NUTRITIVE AND NON-NUTRITIVE BLOOD FLOW IN MUSCLE

By

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DECLARATION

This thesis contains no material which has been used for the award of any other degree or graduate diploma in any tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

J.M. Youd

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Effect of muscle contraction on metabolism of 1-MX and L-arginine in the constant flow perfused rat hindlimb.

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CONTENTS

ABBREVIATIONS

A-V	arteriovenous
bpm	beats per minute
BSA	bovine serum albumin
CL	contralateral leg
DL	denervated leg
d.p.m.	disintegrations per minute
EDL	extensor digitorum longus
g	gram
Gastroc	gastrocnemius
HPLC	high performance liquid chromatography
IMGU	insulin-mediated glucose uptake
Ins	insulin
i.p.	*intraperitoneal*
IU	international units
i.v	*intravenous*
KH	Krebs Henseleit
L	litre
LDF	laser Doppler flowmetry
ml	millilitre
min	minute
NE	norepinephrine (noradrenaline)
NIDDM	non-insulin dependent diabetes mellitus
P	probability
PCA	perchloric acid
Plan	plantaris muscle
PO_2	oxygen partial pressure
PaO_2	arterial oxygen partial pressure
PvO_2	venous oxygen partial pressure
R'g	2-deoxy-D-[1-^{3}H]glucose uptake in muscle
s	second

Sal	saline
SE	standard error
Sol	soleus
Tib	tibialis anterior muscle
TNF	tumour necrosis factor α
VO_2	oxygen consumption
2DG	2-deoxy-D-[1-^{3}H]glucose
5-HT	5-hydroxytryptamine (serotonin)
Δ	change in
µl	microlitre
µM	micromolar
µm	micrometre

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ABSTRACT

The idea that two vascular routes exist within, or closely associated with, skeletal muscle (nutritive and non-nutritive blood flow) dates back to early last century (1900's). Recent work in the past decade or two in our laboratory as well as contributions by other researchers has shed more light on the anatomical nature and functional role of the skeletal muscle vasculature and shown how changes to the blood flow distribution within skeletal muscle occur during different physiological states, e.g. exercise and insulin resistance.

Until recently, an effective and non-invasive method to measure the proportion of nutritive to non-nutritive blood flow within muscle did not exist. Such a method would prove invaluable as a general technique to assess the blood flow distribution within skeletal muscle and would have definite clinical application. Recent studies from this research group have focussed on investigating the use of 1-methylxanthine (1-MX) metabolism as an indicator of nutritive flow, or capillary recruitment, within skeletal muscle. It has been shown that capillary recruitment increases during insulin infusion *in vivo* and this increase is blocked when acute insulin resistance is induced by α methyl serotonin, an agent known to direct muscle blood flow to the non-nutritive route.

This project had two main aims. Firstly, to further investigate the use of the 1-MX method in insulin resistant rats *in vivo* (tumour necrosis factor α, TNF), and in hindimb perfusion (sciatic nerve stimulation and sciatic nerve severance). Secondly, to develop a new technique for the measurement of nutritive and non-nutritive blood flow based on laser Doppler flowmetry (LDF), in the hope that concordance between the two methods (1-MX and LDF) would be attained.

Under conditions of insulin resistance *in vivo* induced by 3 hour infusion of TNF (+/- insulin), 1-MX metabolism (capillary flow) and total femoral blood flow were decreased as compared with insulin alone. In rat hindlimb perfusion, sciatic nerve stimulated rats (causing contraction of the calf muscle group) resulted in increased

1-MX metabolism by the working muscles. Sciatic nerve severance, a rat model of insulin resistance, did not cause any changes in 1-MX metabolism and so the insulin resistance observed in this model did not appear to be a caused by changes in blood flow distribution.

Changes seen in LDF signal under a number of conditions were similar to the changes seen in 1-MX metabolism. During rat hindimb blood perfusion, vasoconstrictors known to increase and decrease nutritive and non-nutritive blood flow in skeletal muscle produced corresponding changes in laser Doppler signal. In addition, *in vivo* where insulin is known to increase capillary recruitment as measured by 1-MX metabolism, the LDF signal increased. Epinephrine, which produces large increases in total blood flow to the hindlimb but does not stimulate glucose uptake, produced no change in capillary recruitment or LDF.

Overall, the study was successful and the two main aims were accomplished. Application of the 1-MX method was extended and concordance with LDF was achieved. Both methods, with certain limitations and assumptions would appear to be capable of detecting changes in capillary recruitment or nutritive blood flow in skeletal muscle.

CHAPTER 1

General Introduction

1.1 Early Evidence for Two Circulatory Routes in Skeletal Muscle

Studies by a number of groups, particularly through the middle of last century, led to the idea that two distinct blood flow pathways exist within skeletal muscle (Barcroft 1963, Barlow *et al.* 1961, Pappenheimer 1941, Walder 1953, Walder 1955). Of these two pathways, one was thought to lead to capillaries that were in intimate contact with the muscle fibres while the other flow route went to a region without any significant nutrient delivery. The majority of the evidence available was based around the fact that total blood flow into muscle did not correlate with:

a) metabolic or heat transfer processes or
b) clearance of radioactive substances which were either infused or injected intramuscularly.

One particular study by Pappenheimer, published in 1945 clearly demonstrated that flow and metabolism are not always directly related (Pappenheimer 1941). Using the isolated constant-pressure perfused dog hindlimb or gastrocnemius muscle, Pappenheimer showed that when blood flow was reduced by stimulation of vasoconstrictor nerves, the A-V O_2 difference decreased. Conversely, if blood flow was reduced to the same extent using low dose epinephrine, the A-V O_2 difference increased. Further to this, even though the flow rates were decreased to the same degree, the difference in temperature between the arterial and venous blood was increased with epinephrine but decreased with nerve stimulation (Pappenheimer 1941). Pappenheimer concluded from this work that the oxygen saturation of the venous blood from resting muscle was not simply a measure of metabolic rate, but an indicator of the blood flow heterogeneity within the muscle.

