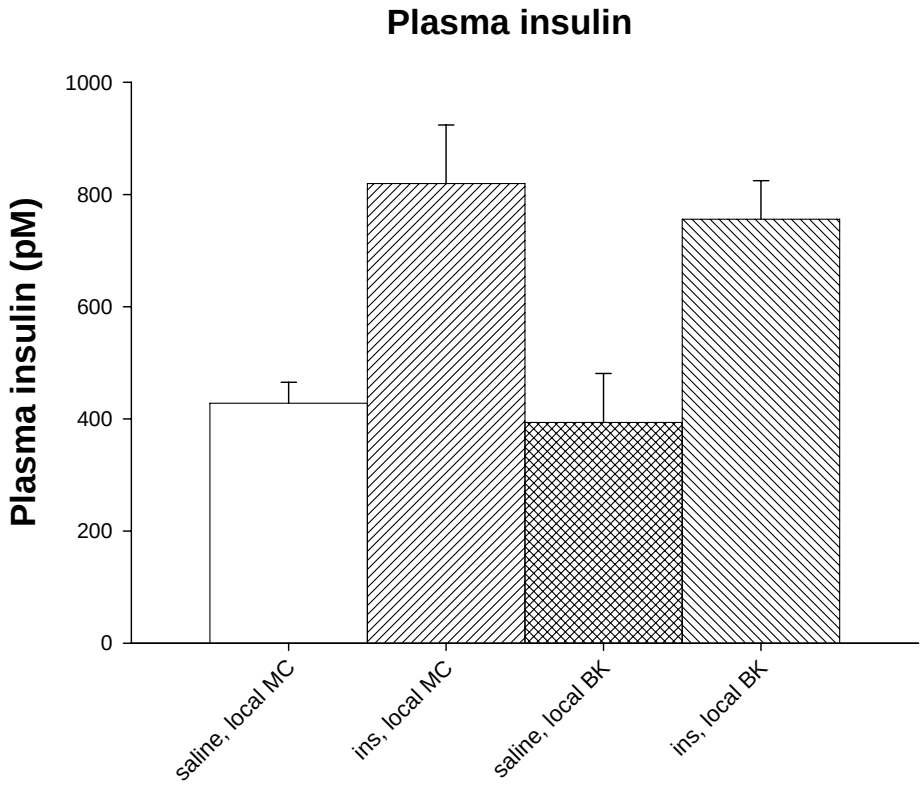


Fig. 10. Effect of local one-leg infusion of MC or BK on plasma insulin levels. MC or BK was infused only in the test leg; saline or insulin was infused systemically. Other details are given in Fig. 4. *, Significantly different ($P < 0.05$) from saline.

4.4 DISCUSSION

The striking finding from this study was that of the two vasodilators, methacholine and bradykinin, only methacholine enhanced insulin-mediated glucose uptake by muscle *in vivo* and that this effect of methacholine was associated with a marked augmentation of capillary recruitment. Thus each vasodilator when infused locally into one leg markedly enhanced bulk flow, but despite this increase, only one of these vasodilators enhanced both capillary recruitment and muscle glucose uptake in the presence of physiologic insulin. Neither vasodilator was able to increase muscle glucose uptake as assessed by 2-DG when infused alone. Thus three important issues emerge from this study. First, it would seem unlikely that bulk flow with or without added insulin controls muscle glucose uptake. Second, enhancement of capillary recruitment by methacholine alone without an accompanying increase in glucose uptake would suggest that capillary recruitment alone is not itself a stimulator of glucose uptake (a corollary herein is that methacholine itself is not a stimulator of muscle glucose uptake). Third, augmentation of capillary recruitment and muscle glucose uptake by methacholine when added with systemic insulin, suggests that capillary recruitment is rate limiting even for physiologic insulin. Thus any intervention that augments capillary recruitment in the presence of elevated insulin (i.e. above basal), will enhance insulin-mediated glucose uptake. Such interventions may include exercise.

There is considerable evidence that the increase in limb blood flow due to insulin is NO-dependent in human subjects [39, 52]. However, attempts to stimulate muscle glucose uptake by increasing limb blood flow with NO-dependent and independent vasodilators has been notably unsuccessful. Thus sodium nitroprusside [41], adenosine [45], and bradykinin [44] which markedly increase limb blood flow, do not increase muscle glucose uptake when infused locally in humans [41]. Similarly when epinephrine [57] is infused systemically in rats *in vivo*, there is no increase in glucose uptake despite increased total flow. In addition, sodium nitroprusside [41] and adenosine [45] fail to ameliorate insulin resistance in insulin resistant patients even though many of these patients show a marked loss of NO-dependent insulin- [267] and cholinergic-mediated

[268] vasodilation. An exception to the general rule that nitro-vasodilators do not increase muscle glucose uptake appears to be the acetylcholine congener, methacholine. Two laboratories have now reported that this agent increases muscle glucose uptake; one to augment insulin in a range of variably responsive individuals [54], the other to increase glucose uptake in insulin resistant hypertensive patients [55] even though nitroprusside in the same patients did not [55]. An explanation for this apparent contradiction may lie with the effect that each vasodilator has on the muscle microvascular blood flow pattern [32]. In rats, insulin and exercise have each been shown to increase capillary recruitment [62], a process that is independent of changes in limb blood flow [13]. Capillary recruitment increases insulin and glucose access to muscle fibers by increasing the proportion of nutritive blood flow. The present study shows that of the two vasodilators, methacholine and bradykinin, only methacholine increases capillary recruitment and augments insulin-mediated glucose uptake.

The findings reported in the present study have only been possible by the use of a novel procedure in the rat whereby local infusion of the vasodilator was made via a catheter placed in the epigastric artery of the test leg (Fig. 1, page 43). To our knowledge this approach has not been used before in rats and has allowed assessment of local effects of the vasodilator either on a background of saline or a hyperinsulinemic euglycemic clamp. The particular advantage of this technique is that systemic effects of each vasodilator were kept to a minimum as a result of the infusion being local and the relatively short biological half-life of the agents. Evidence that systemic effects were minimal can be seen from the unchanged mean arterial pressure and the absence of an effect to increase FBF in the contralateral leg. In contrast, systemic infusion of methacholine at a dose that achieved a lower increase in FBF than that from local infusion caused MAP to fall and blood glucose to rise, resulting in a lower GIR. Thus systemic effects of methacholine have interacted with homeostatic processes and have overridden the local metabolic and hemodynamic effects of methacholine in the muscle.

Clearly an explanation as to why methacholine and not bradykinin has increased capillary recruitment and glucose uptake must focus on the specific site(s) in the

microvasculature at which each acts to decrease hindlimb vascular resistance. As already pointed out by Baron et al. [32] the action of methacholine to vasodilate in large part is through release of the endogenous vasodilator nitric oxide from arterial endothelial cells [269] and its effects are likely to occur at the level of the high resistance small precapillary arteries. This is presumably because of the specific location of receptors for methacholine. The failure of bradykinin to achieve the same outcome as methacholine suggests that the receptors for bradykinin are not located at the level of the high-resistance small precapillary arteries, but rather, at levels where flow that is essentially non-nutritive can be accommodated. There is already evidence that vasodilators such as bradykinin, acetylcholine, histamine and isoproterenol can depress contractile force of exercising muscle perfused at constant flow [270]. The authors of that study concluded that the vasodilators diverted blood flow to connective tissue away from the contracting fibers [270]. The fact that acetylcholine was one of the vasodilators that reduced contractile force suggests that despite the close structural properties with methacholine, the latter reacts with a specific subset of receptors controlling nutritive capillary recruitment and is therefore in this respect similar to insulin [57].

In the present study methacholine alone increased capillary recruitment but did not increase R'g thereby suggesting that capillary recruitment alone is not sufficient to augment the basal insulin effects on muscle R'g. This is consistent with our recent data where we report that capillary recruitment is markedly more sensitive than R'g to insulin [13]. However, in the present study when methacholine was infused on a background of the insulin clamp the increase in R'g due to insulin was augmented. More importantly, methacholine further increased capillary recruitment due to insulin. These findings suggest that the methacholine effect to stimulate capillary recruitment is independent and additive to that of insulin, but as pointed out above, likely to be engaging receptors in the same locality of the microvasculature. It is perhaps pertinent to note that voluntary exercise-training of our rats also augmented insulin-mediated capillary recruitment and insulin-mediated glucose uptake by muscle [271].

Finally, the present findings separate limb blood flow from muscle glucose uptake. Thus both of the vasodilators increased FBF but only methacholine augmented insulin-mediated increase in R'g. This to some degree clarifies the on-going controversy where the role of bulk blood flow as an independent modulator of glucose uptake has been disputed [19, 29, 272] , particularly in relation to vasodilators [41]. Clearly then, the key hemodynamic aspect that controls insulin-mediated glucose uptake by muscle is capillary surface area. Only vasodilators that act to recruit nutritive capillaries can potentiate insulin action to increase glucose uptake. It remains to be further investigated whether flow can independently increase glucose uptake once capillary recruitment is maximal as predicted elsewhere [2].

CHAPTER 5

EFFECT OF NITRIC OXIDE SYNTHASE INHIBITION ON INSULIN-MEDIATED EFFECTS IN MUSCLE

5.1 INTRODUCTION

The results described in the previous two chapters failed to provide direct evidence for the role of NO in insulin action. However, it has been demonstrated by Steinberg and Scherrer that infusion of a nitric oxide synthase inhibitor can completely eliminate insulin-induced increases in total flow to muscle [39, 52]. Effect of NOS inhibition on insulin-mediated glucose uptake is less conclusive. It must be acknowledged that two studies reporting an involvement of nitric oxide in insulin-mediated glucose uptake in rat skeletal muscle have used systemic infusion of L-NAME during a hyperinsulinemic clamp [63, 120]. Shankar and coworkers [121] suggested that central NOS-dependent pathways may control peripheral insulin action and secretion. In humans local infusion of a NOS inhibitor, while abolishing insulin-induced vasodilatation to increase total flow, does not decrease the insulin sensitivity in muscle in most studies [31, 39, 123, 124] with the exception of a study by Steinberg and coworkers [53]. The effect of local infusion of L-NAME on insulin action in muscle in rats has not been assessed.

The present study explores the effect of both local (intra-arterial infusion into one leg) and systemic NOS inhibition on insulin-mediated capillary recruitment and glucose uptake measured by [^{3}H]2-deoxyglucose technique.

5.2 RESEARCH DESIGN AND METHODS

5.2.1 Animals

Male Hooded Wistar rats weighing 280 ± 3 g were raised on a commercial diet as described in chapter 2.1.

5.2.2 Surgical preparation

Details were as essentially described previously in chapter 2.2. In some rats epigastric cannulation was done as described in section 2.3. A schematic diagram is given in chapter 2 (page 43). Once the surgery was completed, a 45-60 min equilibration period was allowed so that leg blood flow and mean arterial pressure could become stable and constant. Rats were then subjected to the protocol A (Fig. 1) or B (Fig. 7).

5.2.3 Experimental protocols

5.2.3a PROTOCOL A: SYSTEMIC INFUSION OF L-NAME

These studies were performed to determine the effect of inhibition of systemic NOS on insulin action in muscle microvasculature. Rats were infused intravenously for two hours with either insulin (3 mU/min/kg), L-NAME (3 mg/kg bolus followed by a continuous infusion of 50 μg/min/kg) or insulin + L-NAME (Fig. 1). Infusion of L-NAME was started 5 min before the insulin clamp. This dose of L-NAME has been found to elevate and maintain a mean arterial pressure of 20-30 mmHg above basal [63]. L-NAME decreases NO production mainly by inhibiting eNOS and nNOS forms [273]. The dose of insulin used in all protocols (3 mU/min/kg) produces plasma insulin concentrations in the normal to high physiological range, and stimulates capillary recruitment with no significant increase in total flow.

5.2.3b PROTOCOL B: LOCAL EPIGASTRIC INFUSION OF L-NAME

Protocol B1: Effects of L-NAME on muscle vasculature and glucose uptake after 1 h of hyperinsulinemic euglycemic clamp

In these experiments L-NAME (Sigma chemicals) infusion (Fig. 7, protocol B1: systemic insulin + local L-NAME) was given locally via the epigastric artery into the test leg for the last hour during a two-hour 3 mU/min/kg insulin clamp. Preliminary experiments were conducted to determine the dose of L-NAME that would produce a decrease in FBF without effects on MAP or heart rate. Some rats received L-NAME alone for an hour.

Protocol B2: Effects of L-NAME on muscle vasculature and glucose uptake before and during hyperinsulinemic euglycemic clamp

Protocol B1 showed that L-NAME had effects on its own and it did not suppress insulin-induced glucose uptake. Therefore, the aim of this protocol was to ensure that the effects of L-NAME occurred prior to insulin-induced vasodilatation and capillary recruitment. L-NAME infusion was started (Fig. 7, protocol B2: local L-NAME + systemic insulin) locally (epigastric artery) into the test leg 15 min before and continued throughout the systemic infusion of insulin for an hour (as a euglycemic insulin clamp with 3 mU/kg/min). Some rats in this protocol received L-NAME alone. The dose of L-NAME used was same as in protocol B1 and it was found that it could be infused for 75 min without any systemic effects.

5.2.4 Blood samples

Arterial samples were taken at the times indicated (Fig. 1 and 7) for blood glucose measurements. The femoral vein of each leg was used for venous sampling, using a 29G insulin syringe (Becton Dickinson). Duplicate venous samples (300 μl) were taken only

on completion of the experiment to prevent alteration of the blood flow in the hindlimb due to sampling, and to minimize the effects of blood loss.

5.2.5 Capillary recruitment

Capillary recruitment was determined by measuring the metabolism of infused 1-MX. Plasma (20 μl) from arterial and leg venous blood samples taken at the end of the experiment was mixed with 80 μl of 0.42M perchloric acid and centrifuged for 10 min. The supernatant was used to determine 1-MX, allopurinol and oxypurinol concentrations by reverse-phase HPLC as previously described in section 2.4. Capillary recruitment, indicated by 1-MX metabolism was calculated from arterio-venous plasma 1-MX difference and multiplied by femoral blood flow [57].

5.2.6 A modified technique developed for 2-deoxyglucose uptake

In protocol B2, insulin clamps were performed for 1 h instead of 2 h and this required 2-DG uptake to be measured over a shorter time period. A modified 2-DG technique was developed where instead of measuring the plasma decay curve, the averaged plasma specific activity of [^{3}H]2-DG was obtained by continuous arterial sampling after giving 2-DG bolus. This enabled (i), a decrease in the labeling period from 45 min to 10 min and (ii), to determine the effect of L-NAME during insulin clamps over short periods (1 h). Thus in protocol A and protocol B1, 50 μCi bolus of [^{3}H]2-DG was administered 45 min prior to the completion of each experiment, (Fig. 1 and 7), while in protocol B2, (Fig. 7), the same bolus of [^{3}H]2-DG was administered 10 min prior to the completion of each experiment. In the modified 2-DG technique glucose uptake was measured over a period of 10 min at the end of the one-hour insulin clamp. At the conclusion of the experiment in both protocols, the soleus, plantaris, gastrocnemius white, gastrocnemius red, EDL and tibialis muscles were removed, clamp frozen in liquid nitrogen and stored at -20°C to be assayed for 2-DG uptake as described in section 2.6. A glucose analyzer (Model 2300 Stat plus, Yellow Springs Instruments, Yellow Springs OH) was used to determine whole blood glucose (by the glucose oxidase method) during the insulin clamp.

5.2.7 Data analysis

All data are expressed as means ± SEM. Data analysis was done as described in section 2.9.

5.2.8 Statistical analysis

Repeated measures two-way analysis of variance was used to test the hypothesis that there was no difference among treatment groups for femoral blood flow, blood pressure, heart rate, vascular resistance, 1-MX, R'g and GIR concentrations throughout the time course. When a significant difference ($P < 0.05$) was found, pair wise comparisons by the Student-Newman-Keuls test were used to determine at which individual times the differences were significant. All tests were performed using the SigmaStat™ statistical program (Jandel Software Corp.).