A number of other studies, where workers attempted to measure muscle blood flow using the clearance of radioactive markers, led to similar conclusions to those of

Pappenheimer (Barlow *et al.* 1958, Walder 1955). Since the effects observed were not consistent with commonly held theories on muscle microcirculation, and were not attributable to direct effects on skeletal muscle metabolism, an explanation for the heterogeneity of isotope clearance rates was required. One postulation was that skeletal muscle contained arteriovenous anastomoses, similar to those seen in rabbit's ears, and cat's stomachs, which were 50 to 100 μm in diameter. However, further work denied the presence of large shunts in skeletal muscle. This evidence included failure to pass injected wax microspheres of 20, 30 or 40 μm either under basal conditions or during stimulation of vasodilator nerves (Piiper and Rosell 1961). Also, an extensive microscopic examination of skeletal muscle by Hammersen in 1970 did not find any evidence of arteriovenous shunts in muscle from a number of mammalian species including dogs, monkeys and rabbits (Hammersen 1970), even though another researcher claimed they existed in human skeletal muscle vascular beds (Saunders *et al.* 1957).

1.2 Location of Non-Nutritive Capillaries

Since published data still suggested the presence of a non-nutritive flow route for blood flow within skeletal muscle, there must be a way of limiting nutrient exchange between these non-nutritive capillaries and the muscle fibres. However, at this point in time, the possibility that these shunt vessels were not located within the skeletal muscle, but away from the majority of the muscle capillaries (nutritive capillaries) had not been considered. It seems that Zweifach and Metz (Zweifach and Metz 1955) were the first to elude to "preferential capillary channels at the edge of the spino-trapezius muscle of the rat". Later, the idea of a dual circulatory system within skeletal muscle was published (Barlow *et al.* 1959). In this model, one branch of the circulatory system was to provide nutrients and hormones to the muscle cells, and the second part of this circulation was to supply the septa and tendons of the muscle. Grant and Payling Wright lent further support to this notion a decade later, when they produced evidence of "non-nutritive" vessels located in connective tissue rather than intimately associated with muscle fibres (Grant and Wright 1970). Their experiments involved visualising the blood vessels of the tibial tendon of the biceps femoris muscle in the rat while it was under anaesthesia. They reported the existence of numerous capillary channels (within the tibial tendon region) which linked the arterial

blood flow to the venous, thereby bypassing the muscle fibres themselves (Grant and Wright 1970). These capillaries were dilated by acetylcholine and histamine but constricted by norepinephrine or epinephrine and did not respond to body temperature changes, and therefore were unlikely to be directly involved in body temperature regulation.

Studies by Lindbom, Arfors and colleagues, published during the 1980's, focussed on the use of the rabbit tenuissimus muscle. This muscle is ideal for studying the microcirculation in skeletal muscle since it is easily accessed and transparent enough to visualise the vasculature within it (Borgstrom *et al.* 1988, Lindbom and Arfors 1984, Lindbom and Arfors 1985). The authors reported that the transverse arterioles supplied both the capillaries in the muscle tissue proper and in the adjacent connective tissue. A diagram showing the vascular arrangement of the rabbit tenuissimus muscle is shown in Figure 1-1. Thus, the muscle capillary network and the connective tissue capillaries next to them, appear to be functioning in parallel with each other. Using intravital microscopy to measure microvascular blood flow at two points (from the beginning of the transverse arteriole, and at a point immediately after the last terminal arteriole supplying the muscle capillaries but preceding the beginning of the connective tissue vessels) on a number of transverse arterioles, it became clear that it was possible to switch the proportion of flow going to each capillary network (muscle or connective tissue) by altering the physiological conditions. For example, changes in the environmental (muscle bathing solution) oxygen tension were found to alter the proportion of flow to connective tissue. When the oxygen tension was low, 44% of the flow was moving through the connective tissue, whereas if the oxygen tension was increased, 95% of the flow went through these vessels. However, since this muscle is not uniform in thickness and only around 67% of the transverse arterioles supplied the connective tissue, the authors concluded that a conservative estimate of connective tissue blood flow at rest would be approximately 10-20% of total muscle blood flow.

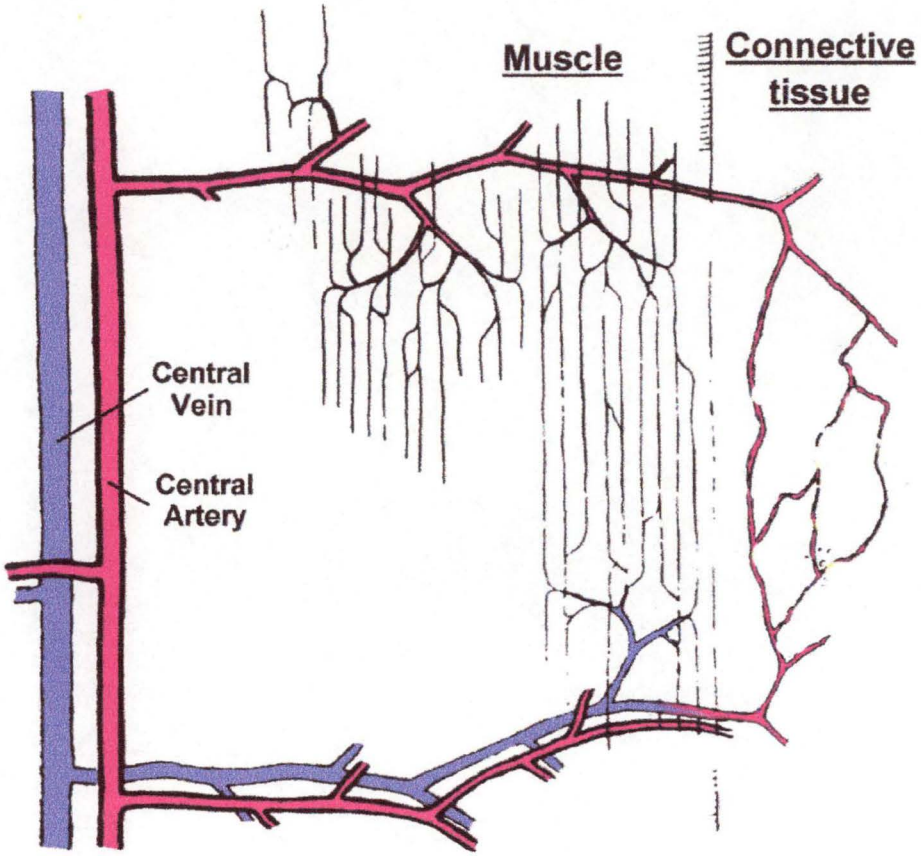


Figure 1.1 Vascular arrangement of the rabbit tenuissimus muscle.
The transverse arteriole provides blood to both the capillaries in the muscle tissue proper and the adjacent connective tissue (modified from Borgstrom *et al.* 1988).