5.3 RESULTS

5.3.1 RESULTS: SYSTEMIC INFUSION OF L-NAME

5.3.1a Hemodynamic effects

Systemic L-NAME infusion was given by itself with saline or with insulin clamp at 3 mU/min/kg (protocol A, Fig. 1). Infusion caused an immediate increase in mean arterial pressure in both L-NAME and insulin + L-NAME groups (L-NAME, 112 ± 3.0 to 156 ± 3 and insulin + L-NAME, 111 ± 2.0 to 129 ± 7.0 mmHg, $P < 0.05$, Fig. 2A). This increase in MAP was accompanied by no change in heart rate (L-NAME, 360 ± 17 to 340 ± 20 and insulin + L-NAME, 354 ± 17 to 300 ± 33 beats/min, Fig. 2B). The systemic infusion of L-NAME had no effect on FBF (L-NAME, 1.4 ± 0.2 to 0.9 ± 0.2 and insulin + L-NAME, 1.0 ± 0.2 to 1.0 ± 0.1 ml/min, Fig. 3A). Vascular resistance increased as a result of infusion of L-NAME (L-NAME, 88 ± 20 to 146 ± 13 and insulin + L-NAME, 121 ± 20 to 146 ± 24 R.U., Fig. 3B). Insulin infusion by itself did not have any significant effect on

MAP and heart rate. Insulin infusion alone increased the FBF and decreased the vascular resistance towards the end (not significant). (Elsewhere in this thesis (chapter 3) insulin infusion caused a significant increase in total flow. This may have resulted from the rats being fasted in that study (chapter 3) while being fed in this study. The dose of insulin used in both studies was physiological and it showed variable effects on total flow).

5.3.1b Glucose metabolism

Blood glucose levels in the all the groups were constant (Fig. 4A). The blood glucose values at 0 min were 4.8 ± 0.5 (L-NAME), 4.5 ± 0.2 (insulin) and 4.0 ± 0.3 mM (insulin + L-NAME) and at 120 min, 5.0 ± 0.4 (L-NAME), 5.6 ± 0.1 (insulin), and 4.3 ± 0.1 mM (insulin + L-NAME). The glucose infusion rate (Fig. 4B) required to maintain euglycemia during insulin + L-NAME reached a plateau at 14.6 ± 1.8 mg/min/kg which was not significantly different from insulin infused alone (12.6 ± 1.8 mg/min/kg).

Fig. 5B shows combined data for R'g or 2-deoxyglucose uptake for six individual muscles of the lower leg. There was an increase with insulin infusion (L-NAME 4.0 ± 0.3, insulin 8.3 ± 0.9 μg/g/min). L-NAME + insulin did not affect the insulin-mediated increase in R'g of any of the individual muscles (Fig. 5A) or of the combined group (9.8 ± 1.7 μg/g/min).

5.3.1c Capillary recruitment

No significant differences were found between the experimental groups in arterial plasma concentrations of 1-MX (L-NAME, 25 ± 1.0 μM; insulin, 22 ± 2.0 μM; insulin ± L-NAME, 22 ± 1.7 μM) or oxypurinol, the metabolite of allopurinol and inhibitor of xanthine oxidase (L-NAME, 4.5 ± 0.4 μM; insulin, 4.8 ± 0.6 μM; insulin ± L-NAME, 4.9 ± 0.3 μM). Insulin infusion significantly increased capillary recruitment from 5.0 ± 0.5 (L-NAME), to 9.4 ± 0.8 nmol/min ($P < 0.05$) as judged from 1-MX metabolism (Fig. 6). 1-MX metabolism was decreased to 7.2 ± 0.7 nmol/min when L-NAME was infused with insulin (Fig. 6). This decrease was not statistically significant from insulin alone (n.s., P =

0.17) or L-NAME alone (n.s., P = 0.27) suggesting that the presence of L-NAME caused a partial inhibitory effect on insulin-mediated capillary recruitment. L-NAME had no effect on 1-MX metabolism as compared to saline (saline 5.1 ± 0.6 from non-fasted rats, data not shown in graph).

5.3.2 RESULTS: LOCAL EPIGASTRIC INFUSION OF L-NAME

Local L-NAME infusion in both B1 (systemic insulin + local L-NAME) and B2 (local L-NAME + systemic insulin) protocols (Fig. 7) produced essentially the same effects on hyperinsulinemic euglycemic clamps with the effects being stronger with L-NAME pre-infusion (protocol B2). Therefore, though both protocols are included in most graphs, in the text local infusion of L-NAME refers to the local L-NAME + systemic insulin (protocol B2) unless stated otherwise.

5.3.2a Hemodynamic effects

The dose of L-NAME infused was determined in preliminary experiments (data not shown) that would decrease FBF in the test leg without changes in FBF in the contralateral control leg, heart rate or MAP. Fig. 8 shows the mean arterial pressure and heart rate. Fig. 9 shows the change in FBF and vascular resistance for both control and test legs in saline and insulin groups where L-NAME was infused locally via the epigastric artery of the test leg and measurements were made in both legs, while animals were receiving saline infusion systemically or were under hyperinsulinemic euglycemic clamps at 3 mU/kg/min. L-NAME decreased FBF with or without insulin only in the test leg ($P < 0.05$, Fig. 9A). The values for MAP (Fig. 8A) before commencement of L-NAME infusion were 101 ± 2.0 (saline) and 104 ± 2.0 mmHg (insulin). After 75 min of L-NAME infusion there was no significant change in MAP and the values were 107 ± 3.5 (saline) and 103 ± 2.0 mmHg (insulin). Consequently, the calculated vascular resistance in the leg receiving L-NAME was found to increase (Fig. 9B).

5.3.2b Glucose metabolism

Blood glucose levels for the two groups involving L-NAME infusion with saline or insulin infused systemically were constant (Fig. 10A). The blood glucose values at 0 min were 4.3 ± 0.3 (saline) and 4.5 ± 0.4 mM (insulin) and at 75 min, 4.2 ± 0.3 (saline), 4.8 ± 0.4 mM (insulin). The glucose infusion rate (Fig. 10B) required to maintain euglycemia during insulin reached a plateau at 14.9 ± 1.6 mg/kg/min.

Figure 11 shows data for R'g of individual muscles of the lower leg. L-NAME alone (saline background) had no effect on R'g of any of the muscles from the leg into which it was infused (control leg, 5.9 ± 1.0 and test leg 5.5 ± 0.7μg/g/min, Fig. 12A). L-NAME (insulin clamp background), did not affect the insulin-mediated increase in R'g of any of the individual muscles (Fig. 11) or of the combined group (control leg, 15.3 ± 1.3 and test leg, 16.6 ± 1.5 μg/g/min, Fig. 12A) of the leg into which it was infused. For the muscle combination, insulin increased R'g ~ 2.5-fold from 5.9 ± 1.0 to 15.3 ± 1.3 μg/g/min (Fig. 12A).

5.3.2c Capillary recruitment

No significant difference was found between the two experimental groups (saline and insulin) in arterial plasma concentrations of 1-MX (saline ± local L-NAME, 19 ± 1.0 μM; insulin ± local L-NAME, 19 ± 1.0 μM) or oxypurinol, the metabolite of allopurinol and inhibitor of xanthine oxidase (saline ± local L-NAME, 6.0 ± 0.8 μM; insulin ± L-NAME, 6.0 ± 1.0 μM). Systemic insulin infusion increased 1-MX metabolism. This was significant when the legs were compared between saline and insulin groups (control legs- $P < 0.02$, test legs- $P < 0.02$; Fig. 12B). Local infusion of L-NAME led to similar decreases in 1-MX metabolism in test legs in saline infused (6.3 ± 0.3 control leg; 4.6 ± 0.5 nmol/min test leg, $P = 0.057$; Fig. 12B) and insulin infused rats (9.5 ± 1.0 control leg; 7.8 ± 0.8 nmol/min test leg, $P = 0.053$; Fig. 12B). Infusing L-NAME before or after insulin did not affect the resulting 1-MX metabolism indicating that the presence of L-NAME did not prevent insulin to recruit capillaries.

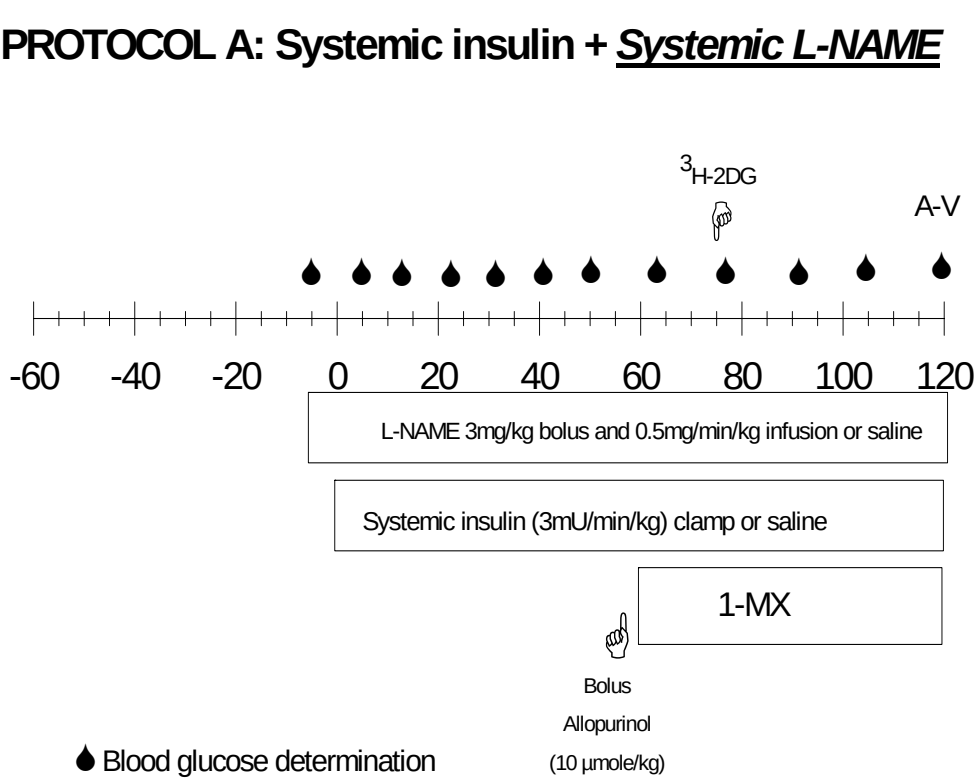


Fig. 1. Study design for systemic infusion of L-NAME with systemic insulin. Arterial and venous samples were collected at times indicated as A-V for HPLC analysis and plasma glucose determination. Arterial blood glucose were determined at time ♦. Venous infusion periods are indicated by bars. Bolus infusion periods are indicated by ☞. Ins + L-NAME, n = 5; L-NAME n= 3; and insulin n = 7.

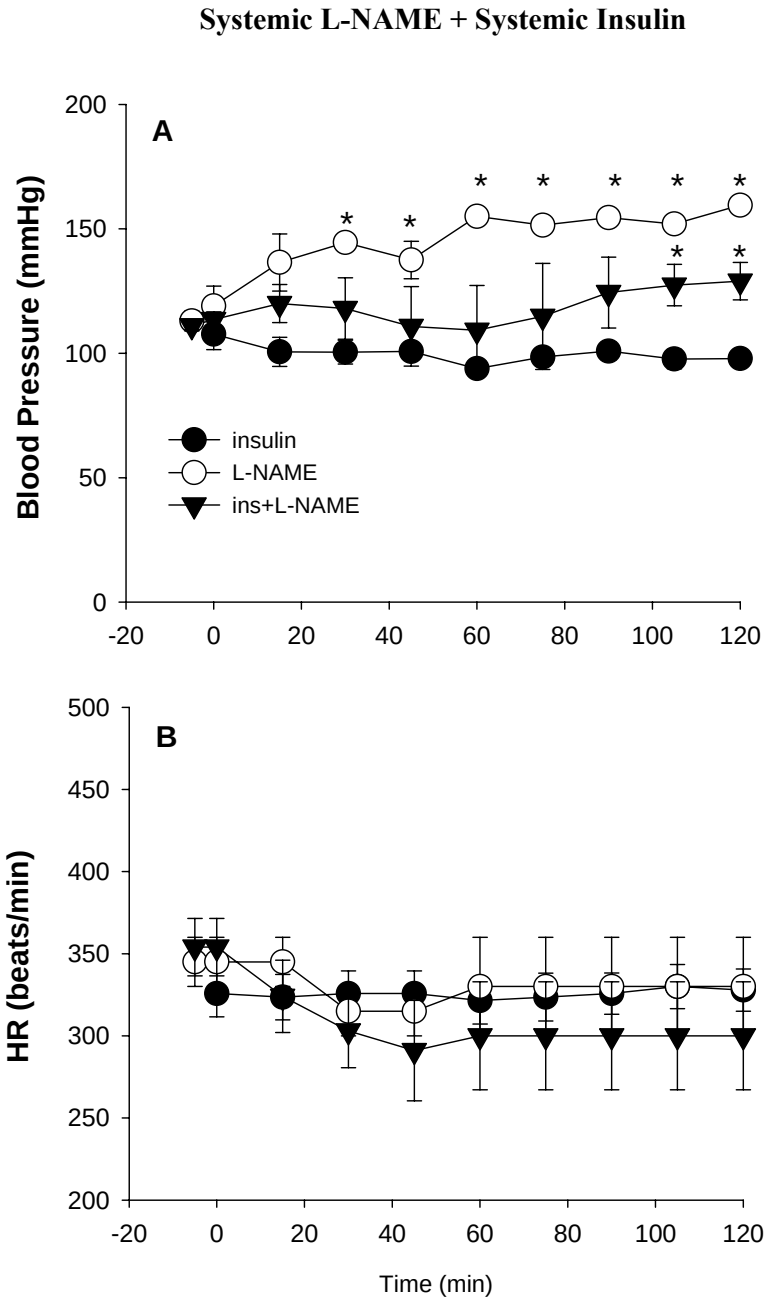


Fig. 2. Mean arterial pressure (A) and heart rate (B) for L-NAME, insulin, insulin + L-NAME treated rats. Data were collected from 5s sub-samples each 15 minutes. Values are means ± SEM. Significant values from insulin are indicated by *, $P < 0.05$.

Systemic L-NAME + Systemic Insulin

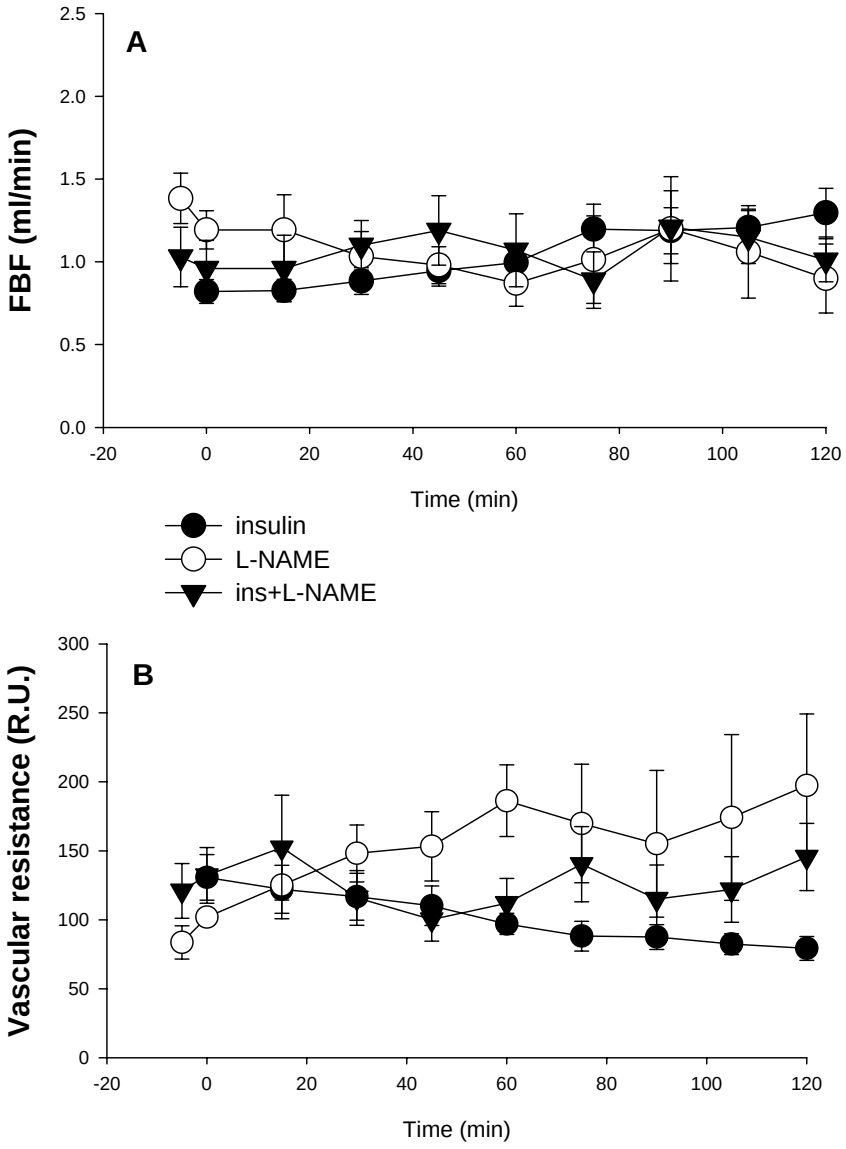


Fig. 3. Femoral blood flow (A) and vascular resistance (B) for L-NAME, insulin, insulin + L-NAME treated rats. Values are means ± SEM.