Further work from these researchers using the rabbit tenuissimus muscle revealed that topically applied isoproterenol (β-adrenergic agent) led to redistribution of flow from muscle into the adjacent connective tissue (Borgstrom *et al.* 1988). This observation was pivotal, since it proved that blood flow between the two vascular routes was controlled by vasoactive agents (Clark *et al.* 1998).

One question, which comes to mind at this point, is whether other skeletal muscles, which are cylindrical in nature, display the same vascular arrangement as the rabbit tenuissimus muscle. Myrhage and Eriksson have shown that the vascular anatomy of the tenuissimus muscle exists as a basic unit in hindleg musculature. In fact, this arrangement of the vasculature has been seen in several muscles of different species, including the rat (Eriksson and Myrhage 1972, Grant and Wright 1970), cat (Myrhage and Eriksson 1980), and monkey (Hammersen 1970). Furthermore, recent studies from our laboratory suggest that these connective tissue vessels may be nutritive for connective tissue and the associated fat adipocytes, even though they are non-nutritive for the muscle itself (Clerk *et al.* 2000). This is possible since fat cells have significantly lower metabolic requirements than muscle but higher potential for hydrolysing circulating triglyceride.

1.3 Vasoconstrictors are Capable of Controlling Muscle Metabolism

Work from this group over the past decade using the perfused rat hindlimb technique has produced new insights into the control of skeletal muscle metabolism. In particular, the discovery that vasoactive agents are capable of altering skeletal muscle metabolism and performance by their effects upon the vasculature. Most of these substances are vasoconstrictors which act to increase the perfusion pressure in the perfused rat hindlimb and they have been classified into one of two types, depending on whether they generally increase (Type A) or decrease (Type B) muscle metabolism. Type A vasoconstrictors, for example: angiotensin II, low dose norepinephrine (< 100 nM), vasopressin, low dose vanilloids and low frequency nerve stimulation (listed in Table 1-1), result in increased oxygen consumption (Colquhoun *et al.* 1988), glycerol (Clark *et al.* 1994), lactate (Hettiarachchi *et al.* 1992), urate and uracil (Clark *et al.* 1990) release (see Table 1-2). In addition, some Type A vasoconstrictors such as angiotensin II, are capable of increasing aerobic tension development, contraction-mediated oxygen uptake and 2-deoxyglucose uptake by plantaris, gastrocnemius red and white muscles during electrical tetanic stimulation of the hindlimb (Rattigan *et al.* 1996).

Table 1-1 Classification of vasoconstrictors into Type A or Type B categories using their effects in the constant-flow perfused rat hindlimb (modified from Clark *et al.* 1995).

Type A Vasconstrictors	Type B Vasoconstrictors
Low dose norepinephrine (<100 nM)	High dose norepinephrine (> 100 nM)
Angiotensin II	Serotonin
Phenylephrine	Capsaicin (> 1.0 μM)
Vasopressin	Sympathetic nerve stimulation (> 4 Hz)
Capsaicin (< 1.0 μM)	
Sympathetic nerve stimulation (< 4 Hz)	

Those vasoconstrictors, which are capable of producing a similar increase in perfusion pressure but with an accompanying decrease in metabolism, are referred to as Type B vasoconstrictors (examples listed in Table 1-1). These vasconstrictors result in decreased oxygen consumption (Dora *et al.* 1991), glycerol (Clark *et al.* 1995), lactate (Hettiarachchi *et al.* 1992), urate and uracil efflux (Table 1-2) (Clark *et al.* 1995). Among those to be classified as Type B vasoconstrictors include serotonin (5-HT), high dose norepinephrine (> 100 nM), high frequency sciatic nerve stimulation and high dose vanilloids (Clark *et al.* 1995). Type B vasoconstrictors also decrease insulin-mediated glucose uptake (Rattigan *et al.* 1993, Rattigan *et al.* 1995), aerobic tension development and contraction-mediated oxygen uptake (Dora *et al.* 1994).

Table 1-2 Summary of the effects of Type A and B vasoconstrictors in the constant-flow perfused rat hindlimb.

	Type A vasoconstriction	Type B vasoconstriction
Perfusion pressure	↑	↑
Oxygen uptake	↑	↑
Lactate efflux	↑	↓
Glycerol efflux	↑	↓
Urate/uracil efflux	↑	↓
Insulin mediated glucose uptake	↑	↓
Aerobic muscle contraction	↑	↓
Anaerobic muscle contraction	–	–
Perfusate distribution volume	↑	↓
Flow & volume in tendon vessels	↓	↑
Metabolism of infused 1-MX	↑	↓

Since all perfusions were performed under conditions of constant total flow, the effects observed are solely due to the vascular action of the vasoconstrictor to switch flow from one route into another, as opposed to direct actions on the skeletal muscle. To prove this point, the metabolic effects were shown to be reversed by infusion of a vasodilator to abolish the increased pressure (Colquhoun *et al.* 1990, Colquhoun *et al.* 1988, Hettiarachchi *et al.* 1992, Rattigan *et al.* 1995) even though different vasoconstrictors used different mechanisms of action on the vasculature. To further emphasise that the metabolic action of the vasoconstrictors was simply due to their ability to vasoconstrict the vessels, isolated incubated muscles were used. These muscles rely on diffusion to obtain their nutrients and oxygen from the surroundings. Therefore, when Type A and B vasoconstrictors were added to their incubation

buffer, there was no effect on muscle insulin-mediated glucose uptake or contractility (Dora *et al.* 1994, Rattigan *et al.* 1993, Rattigan *et al.* 1995).

Another important point to note is that Type A and B vasoconstrictors appear to act at different vascular receptor sites. In addition, the sites responsible for Type A and B vasoconstriction can be distinguished by their metabolic requirements for constriction. Type A vasoconstrictors rely on the presence of extracellular calcium and removal of this, or inhibition of oxidative metabolism (using cyanide, azide or anoxic perfusion) abolishes their action, whereas Type B vasoconstrictors are not affected by these conditions (Clark *et al.* 1994, Dora *et al.* 1992).