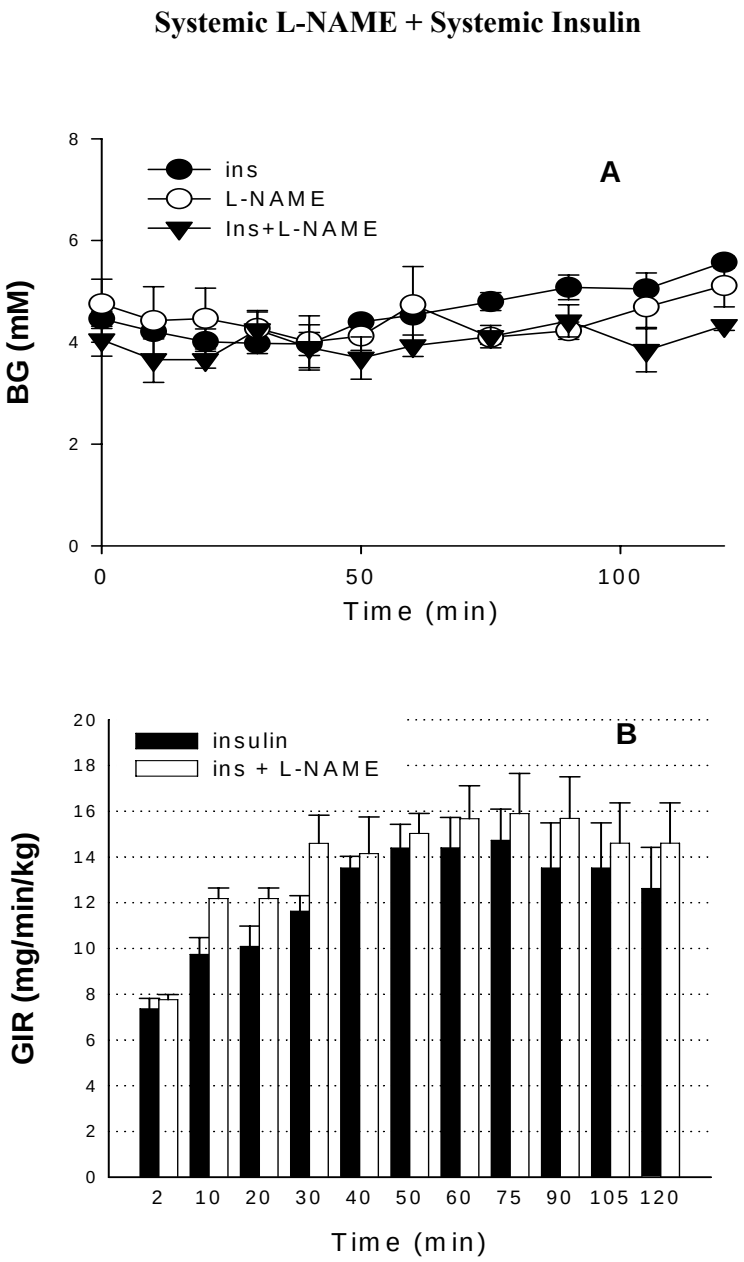


Fig. 4. Blood glucose (A) and GIR (B) for L-NAME, insulin, insulin + L-NAME treated rats.

Systemic L-NAME + Systemic Insulin

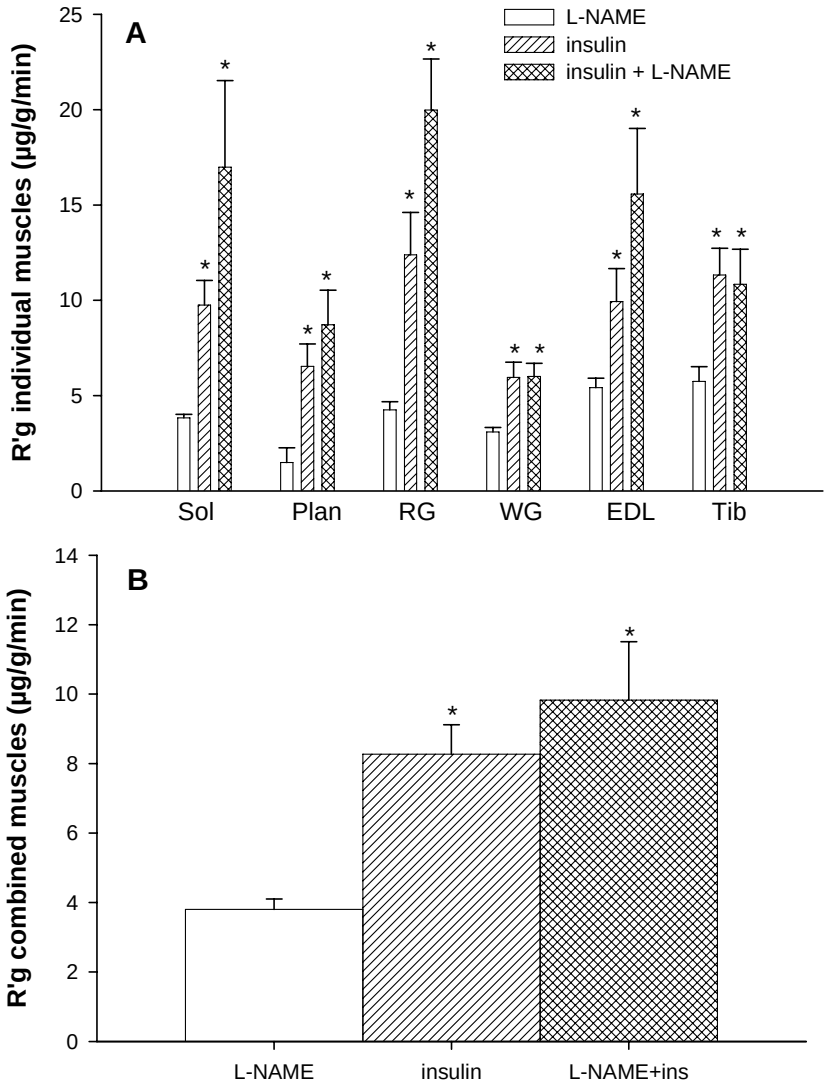


Fig. 5. Individual muscles (A) and combined (B) muscle 2-deoxyglucose uptake as a result of systemic infusion of saline, L-NAME, insulin, insulin + L-NAME treated rats. Significant values from L-NAME are indicated by *, $P < 0.05$.

Systemic L-NAME + Systemic Insulin

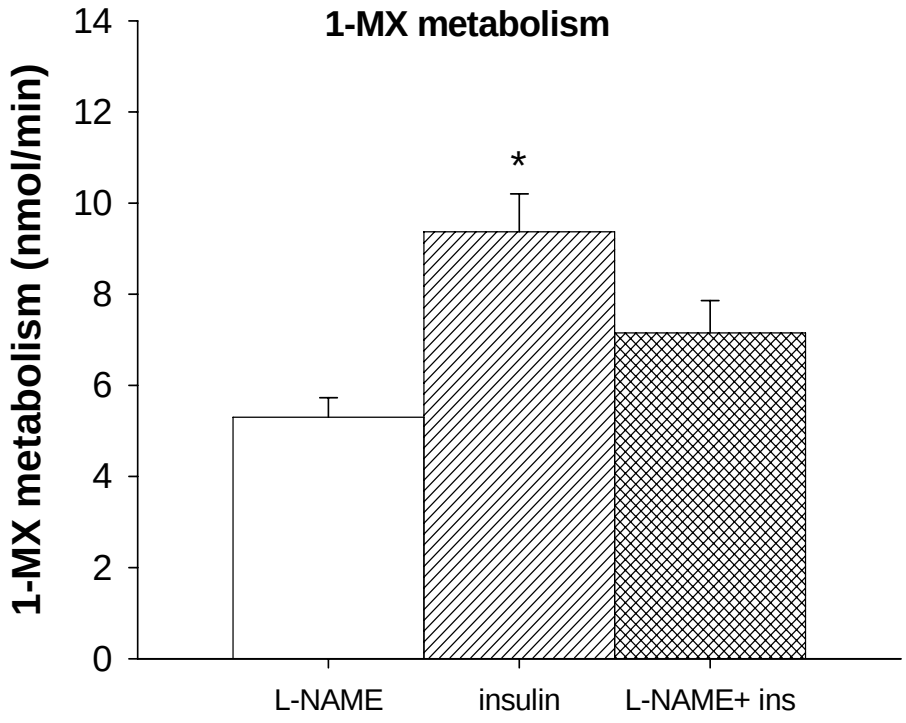


Fig. 6. 1-MX metabolism as a result of systemic infusion of L-NAME, insulin, insulin + L-NAME treated rats. Significant values from L-NAME are indicated by *, $P < 0.05$.

Local L-NAME in one leg with systemic insulin

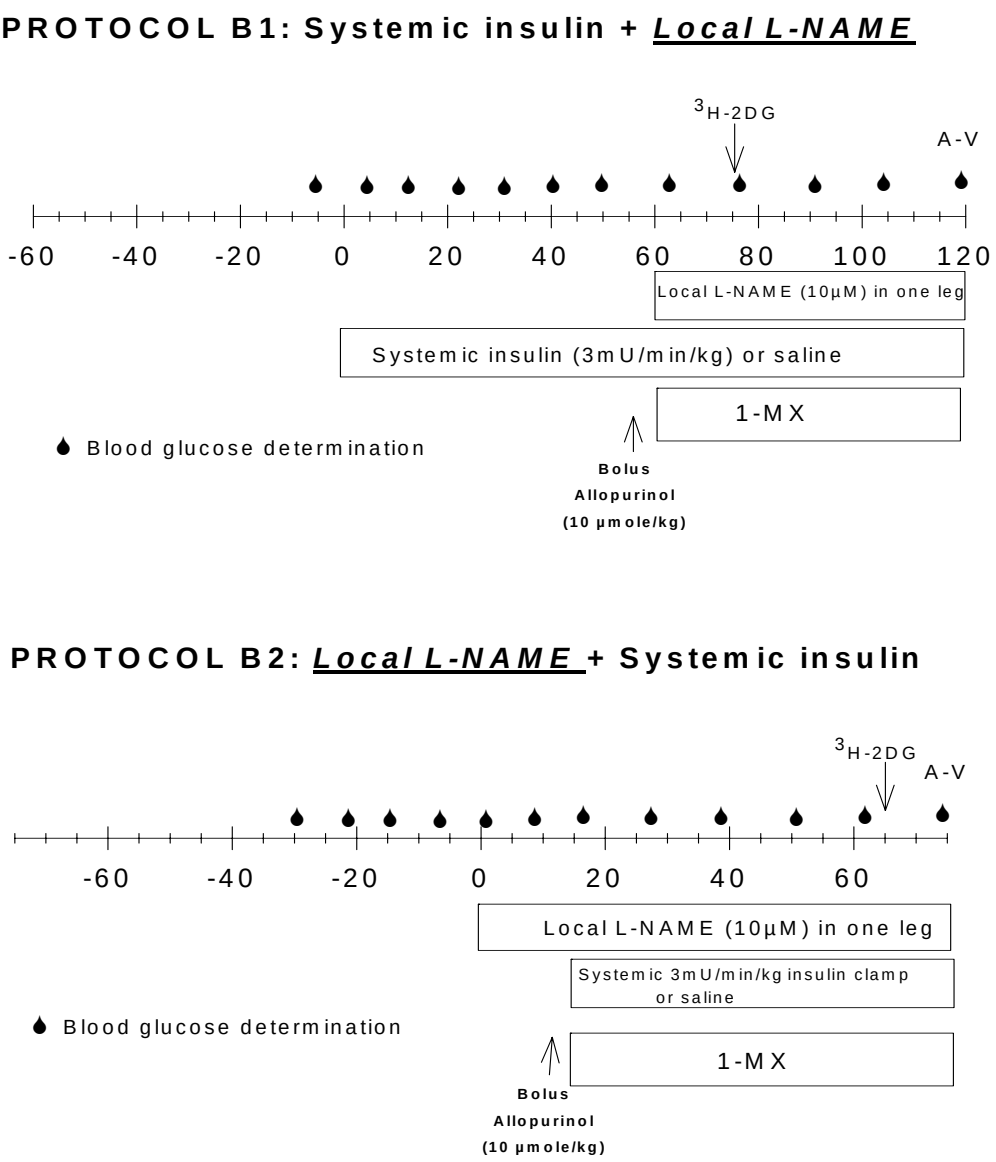


Fig. 7. Study design for protocol B1 (local L-NAME + systemic insulin) and B2 (systemic insulin + local L-NAME). Arterial and venous samples were collected at times indicated as A-V for HPLC analysis and plasma glucose determination. Arterial blood glucose were determined at time ♦. Venous infusion periods are indicated by bars. Bolus infusion periods are indicated by ↓. n = 5-7.

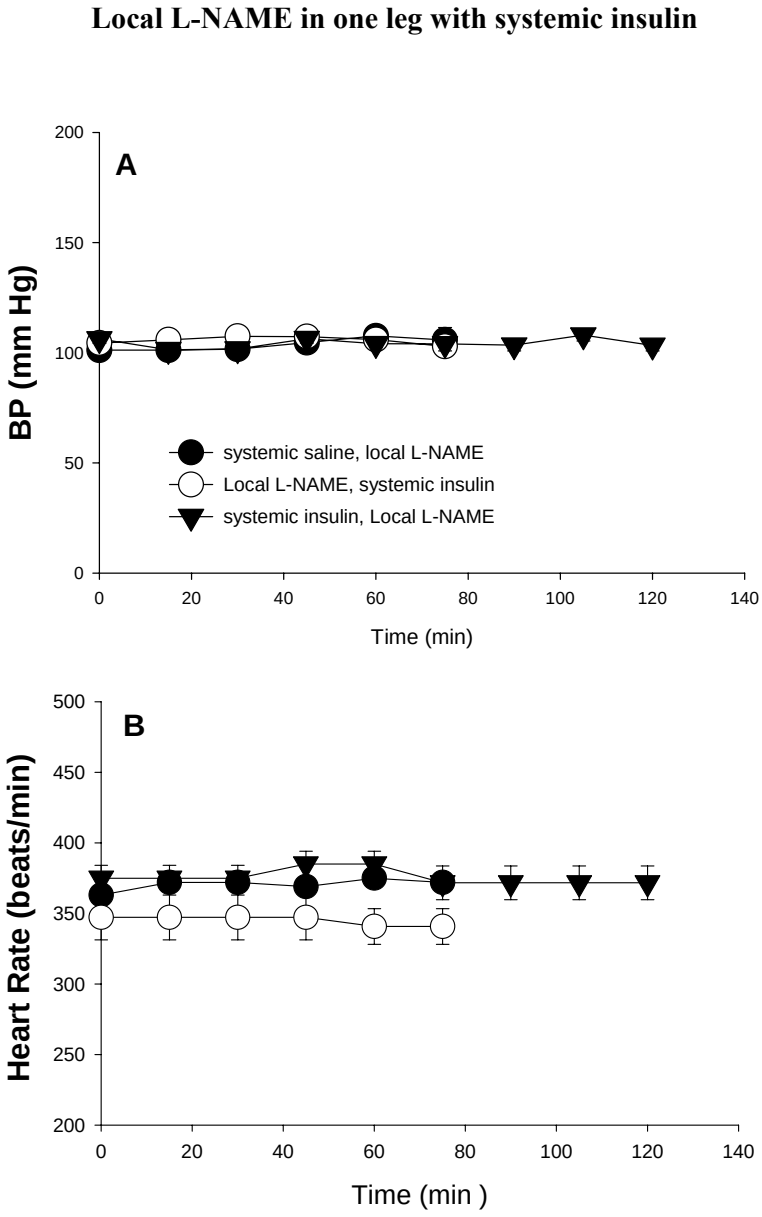


Fig. 8. MAP (A) and HR (B) as a result of local infusion of L-NAME via the epigastric artery into one leg before and after systemic infusion of insulin.

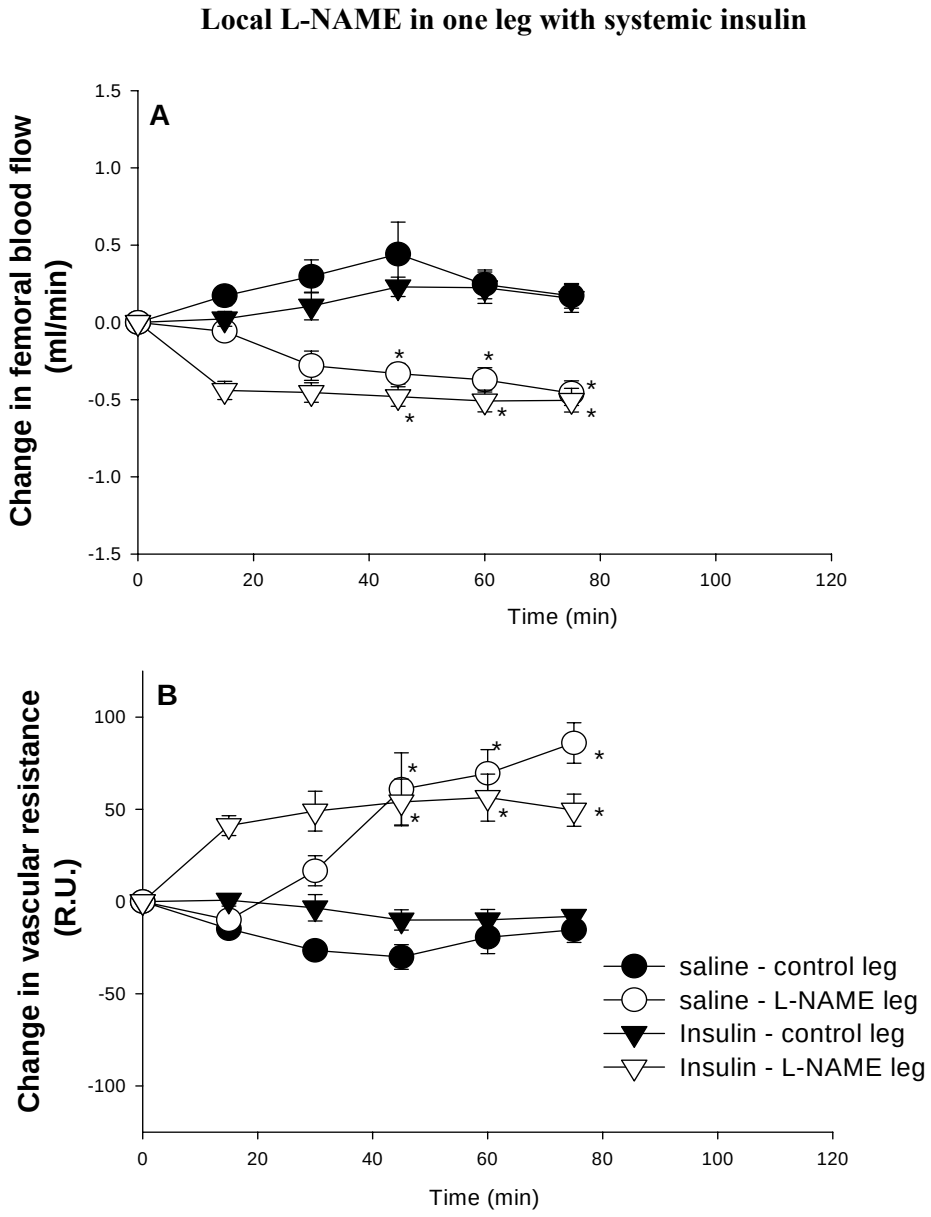


Fig. 9. Change in FBF (A) and VR (B) as a result of local infusion of L-NAME via the epigastric artery into one leg before systemic infusion of insulin. *, Significantly different from control leg ($P < 0.05$).

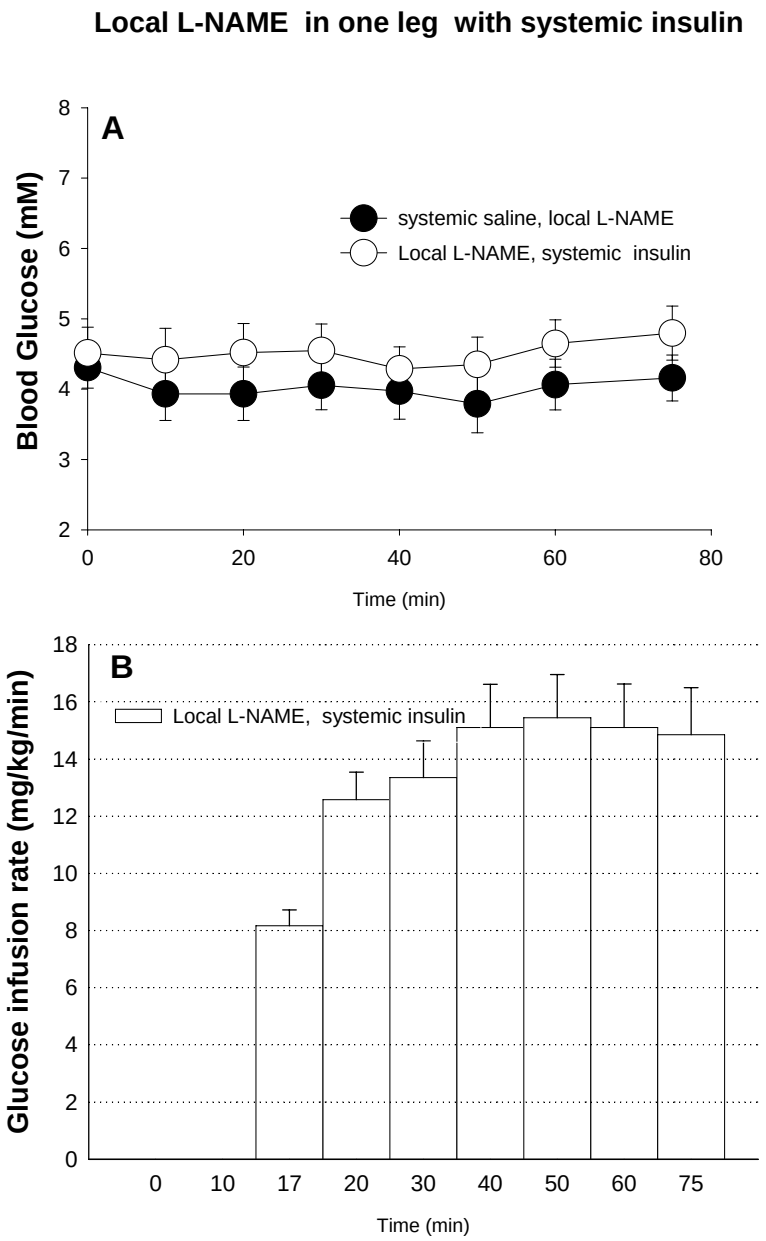


Fig. 10. Blood glucose (A) and glucose infusion rate (B) as a result of local infusion of L-NAME via the epigastric artery into one leg before systemic infusion of insulin.

Local L-NAME in one leg with systemic insulin

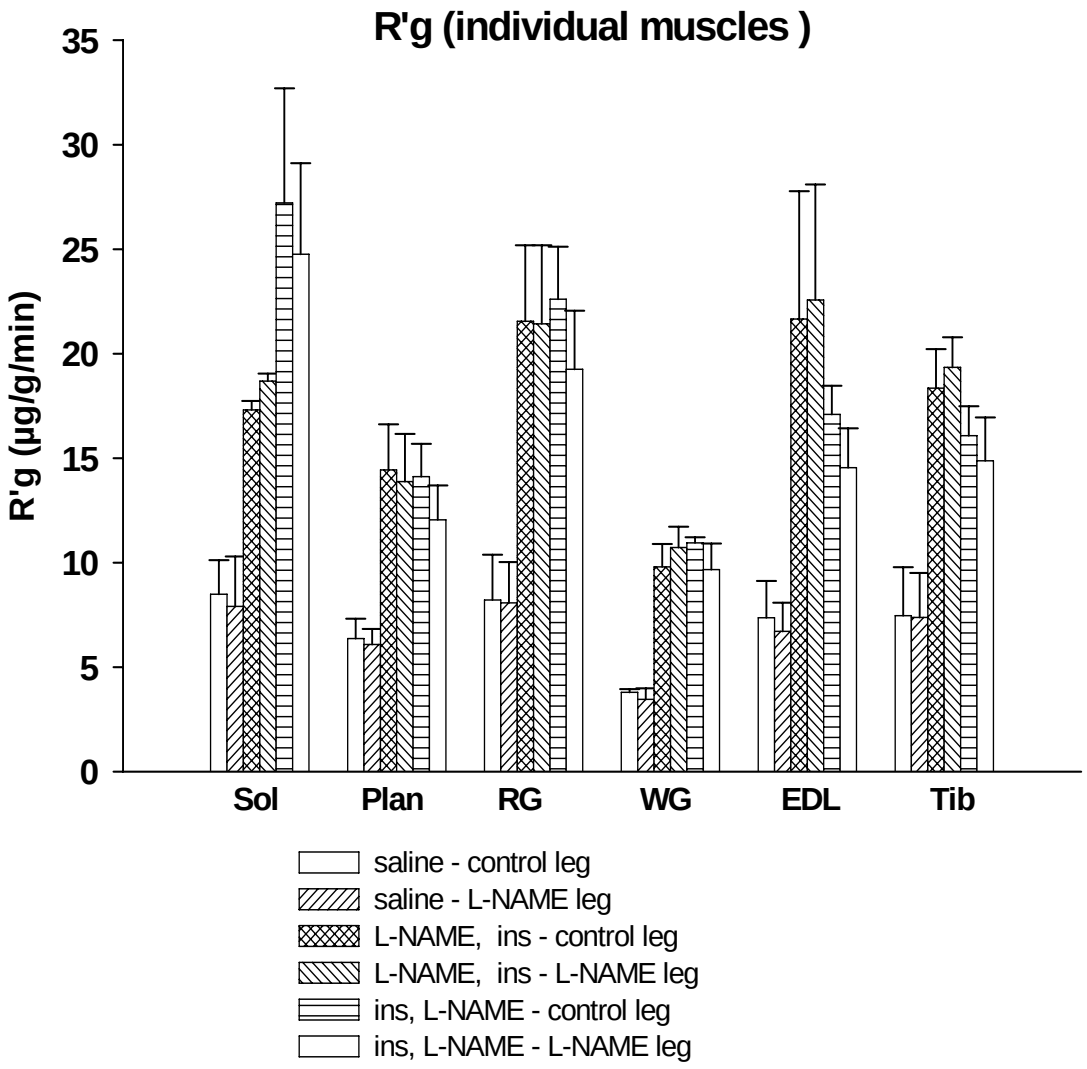


Fig. 11. R'g calculated from [^{3}H]2-DG uptake for the 6 individual muscles (soleus, plantaris, red gastrocnemius, white gastrocnemius, EDL and tibialis) as a result of local infusion of L-NAME via the epigastric artery into one leg (test leg) before (L-NAME, ins) and after (ins, L-NAME) systemic infusion of insulin.

Local L-NAME in one leg with systemic insulin

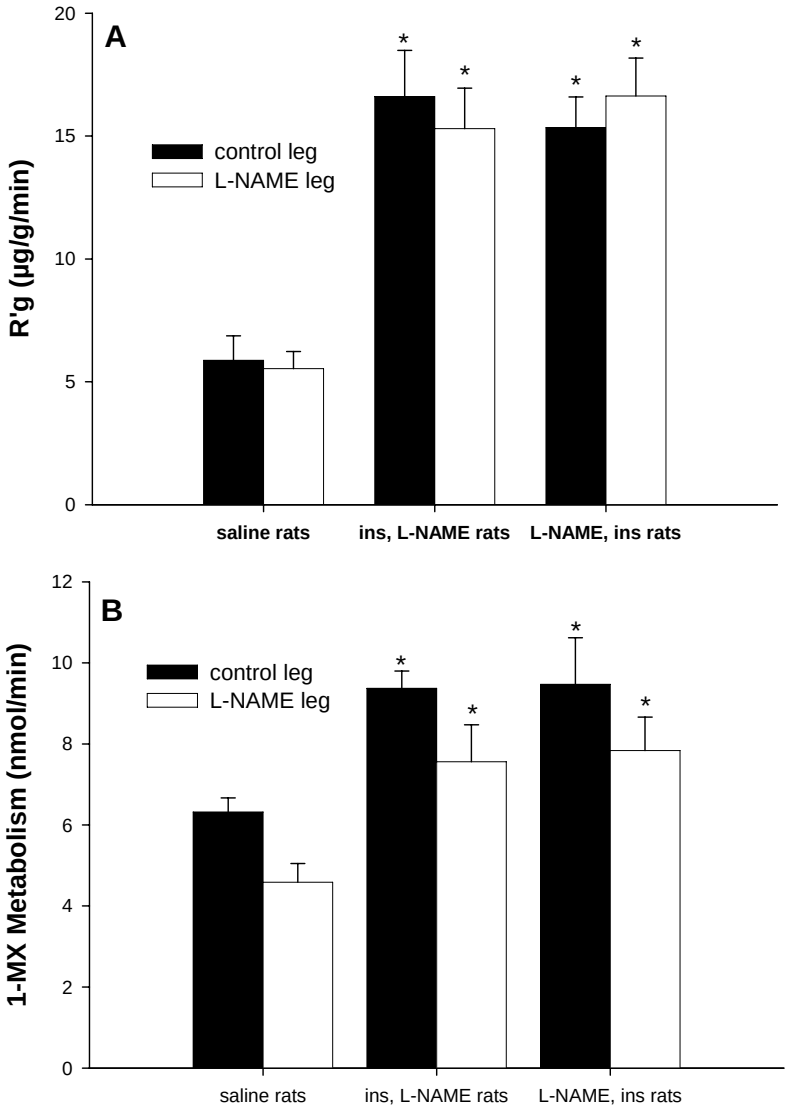


Fig. 12. Combined muscle 2-deoxyglucose uptake (A) and 1-MX metabolism (B) as a result of local one-leg infusion of L-NAME via the epigastric artery into one leg before (L-NAME, ins) and after (ins, L-NAME) systemic infusion of insulin. *, Significantly different ($P < 0.05$) from corresponding leg in saline group.

5.4 DISCUSSION

The present study found no evidence for involvement of NO in mediating insulin-mediated skeletal muscle glucose uptake in rats *in vivo*. This conclusion is based on findings that neither local nor systemic acute infusion of NOS inhibitor L-NAME reduced insulin-mediated glucose uptake measured by 2-deoxyglucose method. Another important finding was that while local infusion of L-NAME in one leg did not prevent insulin-mediated capillary recruitment in muscle, systemic infusion may have partially inhibited it.

When given locally in one leg the systemic effects of L-NAME were kept to a minimum, which facilitated observation of the direct local NOS inhibitory effect of L-NAME on insulin action. Evidence that systemic effects were minimal can be seen from the unchanged blood pressure, heart rate and the absence of an effect to decrease FBF in the contralateral leg. In contrast, systemic infusion of L-NAME with or without insulin, at a dose that did not achieve a decrease in FBF similar to local infusion, induced a pressor response causing acute hypertension. This suggested that the NOS inhibitory action of systemic L-NAME may be related to mechanisms that are not affected during local L-NAME infusion.