In an attempt to explain the opposite metabolic effects of Type A and Type B vasoconstrictors, work focussed on looking at the skeletal muscle vasculature. One possible explanation for the differing metabolic effects seen, could be that flow switches between red and white muscles, skin, adipose tissue and bone, which all have differing metabolic requirements. However, studies using fluorescent microspheres to determine blood flow distribution between tissues, show that Type B-mediated inhibition of oxygen consumption did not correlate with any changes to perfusate flow distribution, either between different muscle fibre types or between muscle and bone, skin or adipose tissue (Rattigan *et al.* 1997a). It was concluded from this that the changes in vascular flow route seen with Type B vasoconstriction are likely to be occurring within the muscle tissue itself leading to reduction in functional capillary surface area available for nutrient and gas exchange (Rattigan *et al.* 1995).

1.4 Possible Reasons for the Existence of Two Flow Routes in Skeletal Muscle

1.4.1 Under Resting Conditions:

One important area, is the consequences of, and possible reasons for, having a second (non-nutritive) flow route exist within skeletal muscle. In order to answer this question we must consider the possible functions of such a system. One possibility is that regulation of the proportion of total flow that passes through the nutritive and non-nutritive route could be involved in control of the body's basal metabolic rate

(Clark *et al.* 2000). So, basal energy consumption (as measured by oxygen uptake and lactate output) would increase as the proportion of total flow that is nutritive increases. Opposingly, basal metabolism would decrease as the proportion of nutritive flow decreases (Clark *et al.* 2000). As mentioned above, there are a number of vasomodulators, which act at specific sites along the vascular tree of muscle, thereby controlling the proportion of nutritive to non-nutritive flow within the muscle tissue. Under conditions of constant total flow, such as that seen in the constant-flow perfused rat hindlimb, vasoconstriction leading to reduced flow within the nutritive route would conversely result in a proportional increase in non-nutritive flow and vice versa. In a more physiological situation where constant pressure predominates, vasoconstriction of the non-nutritive route, for example, may not necessarily increase flow in the nutritive pathway, but it is likely that the proportion of nutritive flow will increase (Clark *et al.* 2000). In this situation, basal energy consumption will not increase whereas if vasoconstrictor activity is at sites leading to nutritive flow routes, then basal metabolism will decrease. Knowing this, it seems likely that when blood pressure is maintained constant *in vivo*, basal metabolism can be controlled, to an extent, by vasoconstrictors controlling blood flow entry into the nutritive route. Further to this, vasodilators that control access to the nutritive flow route by opposing this vasoconstriction will play a major part in the basal metabolic rate for resting muscle. One possible reason for the existence of this non-nutritive route could be because it allows conservation of energy expenditure by the organism, thus conferring an evolutionary advantage over others of the same species. Yet to be examined is whether thyroid hormones and other agents capable of altering resting metabolism in muscle are able to do so by altering the proportion of nutritive to non-nutritive flow as well as by direct effects upon muscle metabolism (Clark *et al.* 2000).

Another possible reason for having two vascular flow routes within muscle is fuel partitioning. As mentioned briefly earlier, the non-nutritive vessels appear to be supplying blood flow to connective tissue and the adipocytes associated with this area (Clerk *et al.* 2000). In a situation where a high proportion of nutritive flow predominates, hormones and nutrients are supplied to the muscle cells and the overall metabolism of the muscle is elevated. Conversely, when flow is largely non-nutritive in nature, nutrients are delivered mainly to the connective tissue and fat cells of the muscle. By supplying these cells with an abundance of nutrients, glucose, insulin and

triglycerides, the flow distribution is favouring the growth of fat cells and an overall lower basal metabolic rate. Furthermore, long term maintenance of this predominantly non-nutritive flow could lead to extensive fat cell deposition (Clerk *et al.* 2000).

1.4.2 During exercise:

On commencement of exercise, an increase in skeletal muscle blood flow occurs to supply adequate nutrients to the muscle while it is working, and to remove the waste products released. It is possible that the non-nutritive flow route provides a flow reserve, which when required can be used to amplify nutrient delivery and the exit of wastes (Clark *et al.* 1998). It is well accepted that during exercise, the blood flow to skeletal muscle increases under the influence of elevated sympathetic nervous system activity and elevated cardiac output by diversion of blood flow away from organs and tissues not immediately requiring a large amount of flow (e.g. gut), to the skeletal muscle (Segal 1992). This described amplification of blood flow would be over and above the increases in total flow seen during exercise. At this point in time the ratio of nutritive to non-nutritive flow in muscle at rest is unclear. From the data of Lindbom and Arfors using the rabbit tenuissimus muscle model (Lindbom and Arfors 1984), the ratio of nutritive:non-nutritive flow could be as high as 30:70 at rest. However, a more conservative position was reached by these authors stating that the ratio was closer to 80:20. Other work using the hydrogen clearance method suggests that the nutritive:non-nutritive flow ratio is as low as 0.16 in muscle at rest (Harrison *et al.* 1990).

Since a number of authors claim that not all capillaries are perfused under resting conditions (Harrison *et al.* 1990), it would be possible for an increase in the nutritive to non-nutritive flow ratio to occur during exercise, independent of changes in non-nutritive flow, simply by increasing total flow (Clark *et al.* 1998). When exercise reaches a maximal state, nutritive flow is likely to be at its maximum capacity and non-nutritive flow would be minimal. A study by Harrison et al (1990) discusses the possibility that the non-nutritive flow route provides a flow reserve that can be recruited during exercise. As such, this flow reserve allows for amplification of nutrient (O_2, glucose etc) delivery and product (lactate, H^+ etc) removal (Clark *et al.*

1998, Harrison *et al.* 1990). A schematic diagram of the possible amplification which can occur within the two vascular flow pathways during exercise is shown in Figure 1-2. In Figure 1-2(i), we assume that the nutritive:non-nutritive flow ratio is 50:50 at rest. As exercise starts, the ratio changes as a result of three influences in particular. Firstly, sympathetic nervous system vasoconstrictor activity increases leading to a change in ratio from 50:50 to 95:5 resulting in a nutritive amplification of 1.9 (via a Type A response, Clark *et al.* 1995). Secondly, total blood flow to the muscle increases by at least 3-fold due to increased cardiac output during exercise giving a total amplification of 5.7 (3 x 1.9). Thirdly, metabolic vasodilators released at the onset and throughout the duration of the exercise also contribute to increased blood flow to muscle nutritive capillaries (Clark *et al.* 1998). If the true resting situation is more like that depicted in Figure 1-2(ii), as predicted by Lindbom and Arfors (1984) and Harrison *et al.* (1990), as well as work from our laboratory in the perfused rat hindlimb, then the potential for amplification becomes even larger.