It is almost seventy years since the first reports of insulin increasing the total flow in skeletal muscle [274]. Since then, a number of studies have substantiated this action of insulin and demonstrated that NO-dependent mechanisms contribute to it [39, 52, 120]. In the present study, a physiologic dose of insulin was used which displayed a trend to increase FBF (not significant). Local L-NAME by itself decreased the FBF significantly while systemic L-NAME did not have any effect of FBF.

It has been reported previously that insulin also acts on muscle microvasculature causing capillary recruitment which contributes to muscle glucose uptake by increasing the delivery of nutrients and of insulin itself to the muscle [57]; that this action of insulin correlates more closely than glucose uptake [57]; and that insulin recruits capillaries prior

to any effect on blood flow in the human forearm [68]. In the current study, it was found that insulin at physiological doses recruited capillaries as indicated by l-MX metabolism. Local infusion of L-NAME reduced the capillary recruitment in the test leg compared to the control leg, in both saline and insulin groups. An important observation here was that the insulin was still able to significantly recruit capillaries when the two test legs (L-NAME) were compared between saline and insulin groups. Thus, local infusion of L-NAME did not prevent insulin action on rat muscle microvasculature. On the other hand, while systemic infusion of L-NAME alone had no effect on 1-MX metabolism (as compared to saline), L-NAME infused systemically during euglycemic insulin clamp partially blocked insulin's effect on capillary recruitment. This indicates that the additional mechanisms activated during systemic L-NAME contribute importantly to its inhibitory effect on insulin. Unfortunately, the lack of widespread use of methods to measure microvascular recruitment in anesthetized rats limits the number of studies available for comparison. The only study where the effect of NOS inhibition has been studied on insulin action on muscle microvasculature is by Vincent et al [63]. They have reported that L-NAME infusion completely abolished insulin-mediated (10mU/min/kg) capillary recruitment. The comparison between this and Vincent's study [63] is drawn later.

It has been suggested by Lautt and coworkers [275] that NOS inhibition by L-NAME blocks the release of a hepatic hormone in response to insulin. This putative hepatic insulin sensitizing substance (HISS) is thought to amplify the skeletal muscle response to insulin [276]. They have demonstrated using a rapid insulin sensitivity test that low doses (1 mg/kg) of intraportal but not intravenous L-NAME produced insulin resistance. This was reversed by intraportal (but not intravenous) administration of NO donors, indicating that the site of NO action controlling insulin sensitivity is hepatic [277]. It is possible that in the present study, the partial inhibitory effect of systemic L-NAME on insulin-mediated capillary recruitment is due to inhibition of HISS-dependent effect of insulin. The hepatic effect of L-NAME may have played a role in the current study of systemic L-NAME.

Another mechanism activated by systemic L-NAME that could potentially contribute to its effect on capillary recruitment is the sympathetic nervous system [278]. It has been suggested that the pressor response of L-NAME is not due entirely to the inhibition of endogenous endothelial NO synthesis [279]. L-NAME may stimulate release of epinephrine from adrenal medulla [280, 281] which could oppose insulin's effect to recruit nutritive capillaries. It has been demonstrated previously that epinephrine increases non-nutritive flow [57]. However, in the present study epinephrine concentrations were not measured. Alternatively, it has been suggested that central NOS-dependent pathways may control peripheral insulin action [121]. Regardless of the mechanism, NO at least in part indirectly contributes to insulin's microvascular effect in muscle.

Blood glucose concentrations remained stable throughout the course of the experiment in all the groups. In the insulin group, blood glucose concentration was maintained at or around basal by infusing a 30% glucose solution. Systemic L-NAME did not affect the GIR when infused with the insulin clamp indicating that NOS inhibition had no effect on whole body insulin sensitivity.

Another important finding, which this study highlights, is that neither local nor systemic infusion of L-NAME affected insulin-mediated skeletal muscle 2-DG uptake. This is the only study where the effect of local NOS inhibition is studied on insulin action in rats. Effects of local NOS inhibition have been studied solely in humans and the majority of the workers report that NOS inhibition had no effect on the insulin sensitivity (muscle glucose uptake or GIR). It is important to note, however, that in the only study in humans reporting an inhibitory effect of a NOS inhibitor (L-NMMA) on insulin-mediated glucose uptake, by Steinberg and coworkers [53], the degree of the observed inhibition was mild (~25%) and the dose of L-NMMA used was much higher than another study published at the same time by Scherrer et al [39], who did not observe any inhibition of insulin-mediated glucose uptake. It is worth mentioning that the dose of local intra-arterial L-NMMA used by Scherrer and coworkers [39] abolished insulin-induced vasodilatation, not only in the infused, but also in the contralateral forearm, suggesting

that L-NMMA had systemic effects. In the present study, the local infusion of L-NAME did not affect the blood flow in contralateral hindlimb.

The results of the local infusion of L-NAME are in agreement with two other groups [31, 122] reporting that acute local NOS inhibition had no effect on glucose disposal during hyperinsulinemic euglycemic clamp in humans. None of the aforementioned studies looked at the radioactive 2-DG uptake during insulin clamps.

In the present study, L-NAME was infused both prior to and during the insulin clamp to find out whether reversing the order of infusion affected the results. However, both the protocols showed the same results indicating that NO is not involved in the induction or maintenance of capillary recruitment.

The finding that systemic infusion of L-NAME had no effect on insulin-mediated muscle 2-DG uptake was unexpected and surprising because Vincent et al [63] have previously reported that systemic L-NAME did reduce the insulin-mediated glucose uptake although they measured this from FBF and arterio-venous difference rather than the 2-DG method [63]. Another study by Roy et al [120] found that systemic L-NAME infusion during an insulin clamp in rats significantly blunted the whole body glucose disposal (-16%) and muscle 2-DG uptake (-30%). Strain-, methodological- and dose-related differences could have accounted for the discrepancy observed between the studies. Vincent's study reported a complete inhibition of insulin-mediated capillary recruitment with partial inhibition of insulin-mediated glucose uptake. In comparison, the present study demonstrates partial inhibition of insulin-mediated capillary recruitment with no inhibition of insulin-mediated glucose uptake.

Strain differences between these experiments could have implications on the results as differences in insulin sensitivity between two different strain of rats has been reported [282]. The studies done by Vincent [63] and Roy et al [120] utilized Sprague-Dawley rats while in the present study Hooded Wistar rats were used.

In addition, the present study measured glucose uptake by the radioactive 2-deoxyglucose uptake method which includes measurement from six individual hindleg muscles and is thus speculated to be more accurate. Hindleg glucose uptake is the product of FBF and plasma arterio-venous glucose difference. Given that the glucose extraction is only around 5% across the leg, an error in either would be potentiated significantly. Another difference between the two methods is that whereas 2-DG is measured only in lower leg muscles, hindleg glucose uptake is measured across the whole leg. Also, a higher dose of insulin (10 mU/min/kg) was used in the study by Vincent et al [63].

A dose- and time-dependent effect of L-NAME on insulin sensitivity has been reported by Lautt and coworkers [277]. They showed by using a rapid insulin sensitivity test in Sprague-Dawley rats, that high (2.5-5 mg/kg) but not lower (1 mg/kg) doses of intravenous L-NAME produced a significant insulin resistance. Effect of high dose (2.5 mg/kg) lasted 2 h whereas the low dose (1 mg/kg) effect wore off in 1 h. It is difficult to draw a comparison between their study and the present study because of different methodology and strains of rats used.

In accordance with our findings, another group demonstrated [123] that acute systemic NOS inhibition by L-NAME had no effect on glucose disposal during a hyperinsulinemic euglycemic clamp in rats. Moreover, oral ingestion of L-NAME has been reported to produce hypertension but no alteration in oral glucose tolerance [127]. The only previous report [125] of acute systemic NOS inhibition in humans found an increase in insulin sensitivity (whole body glucose uptake) after L-NMMA administration at a much lower dose as compared with others [53] but a dose that still produced hypertension and bradycardia.

Thus, by comparing local and systemic effects of L-NAME on insulin action it appears that nitric oxide inhibition by L-NAME is not solely due to a direct inhibitory effect of L-NAME but involves additional mechanisms acting only during systemic L-NAME infusion. Local intra-arterial L-NAME infusion at the dose used in this study had purely local effects, because, vasoconstrictor effects were limited to the infused leg, and

effects on mean arterial pressure or heart rate were lacking. Local L-NAME infusion alone caused a profound vasoconstriction and reduced the capillary perfusion.

The only NO-dependent insulin effect on muscle microvasculature noticed in the current study was a partial inhibition of insulin-mediated capillary recruitment by systemic L-NAME, while local infusion of L-NAME did not block insulin-mediated capillary recruitment or glucose uptake. It is possible that the microvascular action of insulin is not mediated locally in the muscle, but perhaps by systemic (hepatic) or central neural factors. Cardillo et al, and others [31, 101, 283-285] have demonstrated a vasodilatory response to systemic but not to local hyperinsulinemia in the human forearm. This indicates that mechanisms other than NO are likely to play a role in insulin's action on muscle microvasculature and glucose uptake. It seems that insulin-mediated vasodilatation (increase in total flow as reported by [39, 52, 120]) is NO-dependent but insulin-mediated microvascular effects are not. An impaired vascular responsiveness to insulin may contribute to insulin resistance. Thus, a knowledge of other mechanisms underlying insulin-mediated capillary recruitment could be helpful in treating insulin resistance.

CHAPTER 6

EFFECT OF LOCAL BLOCKADE OF ENDOTHELIUM-DEPENDENT HYPERPOLARIZATION FACTOR ON INSULIN ACTION IN MUSCLE USING TETRAETHYLAMMMONIUM

6.1 INTRODUCTION

A number of *in vivo* studies have investigated the mechanism of insulin-induced vasodilation. Combined results from chapters 3, 4 and 5 indicate that NO is not directly involved in insulin-mediated action in muscle microvasculature. However, there is evidence for the participation of adenosine [93] and prostacyclin [94-96]. Moreover, some evidence supports the hypothesis of an additional pathway resistant to NOS and cyclooxygenase inhibition which results in vascular smooth muscle relaxation mediated by a possible endothelium-derived hyperpolarization factor [105, 200]. Although the precise nature of EDHF still remains controversial, a common element is its role in the activation of calcium-dependent potassium (K_{Ca}) channels which can be inhibited by tetraethylammonium (TEA), charybdotoxin, iberiotoxin and apamin [168, 169, 172].

It is not possible to measure hyperpolarization directly in *in vivo* [286]. An indirect involvement of a hyperpolarizing factor can be obtained by using the above mentioned potassium channel antagonists. A role of potassium channels in insulin-induced vasodilatation has been explored in few studies *in vitro* in which vascular hyperpolarization can be directly measured. Recent *in vitro* experiments have demonstrated that blockade of K_{Ca} channels by charybdotoxin blunts insulin-induced vasodilatation in rat mesenteric arteries [231]. Blockade of K_{Ca} channels by TEA attenuated the insulin-mediated vasodilatation of human vessels [232]. However, the data from *in vitro* studies using resistance arterioles is conflicting though hyperpolarization in

one way or another seems to play a role in insulin-mediated vasodilatation [77, 94, 116, 168]. Opening of K_{Ca} channels causes potassium efflux and hyperpolarization of vascular smooth muscle cells. This in turn leads to closure of voltage-dependent calcium channels, resulting in reduction of calcium influx and vasodilatation [171, 200, 208]. Nitric oxide is also capable of hyperpolarizing smooth muscle. It has been suggested that activation of calcium-dependent potassium channels plays an important role in mediating the vasorelaxation caused by NO [171, 234, 235]. Attenuated EDHF-mediated responses have been noticed with no or minor alteration in NO-dependent responses in insulin resistant rats [237-241].

The only one *in vivo* study reported so far using TEA argues against a role for calcium-dependent potassium channels in insulin-mediated increase in total flow and glucose uptake in humans *in vivo* [242]. The role of K_{Ca} channels in the macro and microvascular response to insulin has not been investigated in rats *in vivo*. The present study was undertaken to see whether these channels contributes to insulin-mediated capillary recruitment and glucose uptake in muscle.

6.2 RESEARCH DESIGN AND METHODS

6.2.1 Animals

Male Hooded Wistar rats weighing 280 ± 3 were raised on a commercial diet as described in chapter 2.1.

6.2.2 Surgical preparation

Details were as essentially described previously in chapter 2.2. In these rats epigastric cannulation was done as described in section 2.3. A schematic diagram is given in chapter 2 (page 43). Once the surgery was completed, a 45-60 min equilibration period was allowed so that leg blood flow and mean arterial pressure could become stable and constant. Rats were then subjected to the protocol (Fig. 1) where they were infused

locally (epigastric artery) into the test leg with tetraethylammonium chloride starting 15 minutes before and continued with systemic saline or insulin (as a euglycemic insulin clamp with 3 mU/kg/min) for 1 h. Preliminary experiments were conducted to determine the dose of TEA that would produce a decrease in FBF without effects on MAP or heart rate. TEA was administered at an infusion rate of 10 μl/min, calculated to lead to a local plasma concentration of 0.5 mM. Low dose TEA (0.2-3 mM) selectively blocks K_{Ca} channels *in vitro* [202, 228, 229], whereas TEA loses its specificity at high doses (>5 mM) [230] and can inhibit potassium-, ATP-, and voltage-dependent channels [287].

6.2.3 Blood samples

Arterial samples were taken at the times indicated (Fig. 1) for blood glucose measurements. The femoral vein of each leg was used for venous sampling, using a 29G insulin syringe (Becton Dickinson). Duplicate venous samples (300 μl) were taken only on completion of the experiment (total time 75 min) to prevent alteration of the blood flow from the hindlimb due to sampling, and to minimize the effects of blood loss.

6.2.4 Capillary recruitment

Capillary recruitment was determined by measuring the metabolism of infused 1-MX.

Plasma (20 μl) from arterial and leg venous blood samples taken at the end of the experiment was mixed with 80 μl of 0.42M perchloric acid and centrifuged for 10 min. The supernatant was used to determine 1-MX, allopurinol and oxypurinol concentrations by reverse-phase HPLC as previously described in section 2.4. Capillary recruitment, expressed as 1-MX metabolism was calculated from arterio-venous plasma 1-MX difference and multiplied by femoral blood flow.

Ten min prior to the completion of each experiment, 50 μCi bolus of [^{3}H]2-DG was administered (Fig. 1). At the conclusion of the experiment in both protocols, the soleus,

plantaris, gastrocnemius white, gastrocnemius red, EDL and tibialis muscles were removed, clamp frozen in liquid nitrogen and stored at -20°C to be assayed for 2-DG uptake as described in section 2.6. A glucose analyzer was used to determine whole blood glucose (by the glucose oxidase method) during the insulin clamp.

6.2.5 Expression of results

All data are expressed as means ± SEM. Mean femoral blood flow, mean heart rate and mean arterial pressure were calculated from 5 second sub-samples of the data, representing approximately 500 flow and pressure measurements every 15 minutes. Vascular resistance in the hindleg was calculated as mean arterial pressure in millimetres of mercury divided by femoral blood flow in millilitres per minute and expressed as resistance units (RUs).

6.2.6 Statistical analysis

Repeated measures two-way analysis of variance was used to test the hypothesis that there was no difference among treatment groups for femoral blood flow, blood pressure, heart rate, vascular resistance, 1-MX, R'g and GIR concentrations throughout the time course. When a significant difference ($P < 0.05$) was found, pair wise comparisons by the Student-Newman-Keuls test were used to determine at which individual times the differences were significant. All tests were performed using the SigmaStat™ statistical program (Jandel Software Corp.).