High sympathetic vasoconstrictor activity (in the region of • 5 Hz), reminiscent of a Type B vasoconstrictor effect, is reported to increase non-nutritive flow, while decreasing the nutritive flow (Hall *et al.* 1997, Mulvany and Aalkjaer 1990). Furthermore, Type B vasoconstrictors decrease aerobic muscle contractility (Dora *et al.* 1994). Thus, Figure 1-2 includes the proposed scenario when high sympathetic vasoconstrictor activity is present in combination with exercise. The result of this situation would be decreased nutritive blood flow and a corresponding decline in performance (Clark *et al.* 1998). To compound this negative situation, metabolic vasodilators do not affect these vasoconstrictor sites (Dora *et al.* 1994).

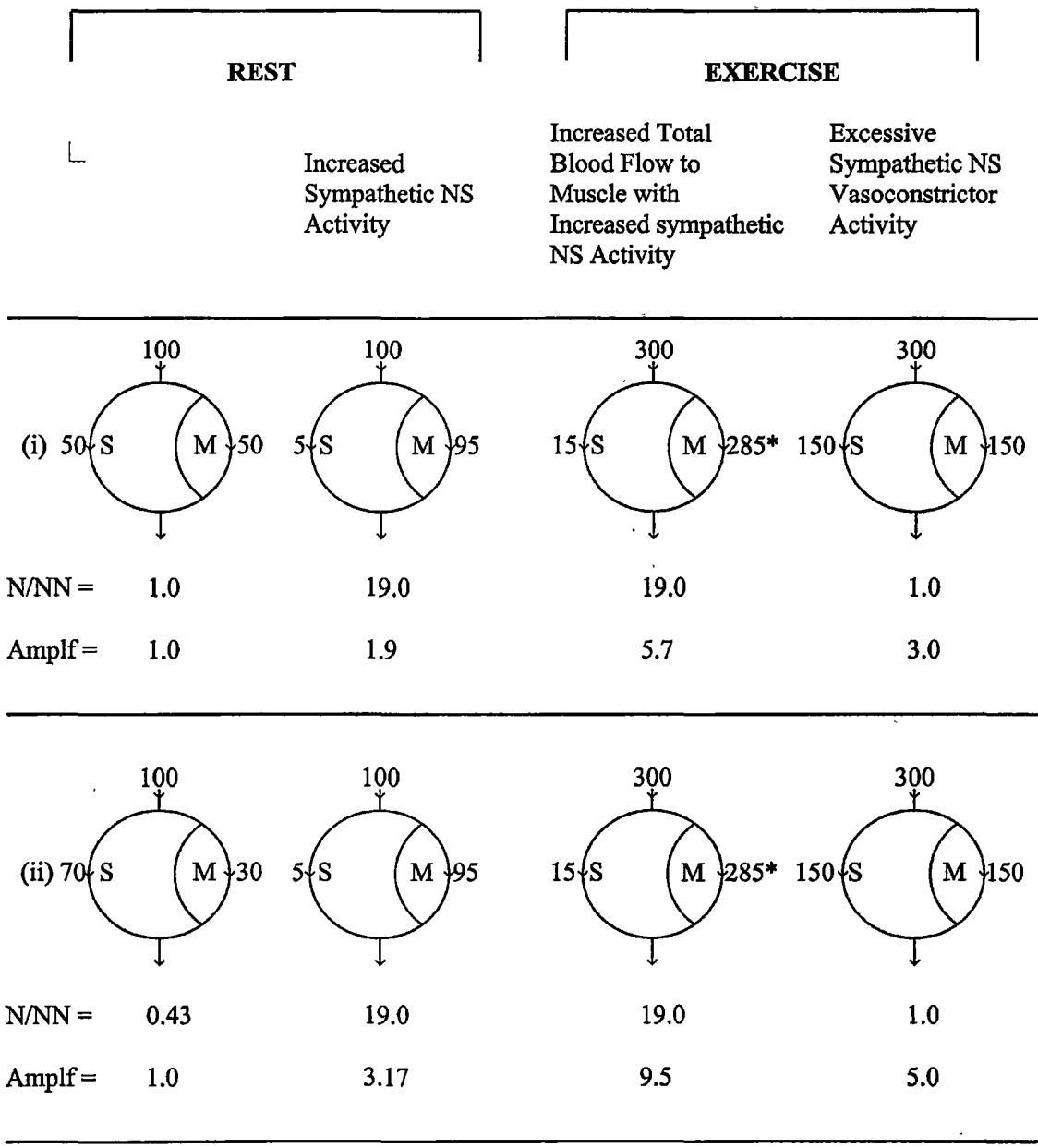


Figure 1-2 Possible method of amplifying nutrient delivery during exercise.

S = shunt or non-nutritive route; M = muscle nutritive capillaries; N/NN = nutritive/non-nutritive flow ratio; Amplf = amplification of muscle nutritive flow relative to basal nutritive flow. The numbers represent flow rates in arbitrary units of measurement. The N/NN was set at 1.0 (i) or 0.43 (ii) based on data from Lindbom and Arfors (1984). * Note that the release of metabolic vasodilators during exercise is partly responsible for the increase in M from 95 to 285 (Figure reproduced from Clark *et al.* 1998)

Since blood flow to muscle remains elevated even after exercise has ceased, the proportion of nutritive to non-nutritive flow may play an important role in the recovery of muscle from exercise by allowing removal of waste products and supply of required nutrients to replenish the depleted energy stores. There have been a number of reports where post exercise metabolism is significantly elevated for extended periods (Bahr and Maehlum 1986, Bahr and Sejersted 1991, Borsheim *et al.* 1994, Ullrich and Yeater 1997). This phenomenon has not been explained by others and may result from exercise induced increases in the proportion of nutritive:non-nutritive flow, thus resetting the basal metabolic rate.

1.5 Non-Nutritive Flow Route Measurement

A number of research groups, including our own, have attempted to measure nutritive and non-nutritive flow. Friedman appears to be the first to attempt the measurement of non-nutritive flow and his method involved using indicator dilution patterns of infused marker substances to determine differing blood flow volumes (Friedman 1966, Friedman 1968, Friedman 1971). Radioactively labelled albumin was used to determine total blood flow and ^{86}Rb used to derive the volume of non-nutritional blood flow. This method relied on two assumptions. Firstly, that rubidium was unable to exchange with the tissue while passing through the non-nutritive route, where extraction was low, and secondly, that capillary permeability to rubidium was unlimited. Friedman estimated from this work that the volume of non-nutritive blood flow in the whole leg of a dog was 75% of the total blood volume (Friedman 1966). One limitation to this work however, was that Friedman did not take into account the blood flow to the skin of the leg. A number of approaches by others have involved measurement of nutritive flow and total flow by studies determining the removal of intramuscularly injected radioactive markers, and then estimating that non-nutritive flow made up the difference (Clark *et al.* 2000).

One of the main problems in the search for a method to measure non-nutritive flow is the fact that little evidence is available as to the characteristics of the non-nutritive vessels, thus making it difficult to devise methods capable of distinguishing flow to these vessels from total flow. Since studies by others have pointed toward the muscle-associated connective tissue and tendon vessels as the location for non-

nutritive capillaries (Barlow *et al.* 1961, Grant and Wright 1970), Newman and others from this laboratory measured flow to connective tissue in the constant-flow perfused rat hindlimb (Newman *et al.* 1997). The perfused leg was positioned under the a surface fluorimeter probe with the tibial tendon vessels of the biceps femoris muscle exposed, so that the signal strength could be monitored during infused pulses of fluorescein isothiocyanate dextran. In addition to this work, in a separate experiment the leg was set up in the same way underneath the objective lens of an inverted microscope for photography of the vasculature when pulses of India ink were infused (Newman *et al.* 1997). Steady state measurements were taken during infusion with vehicle, noradrenaline (Type A vasoconstrictor) or serotonin (Type B vasoconstrictor). Under the influence of noradrenaline, perfusion pressure and oxygen uptake both increased as expected, but the fluorescence signal from the tendon vessels decreased. Furthermore, the diameter of the India ink filled vessels diminished indicating that the tendon vessels had indeed decreased in diameter. As expected, serotonin decreased oxygen uptake with an accompanying increase in perfusion pressure. The tendon fluorescence signal increased in association with the serotonin infusion and an increase in tendon vessel diameter was clearly seen using photomicroscopy (Newman *et al.* 1997). Examining a range of concentrations of NE, as well as serotonin, a reciprocal relationship (shown in Figure 1-3) between resting muscle metabolism (as represented by oxygen uptake) as controlled by vasoconstrictors, and flow through muscle tendon vessels was apparent. This study supports earlier studies by Barlow *et al.* (1961) and Grant *et al.* (1970) a number of years ago. Another important point to take from this work is that tendon vessel flow does not stop completely, even when the nutritive flow is very high.

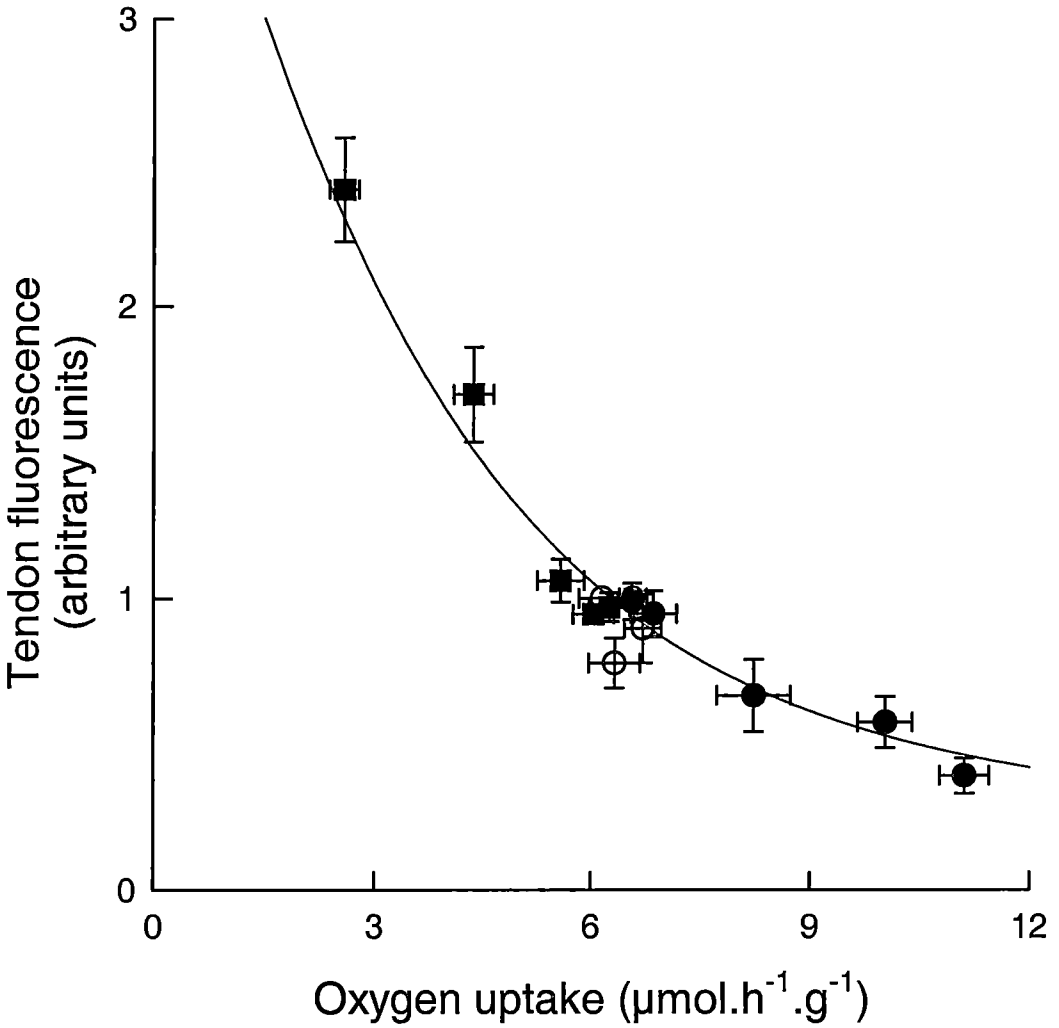


Figure 1-3 Relationship between muscle tendon vessel flow and oxygen uptake (as influenced by infusion of vasoconstrictors).