6.3 RESULTS: EFFECTS OF LOCAL EPIGASTRIC INFUSION OF TEA

6.3.1 Hemodynamic effects

The dose of TEA infused was previously determined to decrease FBF in the test leg without changing FBF in the contralateral control leg, heart rate or MAP. Figure 2 shows the blood pressure and heart rate. Figure 3 shows the change in FBF and vascular resistance for both control and test legs in saline and insulin groups where TEA was infused locally via the epigastric artery of the test leg and measurements were made in both legs, while animals were receiving saline infusion systemically or were under hyperinsulinemic euglycemic clamps at 3 mU/kg/min. Insulin infusion did not increase the FBF significantly when the two control legs were compared between saline and insulin groups ($P = 0.07$). TEA decreased FBF in both saline and insulin groups only in the test leg (test leg significantly different in insulin group from control leg at 45 and 60 min $P < 0.05$, Fig. 3A). The values for MAP (Fig. 2A) before commencement of TEA infusion were 103 ± 4 (saline) and 108 ± 2 mmHg (insulin). At 75 min after TEA infusion there were no significant changes and the values were 107 ± 3 (saline) and 110 ± 1 mmHg (insulin). Consequently, the calculated vascular resistance in the leg receiving TEA was found to increase (Fig. 3B).

6.3.2 Glucose metabolism

Blood glucose levels for the two groups involving TEA infusion with saline or insulin infused systemically were constant (Fig. 4A). The blood glucose values at 0 min were 3.9 ± 0.3 (saline) and 4.4 ± 0.2 mM (insulin) and at 75 min, 4.5 ± 0.2 (saline), 4.8 ± 0.2 mM (insulin). Glucose infusion rate (Fig. 4B) to maintain euglycemia during insulin reached a plateau at 14.4 ± 0.6 mg/kg/min.

Figure 5A shows data for R'g of individual muscles of the lower leg. TEA alone (saline background) had no effect on R'g of any of the muscles from the leg into which it was infused (Fig. 5). TEA (insulin clamp background), attenuated the insulin-mediated

increase in R'g in white gastrocnemius and tibialis muscles ($P < 0.05$) (Fig. 5A) of the leg into which it was infused. For the muscle combination, insulin increased R'g ~ 1.8-fold from 7.8 ± 0.4 to 14.0 ± 1.6 μg/g/min (Fig. 5B). Infusion of TEA in the test leg (insulin group) decreased the glucose uptake to 11.4 ± 1.0 (Fig. 5B).

6.3.3 Capillary recruitment

No significant difference was found between the two experimental groups in arterial plasma concentrations of 1-MX (saline ± local TEA, 20 ± 1.0 μM; insulin ± local TEA, 20 ± 3.0 μM) or oxypurinol, the metabolite of allopurinol and inhibitor of xanthine oxidase (saline ± local TEA, 6.0 ± 0.5 μM; insulin ± TEA, 6.1 ± 0.4 μM). Local infusion of TEA had no effect on capillary recruitment, 5.0 ± 0.8 to 4.5 ± 0.8 nmol/min in the test leg as judged from 1-MX metabolism. Systemic insulin infusion increased 1-MX metabolism. This was significant when control legs were compared ($P < 0.05$; Fig. 6). 1-MX was decreased from 11.3 ± 0.8 to 6.6 ± 1.3 nmol/min ($P < 0.05$) in the test leg when TEA was infused locally on a background of insulin clamp (Fig. 6) indicating a complete inhibition of insulin-mediated capillary recruitment.

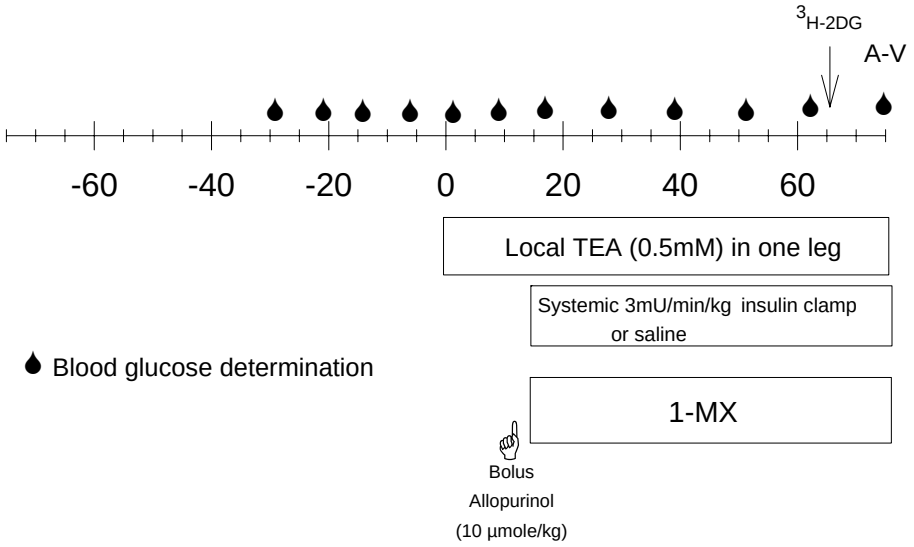


Fig. 1. Study design. The protocol involved the euglycemic clamp at 3 mU/kg/min insulin commencing at time = 0 min and TEA infused into the epigastric artery of the test leg (0.5 mM). Duplicate arterial and femoral venous plasma samples from each hindleg (test and contralateral control) were collected at the end of experiment, for HPLC analysis, and plasma glucose determinations. Systemic venous infusions are indicated by the bars. Bolus systemic injections of allopurinol or 2-DG were made as indicated. Arterial samples for glucose determinations are indicated by ♦. Muscle samples were taken at the end of the experiment for 2-DG. saline ± TEA, n = 5; insulin ± TEA, n = 7.

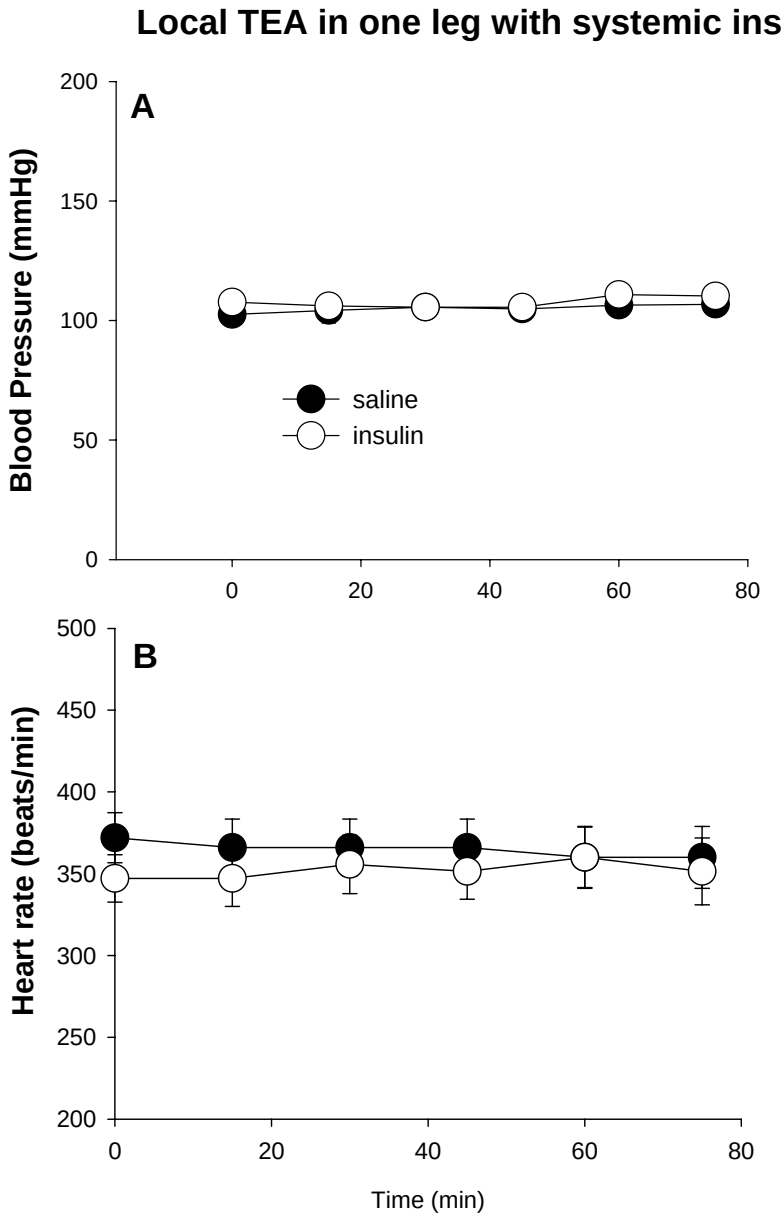


Fig. 2. Mean arterial pressure (A) and heart rate (B) as a result of local infusion of TEA via epigastric artery in one leg with or without systemic insulin. Values are means ± SEM.

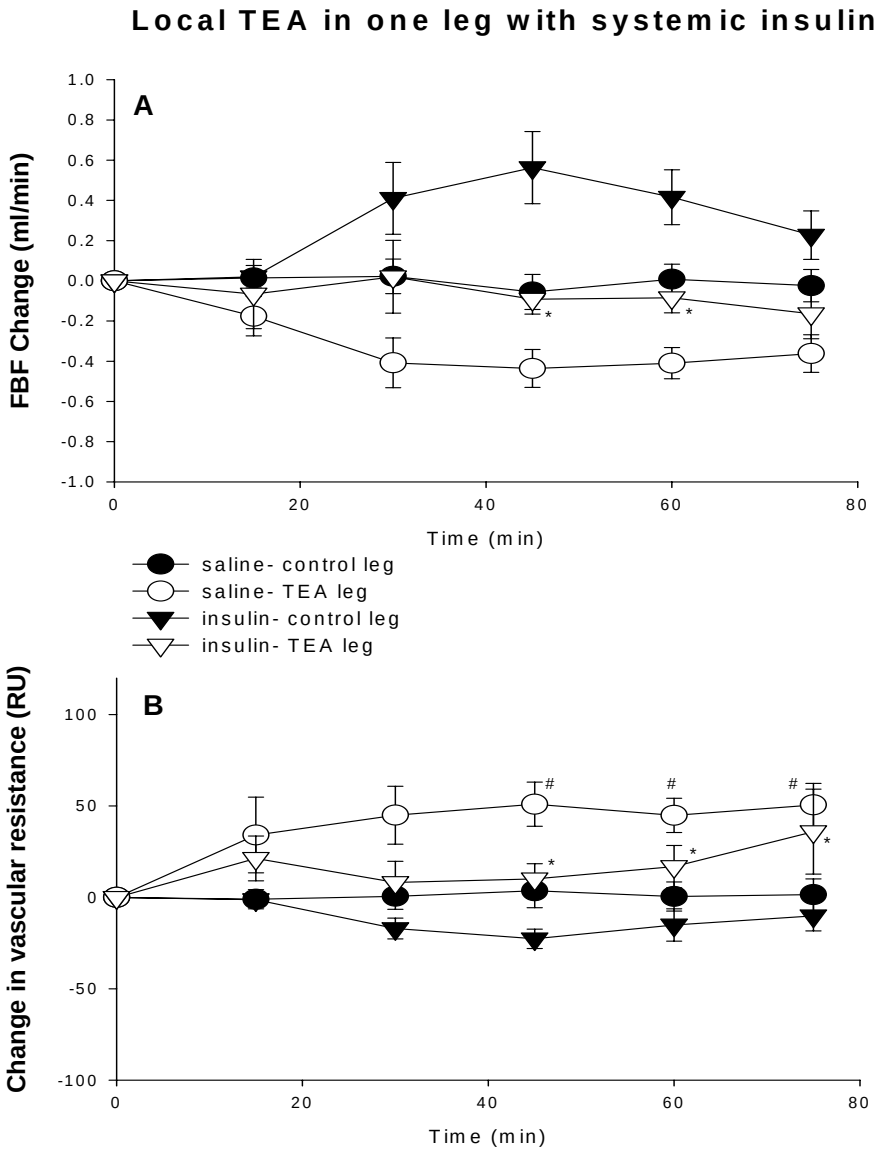


Fig. 3. Change in FBF (A) and VR (B) as a result of local infusion of TEA via the epigastric artery into one leg with or without systemic infusion of insulin. Details are given in Fig. 1. * indicates that insulin-TEA leg is significantly different from insulin-control leg, $P < 0.05$. # indicates that saline-TEA leg is significantly different from saline-control leg, $P < 0.05$.

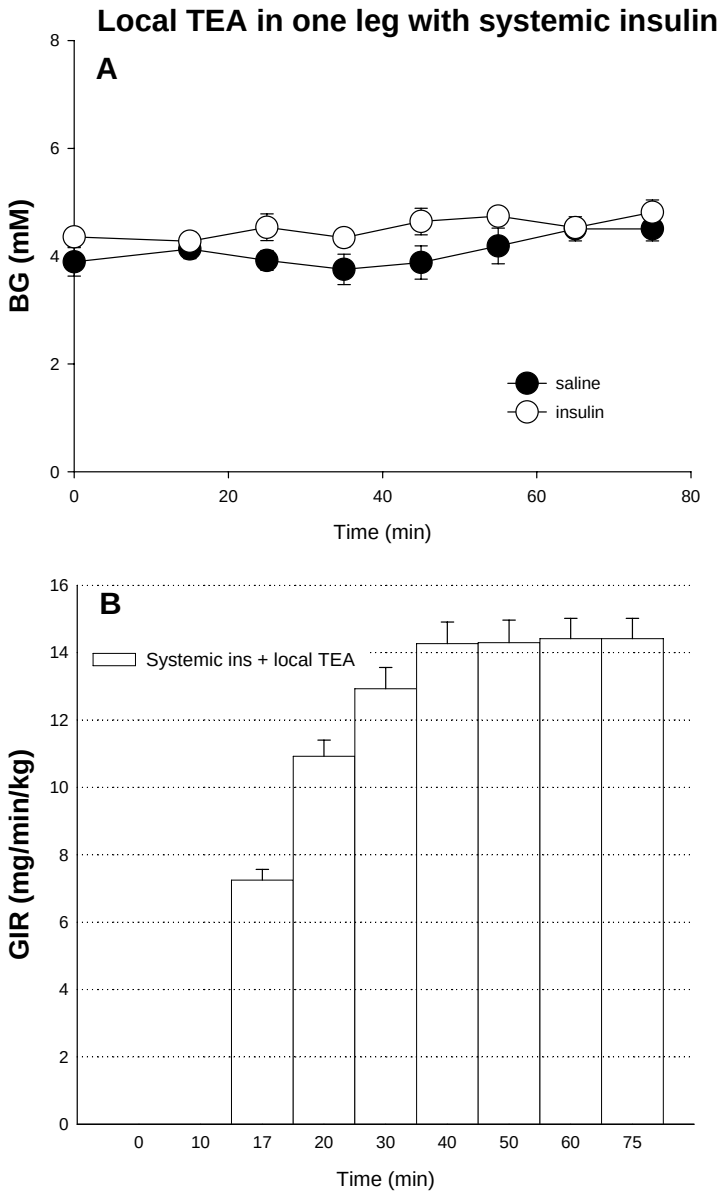


Fig. 4. Blood glucose (A) for saline and insulin treated rats. It also shows glucose infusion rate (B) to maintain blood glucose level at or above basal level during insulin infusion. Values are means ± SEM.