All experiments were conducted using the constant-flow perfused rat hindlimb with the addition of varying concentrations of either noradrenaline (●), serotonin (■) or vehicle (○). Determination of tendon vessel flow was by measuring the fluorescence signal strength of infused fluorescein isothiocyanate-labelled dextran (Mr 150,000) over the tibial tendon region of the biceps femoris muscle. Figure was reproduced from Clark *et al.* (2000) and was constructed using data from Newman *et al.* (1997).

A recently published study by Clerk and others (2000) has provided important information about the non-nutritive flow route and perhaps a method of measuring relative flow in it (Clerk *et al.* 2000). Initially this study was focussed on assessing the effect of low nutritive flow (with corresponding high non-nutritive flow) on the clearance of triglyceride in the form of a chylomicron emulsion. This work was performed in the perfused rat hindlimb and results unexpectedly showed that clearance was increased in a predominantly non-nutritive flow situation. The findings suggest that lipoprotein lipase was more concentrated in the non-nutritive route than the nutritive route (Clerk *et al.* 2000). This begins to make sense in that it has been postulated (as mentioned previously in this introduction) that the non-nutritive flow route is involved in nourishing the fat cells located around the connective tissue of muscle, particularly in the area of the perimysium and epimysium (Myrhage and Eriksson 1980).

The work discussed above provides further evidence that the vessels termed 'non-nutritive' are located in the connective tissue closely associated with each muscle. Evidence suggests that these non-nutritive vessels are low resistance and high capacitance in character and thus capable of carrying a large amount of flow when the muscle is at rest. One point to note is that even though these connective tissue vessels may be larger than the nutrient supplying capillaries within the muscle, they are still incapable of allowing passage of 15 μm microspheres through them (Rattigan *et al.* 1997a).

1.6 Nutritive Flow Route Measurement

A number of early methods to measure nutritive flow in muscle were aimed at determining the proportion of total flow that was nutritive. Some of these techniques included hydrogen washout (Nakamura *et al.* 1972), clearance of ^{133}Xe from an intramuscular injection site (Hudlicka 1969, Kjellmer *et al.* 1967, Sejrsen and Tonnesen 1968), intravital microscopy as discussed earlier in this chapter (Lindbom and Arfors 1984), and local hydrogen clearance with microflow assessment (Harrison *et al.* 1990). Results using most these methods indicated that non-nutritive flow is likely to predominate under resting conditions. Further to this, in some situations, total flow increased with either an increase or no change to nutritive flow (Hudlicka

1973). Freis *et al.* found that during exercise, both nutritive and non-nutritive flow increased (Freis and Schnapper 1958). Another study by Brod *et al.* showed that under conditions of emotional stress, total blood flow increased without any corresponding change in nutritive flow (Brod *et al.* 1966).

1.7 1-Methylxanthine Metabolism and Measurement of Capillary Recruitment

Work in this laboratory over the past few years has been focussed on the search for new methods to measure nutritive and non-nutritive flow in skeletal muscle. While these methods are initially being developed in rats, their future application in the clinical setting for assessment of microvascular function has been kept strongly in mind.

As discussed in Section 1.3, Type A and B vasoconstrictors are capable of changing the capillary flow distribution within skeletal muscle through their action on the vasculature. When nutritive flow is decreased by the addition of a Type B vasoconstrictor, such as serotonin (5-HT), flow is redirected into predominantly non-nutritive vessels where nutrient and gas exchange are limited due to the nature of these vessels. Effectively the capillary exposure has diminished due to the blood flowing predominantly through non-nutritive vessels, rather than through nutritive capillaries where nutrient exchange is free to occur. Knowing this, the next step was to find a way of measuring the capillary flow, thereby assessing changes in nutritive flow under different physiological conditions. One method developed in this laboratory for the measurement of nutritive flow is 1-methylxanthine metabolism. 1-Methylxanthine is a substrate for the capillary endothelial enzyme, xanthine oxidase (XO), which converts it to 1-methylurate. Xanthine oxidase is widely distributed within mammalian tissues, with high levels of expression in the liver and intestine (Parks and Granger 1986), as well as detectable activities in the heart, spleen, kidney and skeletal muscle (Jarasch *et al.* 1986). Immunohistochemical studies by Jarasch *et al.* (1986), and later by Hellsten *et al.* (Hellsten *et al.* 1997), demonstrated that xanthine oxidase is located primarily in the cytoplasm of capillary endothelial cells of a variety of tissues, including skeletal muscle (Jarasch *et al.* 1986). Since the endothelium of larger vessels of skeletal muscle contains only minor amounts of

xanthine oxidase it was reasoned that measurement of 1-MX metabolism by XO could provide a way of determining capillary blood flow within skeletal muscle.

Initial work on the development of the 1-MX metabolism method was performed in the perfused rat hindlimb under conditions of constant total flow (Rattigan *et al.* 1997a). The metabolism of 1-MX under control conditions was compared with the metabolism in the presence of serotonin, a Type B vasoconstrictor. 1-MX itself has no haemodynamic effect upon the perfused rat hindlimb and the sum of the perfusate levels of 1-MX and 1-MU was always quantitative (100 ± 5%) during the duration of the experiment (Rattigan *et al.* 1997a). To check whether the metabolism of 1-MX was solely due to XO, a specific inhibitor of the enzyme, allopurinol, was infused into the hindlimb (Emmerson *et al.* 1987). This inhibitor completely blocked the conversion of 1-MX to 1-MU as did xanthine, a competing substrate for XO (Rattigan *et al.* 1997a). This study showed that 1-MX metabolism decreased in the presence of 5-HT, a vasoconstrictor proposed to decrease muscle metabolism by diverting flow away from the nutritive capillaries of muscle into the larger, non-nutritive capillaries, which are not involved in nutrient exchange with the muscle (Rattigan *et al.* 1997a).

Direct action of 5-HT on XO itself was ruled out, since there was no change to muscle XO *in vitro*, whether 5-HT was present or not in isolated muscle incubations (Rattigan *et al.* 1997a). Kinetics studies of the purified enzyme form milk fat globule indicated the Km for 1-MX to be 7.6 μM. On this basis changes in the rate of metabolism of 1-MX were likely to be indicative of changes in enzyme exposure, possibly capillary recruitment (nutritive flow).