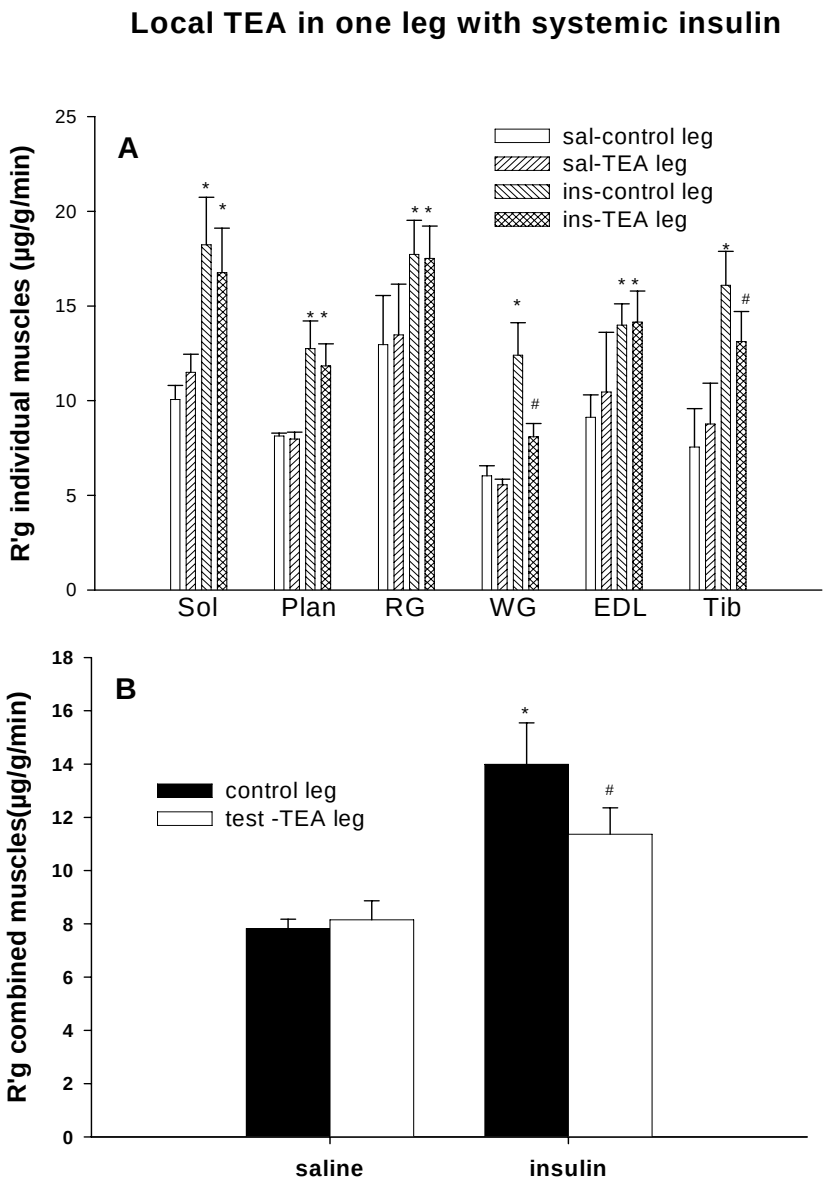


Fig. 5. Effect of local one-leg infusion of TEA on 2-deoxyglucose uptake (R'g) by lower leg muscles. TEA was infused only in the test leg; saline or insulin was infused systemically. *, Significantly different from the control leg (saline); #, significantly different from the contralateral leg ($P < 0.05$). Values are means ± SEM.

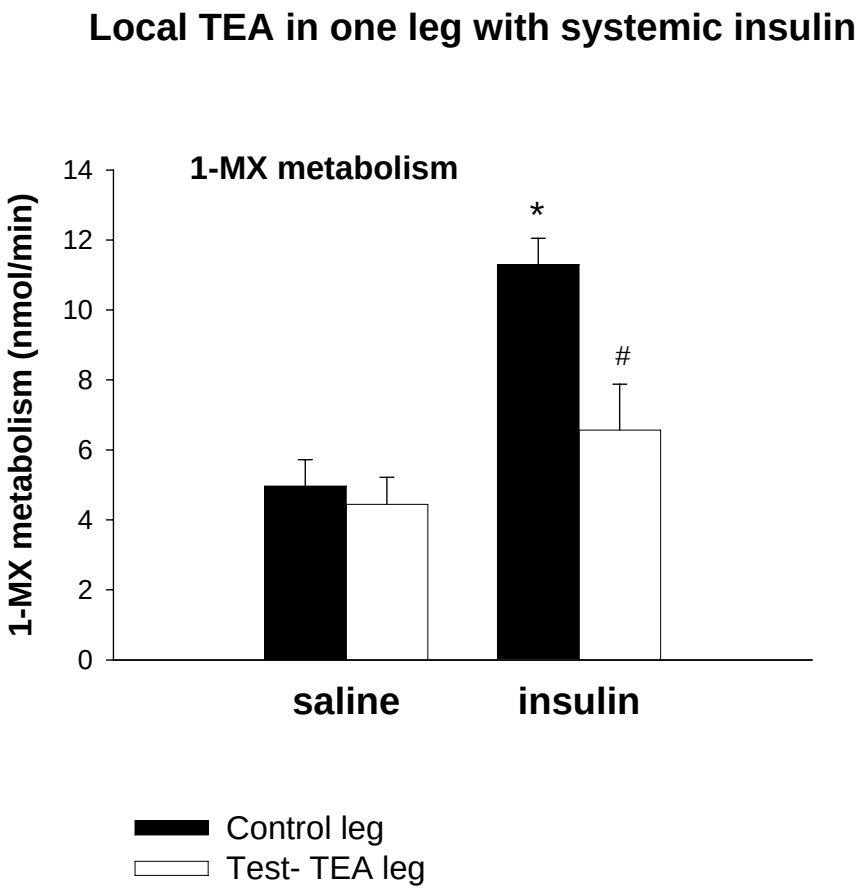


Fig. 6. I-MX metabolism as a result of local infusion of TEA via the epigastric artery into one leg with or without systemic infusion of insulin. Details are given in Fig. 1. *, Significantly different from the control leg (saline); #, significantly different from the contralateral leg ($P < 0.05$). Values are means ± SEM.

6.4 DISCUSSION

The present study explores the role of calcium-dependent potassium (K_{Ca}) channels in insulin-mediated hemodynamic (microvascular) and metabolic effects (glucose uptake) in muscle *in vivo*. Local intra-arterial infusion of a blocker of vascular smooth muscle K_{Ca} channels almost completely blocked capillary recruitment while partially blocking insulin-mediated glucose uptake.

In this study, a hyperinsulinemic euglycemic clamp was combined with local intra-arterial infusion of tetraethylammonium (TEA) in one leg at a dose which decreased the femoral blood flow only in the test leg. TEA antagonizes different types of channels with varying degrees of potency [288] but the low dose used in this study selectively blocks vascular K_{Ca} channels [228, 243, 289]. TEA was infused intra-arterially to obtain local effective concentrations (0.5 mM) in the test hindleg without interference from systemic effects.

There is some evidence indicating the contribution of NO, prostacyclin [94-96] and adenosine [93] to insulin-induced vasodilatation. Other authors have suggested that the relative contribution of these mechanisms to insulin-induced vasodilatation probably depends on the vessel size [77, 168, 290]. The contribution of NO and EDHF to insulin-mediated effects can vary with vessel diameter, with EDHF being more prominent in smaller muscular arteries and arterioles than in larger muscular or conduit vessels [77, 168]. In the present study, TEA blocked the insulin effect on muscle microvasculature. The dose of insulin used did not have a significant effect on total blood flow and thus the effect of TEA on insulin-mediated increase in total flow could not be assessed.

A role for EDHF in insulin-mediated hemodynamic effects has been suggested mainly from studies using isolated vessels. Insulin-induced relaxation of rat mesenteric artery can be abolished by charybdotoxin, (a blocker of large and intermediate K_{Ca} channels) and endothelial denudation but not by L-NAME suggesting a role for large-conductance K_{Ca} channels and EDHF [212, 231]. Izhar and coworkers [232] described an

endothelium independent, nitric oxide independent vasorelaxation of rings from human internal mammary artery and saphenous vein in response to insulin that was attenuated by the potassium channel blocker TEA. However, the high dose of TEA (10 mM) used in their study [232] reflected a non-specific antagonism of all potassium channels [230]. In the current study, a low dose of TEA has been used which selectively blocks large conductance K_{Ca} channels in smooth muscle cells *in vitro* [228, 229, 291] while higher concentrations (>5 mM) can inhibit both ATP and voltage-dependent potassium channels [230].

Three studies report conflicting results in resistance arteries, all using similar concentrations of insulin and of inhibitors. Miller [94] and Chen [116] in separate studies could not find a role for calcium-dependent potassium channels in the vascular response to insulin using TEA, charybdotoxin, and apamin (a blocker of small conductance K_{Ca} channels). In contrast, insulin-induced vasodilatation could be inhibited by tetrabutylammonium chloride, a non-specific potassium channel blocker but not by specific potassium channel blockers (TEA, charybdotoxin, and apamin) suggesting that hyperpolarization via some other K_{Ca} channels is probably involved [168]. McKay et al [77] have also shown that insulin-induced dilatation in hamster cremaster arterioles is NO-dependent in second order but not in third- or fourth-order arterioles while blockade of ATP-sensitive potassium channels by glibenclamide prevented insulin-induced dilatation in both second and fourth-order arterioles. Thus, hyperpolarization in one way or another seems to play a role in insulin-mediated vasodilatation.

In the intact animal, the involvement of K_{Ca} channels in the vasodilator response to insulin is difficult to assess and very few studies have been designed specifically to address this issue. There is only one *in vivo* study (Abbink et al) [242] to date using TEA and glibenclamide, which argues against a role for calcium- and ATP-dependent potassium channels in insulin-mediated increase in total flow and glucose uptake in humans. In contrast, in the present study low dose TEA totally abolished insulin-mediated capillary recruitment and partially blunted insulin-mediated glucose uptake. Dose-, species- and methodological differences could account for the observed differences

between the current study and the study by Abbink et al [242]. The use of specific potassium channel blockers (the toxins- charybdotoxin, iberiotoxin and apamin) is restricted as they are too toxic for *in vivo* use [243]. TEA did not have any significant effect on capillary recruitment or glucose uptake on its own.

This study is the first of its kind pointing towards the role of K_{Ca} channels in insulin-mediated effects in muscle microvasculature *in vivo*. The mechanism of insulin-mediated capillary recruitment in skeletal muscle is still unresolved. A separate study in this thesis (chapter 5) demonstrates that nitric oxide is responsible, in part, for insulin-mediated capillary recruitment. Lack of availability of methods to measure capillary recruitment limits the number of available studies but evidence available from the studies looking at insulin-mediated increase in total flow suggests that NO participates in endothelium-dependent relaxation by insulin. The second mechanism is independent of NO-cGMP and may be mediated by hyperpolarization of vascular smooth muscle cell membrane [292, 293]. This study provides evidence for the involvement of K_{Ca} channels in insulin-mediated capillary recruitment. There is some evidence that potassium is a non-nitric oxide, non-prostanoid endothelium-derived relaxing factor in rat femoral arteries [171, 294].

The current study also looked specifically at the relationship between hemodynamic and metabolic actions of insulin. Significant inhibition of insulin-mediated capillary recruitment and simultaneous attenuation of insulin-mediated glucose uptake confirms the previously observed relationship between capillary recruitment and glucose uptake. This dual inhibition may indicate blood flow redistribution from the nutritive to the nonnutritive network. It has been demonstrated previously that α-methylserotonin [65], TNF-α [66] and T-1032 [295] (chapter 3) completely abolished insulin-mediated increases in capillary recruitment and attenuated ~50% of insulin-mediated glucose uptake. Insulin resistant models including genetically obese Zucker rats [66, 296] and acute administration of Intralipid® and heparin to raise circulating free fatty acids have been shown to have a similar effect [67].

It is important to mention that out of 6 hindleg muscles TEA significantly attenuated insulin-mediated glucose uptake only in the white gastrocnemius and tibialis muscles. White gastrocnemius is made up of white fibers while tibialis is composed of both white and red fibers [297, 298]. As the TEA effect on these muscles does not seem to be fiber-specific, there is no apparent explanation why other muscles did not show an attenuation of insulin-mediated glucose uptake with TEA. Together, the two muscles make the bulk of the hindleg muscles (70%) collected for 2-DG analysis. Inhibition of insulin-mediated capillary recruitment by TEA could also have occurred predominantly in these muscles. The 1-MX method measures capillary recruitment across the whole hindleg. There is no known method to specifically measure capillary recruitment in individual muscles.

TEA decreased the FBF only in the test leg in both the insulin and saline groups. To determine whether the flow effects were responsible for the decrease in glucose uptake in the insulin group, some experiments were done where the FBF was manually reduced (by applying a suture around femoral artery) by 45% only in one leg during systemic insulin clamp. This intervention did not have any effect on 2-DG uptake, as both the legs showed similar 2-DG uptakes (data not shown).

Attenuated EDHF-mediated responses have been reported in various insulin resistant rat models including the fructose-fed rat, the leptin deficient, genetically obese and mildly hypertensive Zucker rat and the Otsuka Long-Evans Tokushima fatty rat [85-87, 138, 159]. Oliveira and coworkers [233] confirmed the contribution of EDHF to the alteration of microvascular reactivity in diabetic rats.

It is concluded that acute local intra-arterial administration of the potassium channel blocker, TEA, induced a state of insulin resistance at microvascular level in muscle. It is proposed that activation of potassium channels on vascular smooth muscle may play a role in insulin-mediated capillary recruitment, and that activation of these channels increases potassium efflux, producing hyperpolarization of smooth muscle cells. This would reduce intracellular calcium and lead to vasorelaxation. Inhibition of K_{Ca} channels by TEA inhibits vascular smooth muscle hyperpolarization. Thus, this study highlights

the importance of K_{Ca} channels in insulin-mediated hemodynamic effects in muscle capillary recruitment. This effect was simultaneously associated with blunting of insulin's metabolic effect. Further investigation into the role of potassium channels will increase our understanding of normal physiology of insulin action and may have important therapeutic implications.

CHAPTER 7

GENERAL DISCUSSION

7.1 Key findings

The work presented in this thesis explored the role of NO and calcium-dependent potassium channels (K_{Ca}) on insulin-mediated capillary recruitment and glucose uptake in muscle. This was examined by infusing test agents systemically or locally during hyperinsulinemic euglycemic clamps in anesthetized rats. A novel technique was successfully developed for this purpose wherein the epigastric artery (a branch of femoral) was cannulated and test substances were infused locally in one leg to avoid any systemic effects. Unexpectedly, inhibiting the degradation of cGMP using systemic infusion of a type 5 phosphodiesterase inhibitor, T-1032, did not potentiate insulin action. On the other hand, enhancing the production of NO and doubling the total flow with local infusion of methacholine (but not bradykinin) in one leg increased the microvascular recruitment and led to potentiation of insulin-mediated glucose uptake. However, both local and systemic infusion of L-NAME had no effect on insulin-mediated glucose uptake while only the systemic L-NAME blunted the insulin-mediated capillary recruitment. Furthermore, local intra-arterial infusion of the K_{Ca} blocker, TEA, significantly reduced insulin-mediated capillary recruitment and attenuated insulin-mediated glucose uptake. Collectively, these findings suggest that other than insulin, methacholine can also induce capillary recruitment and that both systemic NO and local K_{Ca} channels seem to contribute to insulin-mediated capillary recruitment. Moreover, the results further corroborate the previous reports regarding the important contribution of microvascular blood flow to glucose uptake [65-67, 296].

7.2 Insulin mediated capillary recruitment and its effect on glucose uptake

There is substantial evidence from previous studies for the existence of two vascular routes within muscle [69, 70, 73]. One is nutritive, made up of long tortuous capillaries and other is non-nutritive, made up of shorter slightly wider capillaries. Insulin probably distributes the flow from non-nutritive to nutritive areas in skeletal muscle, thus facilitating the access of insulin and glucose to muscle cells. To accomplish this, insulin may have to constrict the terminal arterioles preceding non-nutritive vessels while relaxing the arterioles supplying nutritive vessels. Thus insulin, possibly by a dual vascular action, increases capillary recruitment.

A correlation between capillary recruitment and glucose uptake has been shown [65-67]. In a separate set of studies - α-methyl serotonin, TNF-α and lipid infusion inhibited insulin-mediated increases in total flow and capillary recruitment *in vivo* and impaired ~50% of insulin-mediated glucose uptake. The same correlation was observed in this thesis with T-1032 and TEA studies (chapters 3 and 6). Also, methacholine potentiated insulin-mediated capillary recruitment accompanied by an increase in insulin-mediated glucose uptake (chapter 4).

7.3 Mechanism of insulin action in muscle

Thus, there is evidence that factors affecting insulin-mediated capillary recruitment will alter glucose uptake. Despite an enormous amount of research into the vascular actions of insulin, the mechanism of action of insulin-mediated capillary recruitment is still not certain.

Insulin-mediated capillary recruitment could be the result of a mediator released from endothelial cells, VSMC or myocytes. There is evidence for the involvement of NO [52, 115, 120], hyperpolarization factor [231, 232], adenosine [93] and prostacyclin [94-96]. Insulin may also have direct effect on endothelial cells, VSMC or myocytes. The direct effect of insulin on myocytes could also be important as MIRKO mice (muscle-specific

insulin receptor knockout) [299, 300] exhibited an impaired isolated insulin-mediated glucose uptake despite normal whole body glucose disposal. On the other hand, a direct neural effect of insulin cannot be ruled out.

7.4.1 Local NOS inhibition: no effect on insulin action

It has been raised by a number of workers [85, 86, 126] that nitric oxide (NO) may play a role in muscle glucose uptake under basal conditions, and also during exercise. Most of these researchers regard the effects as direct on muscle and not involving vascular actions. Arguments in favour of such a relationship have been based on findings that NO donors such as sodium nitroprusside increase 2-deoxyglucose (2-DG) uptake in a dose-dependent manner in isolated incubated soleus [301] and EDL [89, 90, 302] muscles. In addition, sodium nitroprusside has been shown to increase the rate of glucose oxidation in incubated soleus muscles [261].

By employing three different strategies- enhancing cGMP levels in muscle or NO production or inhibiting NOS in muscle (chapter 3, 4 and 5) during an insulin clamp we have shown that NO is not involved locally in insulin action in muscle. A striking finding (chapter 4) was that methacholine infusion in one leg enhanced insulin-mediated glucose uptake by muscle *in vivo* and that this effect of methacholine was associated with a marked augmentation of capillary recruitment. This was not solely related to enhanced NO production as another nitro-vasodilator, bradykinin neither increased capillary recruitment nor glucose uptake (chapter 4).

Thus, NO-cGMP pathway does not appear to play a direct role in insulin-mediated capillary recruitment and glucose uptake locally in muscle (Fig. 1, page 151).

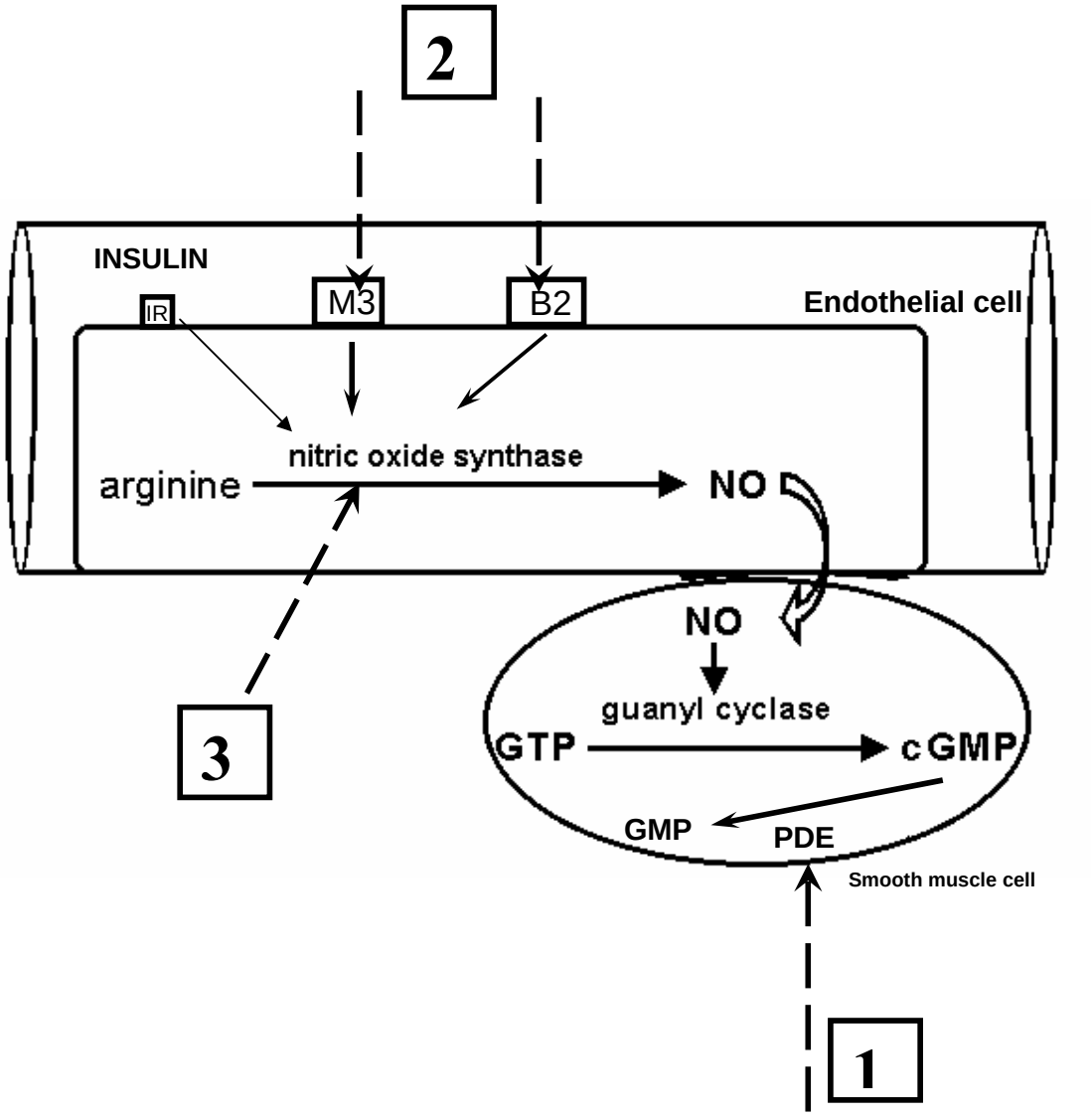


Fig. 1. Results: insulin action on muscle microvasculature is not nitric oxide-dependent locally.

1. Increased muscle cGMP action with T-1032: inhibition of insulin action

2. Increased NO production using methacholine and bradykinin: non-specific increase in insulin action with methacholine only

3. Decreased NO synthesis using nitric oxide synthase inhibitor: no effect on insulin action locally in muscle.

7.4.2 Systemic NOS inhibition: partially blocks insulin-mediated capillary recruitment

The results of gene-deletion studies however, indicate that NO is indirectly involved in insulin action. Both eNOS and nNOS knockout mice were insulin resistant [113, 114]. It has also been reported by Vincent et al that when NO production is blocked by systemic infusion of an inhibitor of NO synthase, insulin-stimulated capillary recruitment is totally blocked and insulin-mediated glucose uptake by muscle is inhibited by approximately 50% [63].

However, results from chapter 5 indicate that systemic NOS inhibition partially inhibits insulin-mediated capillary recruitment and does not block insulin-mediated glucose uptake. This suggests that NOS involvement possibly in tissues other than muscle plays some role in insulin action. Central effects of L-NAME during systemic infusion could have accounted for the partial inhibitory effect on insulin-induced capillary recruitment since local NOS inhibition had no effect on insulin action. Shankar et al [121] reported that central NOS–dependent pathways may control peripheral insulin action and secretion. Intracerebroventricular (ICV) administration of L-NMMA caused hyperglycemia via the induction of defects in insulin secretion and insulin action. They [121] and others [303] have suggested that perhaps one of the efferent signals triggered by insulin in the CNS may be nitrergic in nature. Oboci [304, 305] demonstrated that ICV infusion of insulin suppressed glucose production (impaired by central antagonism of insulin signaling) in the absence of increased circulatory levels of insulin. Increased glycogen synthesis in response to ICV infusion of insulin has also been reported [306]. Thus there is some evidence for the central NO-dependent efferent pathways and for the role of central nervous system in peripheral action of insulin.

On the other hand**,** Lautt and coworkers [276] raised the hypothesis that insulin initiates a parasympathetic reflex in liver that releases acetylcholine, leading to generation of nitric oxide and subsequently to release of a hormone, hepatic insulin sensitizing substance (HISS) which sensitizes the skeletal muscle response to insulin (or has a direct

insulin-like action). They demonstrated [275] that NOS antagonism by L-NAME is caused by blockade of this hepatic parasympathetic reflex that is released in response to insulin. It is possible that the hepatic effect of L-NAME might have played a role in systemic study (chapter 5) but whether this also plays a role in insulin-mediated capillary recruitment remains speculative.

Thus it seems NO is involved in insulin action, but elsewhere, not in the muscle microvasculature. Results from previous studies [39, 52, 120] indicate that NO is responsible for insulin-mediated increases in total flow. This could not be assessed in the current study as the physiological dose of insulin used did not have significant effect on total flow.

7.5 Another mediator involved in insulin action on muscle microvasculature

It has been reported that the effect of insulin on muscle microvasculature is a more sensitive and quicker event than its effect on total blood flow. It was found in the present study (chapters 5 and 6) that physiological insulin (3 mU/min/kg) induced capillary recruitment at 60 minutes, without inducing a significant increase in total flow. This indicates that microvascular recruitment is an early event and more sensitive than total flow at one hour of insulin clamp. Previous reports using contrast enhanced ultrasound [68] demonstrated that while insulin required 120 min to augment total flow, it increased microvascular volume within 30 min in skeletal muscle. This indicates that smaller (3^{rd} to 5^{th} order) arterioles respond quicker to insulin than the larger resistance vessels. It is also reported that capillary recruitment is markedly more sensitive than R'g to insulin [13]. Thus the effect of insulin on microvasculature seems to be more important [307].

Muscle microvasculature may have different sensitivity to mediators such as NO and potassium. It has been suggested that the relative contribution of NO [308] and EDHF [165] to agonist-induced vasodilatation depends on the vascular bed and on the vessel diameter [165]. EDHF plays a more prominent role in smaller muscular arteries and arterioles than in larger muscular or conduit vessels *in vivo* [165, 166] and *in vitro* [1,

164, 166]. Oltman [168] and McKay et al [77] have reported that in microvessels, relaxation to insulin is not mediated by NOS pathways but rather through potassium-dependent mechanisms. McKay et al [77] have reported an increase in insulin sensitivity (vasodilatation) with decreasing vessel size.

The current study (chapter 6) supports the role for calcium-dependent potassium channels (K_{Ca}) in insulin action locally in muscle microvasculature. Infusion of a blocker of K_{Ca} channels tetraethylammonium (TEA) in one leg greatly reduced insulin-mediated capillary recruitment and attenuated insulin-mediated glucose uptake. A low dose of TEA was used which selectively blocks large conductance calcium-dependent potassium channels VSMC *in vitro* [228, 229, 291]. However, the specificity of pharmacological blockade is based on published *in vitro* studies, as the specific effects of the potassium channel blockers during *in vivo* studies are incompletely identified [309].

Recently, a number of *in vitro* studies [94, 116, 168, 212, 231, 232] have indicated the role of potassium in insulin-mediated hemodynamic effects. Conversely, the only *in vivo* study reported so far to examine the role of potassium (K_{Ca} and K_{ATP}) channels in insulin action using TEA and glibenclamide did not find a role for calcium-dependent potassium channels in insulin-mediated increase in total flow and glucose uptake in humans [242]. This study however, used a different methodology compared to the study in chapter 6 and was done in humans.

Thus, results from this thesis indicate that insulin action in muscle is not the result of a single mediator. This thesis provides evidence for involvement of systemic NO and local K_{Ca} channels in insulin-mediated capillary recruitment.

7.6 Insulin action: role of endothelial cells and/or VSMC

Insulin may stimulate endothelial-dependent hyperpolarization of VSMC by stimulating transient increases in intracellular calcium in the endothelial cell via non-selective cation channels and release of calcium from intracellular stores causing

endothelial hyperpolarization [196-199]. The endothelial hyperpolarization induces efflux of K^+ through the endothelial SK_{Ca} and IK_{Ca} channels (calcium-dependent small conductance potassium channels and calcium-dependent intermediate conductance potassium channels). This increases extracellular potassium and causes endothelium-dependent hyperpolarization of underlying smooth muscle cells, inhibiting voltage-activated calcium channels which results in vasorelaxation.

There is some evidence that insulin might not act directly on endothelial cell to cause capillary recruitment. The observations that vascular endothelial cell insulin receptor knock-out (VENIRKO) mice [310] had normal fasting glucose and insulin levels and were not insulin resistant argues against the origin of a dilator from endothelial cells [310], though glucose uptake and effect of insulin on blood flow was not measured in these mice. Furthermore, the results from chapters 3, 4 and 5 also do not support the endothelial origin of NO as a mediator involved in insulin action locally.

However, results from the VENIRKO [310] studies should be interpreted with caution. It is difficult to assess the results of gene-deletion studies since gene-deletion may lead to compensatory developmental changes. Theoretically, if endothelial cells are involved in capillary recruitment, VENIRKO mice [310] will show a total loss of insulin-mediated capillary recruitment and will result in 50% loss of glucose uptake. This should not result in whole body insulin resistance because MIRKO mice [299, 300] with almost total loss of glucose uptake do not result in whole body insulin resistance.

If endothelial cells are not involved then it is possible that insulin acts directly on VSMC to cause vasodilatation via an endothelium independent mechanism. Direct action of insulin on smooth muscle and a decrease in cytosolic calcium concentration in aortic smooth muscle cells in response to insulin has been demonstrated in several studies [79-81, 83]. It has been reported that insulin may activate and translocate sodium potassium ATPase in VSMC leading to hyperpolarization of VSMC [210]. Alternatively, insulin may activate calcium-dependent potassium channels present on VSMC inducing potassium efflux and membrane hyperpolarization [242]. This may reduce the influx from

voltage-dependent calcium channels, decreasing intracellular calcium leading to vasorelaxation (Fig. 4, page 35). In patients with insulin resistance increased intracellular calcium has been reported in VSMC [84].

Thus insulin may not necessarily act via the endothelium. Potassium channels in VSMC may be involved in insulin action.

7.7 Another way of inducing capillary recruitment: Methacholine

Previously, exercise and then insulin have been identified as mediators of capillary recruitment. A striking finding that has come out of this study (chapter 4) is that methacholine can also cause capillary recruitment. Mechanism of methacholine-mediated capillary recruitment could be different from other two, probably involving muscarinic receptors. Methacholine further increased capillary recruitment due to insulin suggesting that the methacholine effect to stimulate capillary recruitment is independent and additive to that of insulin, likely to be engaging receptors in the same locality of the microvasculature.

7.8 Other mechanisms of insulin mediated capillary recruitment?

This study has highlighted that other than NO, potassium movements could also be involved in insulin-mediated capillary recruitment and has opened up a new field to be explored in relation to insulin action. Role of more specific potassium channels in insulin's action in muscle need to be investigated by using specific antagonists viz, 1-EBIO, TRAM 34 or TRAM 39 as blockers of IK_{Ca} [169, 181, 214], and toxins such as apamin to block SK_{Ca} [202], iberiotoxin, a blocker of large conductance calcium-dependent potassium channels (BK_{Ca}) [215], charybdotoxin which blocks both $IK_{Ca,}$ BK_{Ca} and also voltage-sensitive potassium channels [216], and scyllatoxin, a structurally distinct SK_{Ca} inhibitor [217-220]. However, use of these toxins is limited as they are toxic for *in vivo* use. Moreover, role of adenosine and prostacyclin in insulin-mediated capillary recruitment needs to be examined. Thus, there is a possibility that capillary recruitment

mediated by insulin can be modulated by various factors. Since this study also highlights that methacholine could also cause capillary recruitment, the possibility that other muscarinic agonists (such as bethanechol, carbachol) can influence insulin action could be explored. ICV infusion of insulin and effect of ICV NOS and potassium channel antagonism could also be tried.

7.9 Conclusion

In conclusion, the work presented in this thesis explored a number of possible mechanisms of insulin action in muscle. A novel technique was developed to test the agents locally in the leg and to examine their effects on microvascular action of insulin and its relation with glucose uptake.

Insulin-mediated capillary recruitment is unlikely to involve local NO production. It is possible that nitrergic signals generated by insulin in the brain or other sites (liver) remotely stimulate capillary recruitment.

Capillary recruitment in muscle may involve calcium-dependent potassium channels. This might be via EDHF or insulin could act directly on VSMC.

It was found that capillary recruitment can also be induced by an endothelium-dependent nitro-vasodilator methacholine, which also potentiated insulin-mediated glucose uptake.

This study has opened up new areas for further investigation. It is stressed that a reduction in insulin-mediated hemodynamic action in muscle might contribute to insulin resistance. It is anticipated that a better understanding of mechanisms involved in capillary recruitment will be helpful in designing new targets for the treatment of insulin resistance.

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