One shortcoming of the 1-MX method is its inability to measure an increase in nutritive flow in the perfused rat hindlimb as a result of Type A vasoconstriction. Despite significant increases in oxygen consumption in the presence of a Type A vasconstrictor, no significant increase in 1-MX metabolism is observed. A possible reason for this is that during Type A vasoconstriction, the concomitant release of endogenous xanthines inhibits 1-MX metabolism (Rattigan *et al.* 1997a). Xanthine, a

purine released endogenously, is capable of inhibiting 1-MX conversion to 1-MU by competitively inhibiting XO.

An obvious application of the 1-MX method was its use *in vivo*. Most of the recent evidence in the literature supports the idea that insulin is capable of increasing total blood flow to the muscle (Baron 1994). Furthermore, it has been proposed that insulin's action to increase total blood flow could partially account for insulin's ability to stimulate glucose uptake by increasing the delivery of glucose to the muscle cells (Baron and Brechtel 1993b). Rattigan *et al.* (1997) proposed that insulin not only increases total blood flow to the muscle, it also increases the capillary recruitment (nutritive flow) within the muscle itself (Rattigan *et al.* 1997b). To test this hypothesis, 1-MX was infused into anaesthetised rats under control conditions, and in the presence of insulin under euglycaemic hyperinsulinaemic clamp conditions (Rattigan *et al.* 1997b). As with the perfused rat hindlimb, 1-MX alone had no vasoactive action and there were no changes in haemodynamic parameters such as arterial blood pressure, heart rate, femoral blood flow, or hindleg vascular resistance (Rattigan *et al.* 1997b). Results showed that 1-MX metabolism increased in the presence of insulin *in vivo* (Rattigan *et al.* 1997b), again suggestive of increased nutritive flow or capillary recruitment. Also, these changes could not be accounted for by the increase in total blood flow to the hindleg, since similar increases in total flow induced by epinephrine infusion, did not produce any change in 1-MX metabolism. Further to this, insulin itself did not directly affect the activity of XO when incubated with muscles *in vitro* (Rattigan *et al.* 1997b).

A second study *in vivo* by Rattigan *et al.* (1999) focussed on the effect of infusion of a Type B vasoconstrictor, α-methyl serotonin on insulin responsiveness(Rattigan *et al.* 1999). α-Methyl serotonin was used since its actions are restricted to the vasculature and it has no direct effects on muscle metabolism itself (Rattigan *et al.* 1999). In addition to the insulin resistance of glucose uptake, insulin was prevented from increasing 1-MX metabolism when α-methyl serotonin was present (Rattigan *et al.* 1999). This work demonstrates that vasoconstriction by such agents in muscle prevents normal insulin recruitment of capillary flow, resulting in impairment of

muscle glucose uptake and contributing to the insulin resistance observed *in vivo* (Rattigan *et al.* 1999).

Overall, results thus far, have suggested that changes in 1-MX metabolism both in perfused muscle studies and *in vivo* are indicative of changes in capillary recruitment (albeit nutritive flow). Key questions that now emerge are: firstly, is the 1-MX technique robust enough to be more widely applicable and secondly, can another independent method be developed to provide concordance.

1.8 Laser Doppler Flowmetry as a Tool for Measuring Relative Nutritive and Non-Nutritive Flow

One limit to the 1-MX metabolism method is that it does not allow a quantitative assessment of nutritive flow particularly when there is a concomitant increase of endogenously released purines, and is limited to detecting only changes in an indirect manner. There is a need to develop a more direct measurement of nutritive and non-nutritive flow that could also present confirmation of findings using the 1-MX method. Laser Doppler flowmetry could be useful for measuring these changes. A search of the literature shows that most of the work performed using laser Doppler flowmetry (LDF) was focussed on measuring skin microvascular flow under a variety of different situations. These situations include altered blood glucose, blood insulin and C-peptide levels (Forst *et al.* 1998), infused insulin-like growth factor-1 (IGF-1) (Franzeck *et al.* 1995), peripheral nerve transection (Gonzalez-Darder and Segura-Pastor 1994) and diabetes (Stansberry *et al.* 1997). Another application for which LDF has proven useful is assessment of tissue graft perfusion (Erni *et al.* 1996). Of interest to our group was the fact that a number of workers have used LDF in an attempt to assess different parameters of muscle blood flow. Gustafsson *et al.* (1994) investigated the characteristics of the microcirculatory blood flow within skeletal muscle during adenosine infusion by using LDF (Gustafsson *et al.* 1994), while Skjeldal *et al.* (1993) used LDF to evaluate skeletal muscle perfusion before and after acute hindlimb ischaemia in rats (Skjeldal *et al.* 1993). One particular publication by Kuznetsova *et al.* (1998) prompted this laboratory to focus on laser Doppler flowmetry as a possible method for measuring nutritive and non-nutritive flow within skeletal muscle (Kuznetsova *et al.* 1998). In that study, ganglionic-blocked

anaesthetised rats were used to examine the relationship between total flow (as measured by radioactive microsphere entrapment) and apparent muscle nutritive flow (measured by LDF) when a selection of vasoactive agents were administered (Kuznetsova *et al.* 1998). Upon infusion of phenylephrine or angiotensin II, a distinct increase in muscle vascular resistance was seen. Along with this, no change in muscle blood flow occurred, but a significant increase in LDF signal was observed (Kuznetsova *et al.* 1998). Similarly, isoproterenol, a vasodilator, produced a marked increase in muscle blood flow, while muscle vascular resistance and muscle nutritive flow (as measured by LDF) were significantly decreased. Despite other explanations which could account for these results, it appears likely that the two Type A vasconstrictors (phenylephrine and angiotensin II, see Table 1-1 above) have each produced effects *in vivo* involving a selective increase in muscle nutritive flow which are consistent with previous data found using the perfused rat hindlimb. Furthermore, the vasodilator, isoproterenol appears to have increased flow to the non-nutritive route at the expense of flow to the nutritive pathway (Clark *et al.* 2000). Previous data (Colquhoun *et al.* 1990) indicates that Type A vasoconstriction is opposed by isoproterenol in the constant-flow perfused rat hindlimb. This is thought to occur by relaxing constricted sites in the vasculature, which reduce flow to non-nutritive routes. As indicated by Rattigan *et al.* (Rattigan *et al.* 1997a), microspheres do not appear to be suitable for distinguishing between nutritive and non-nutritive routes in muscle. In addition, serotonin, and other agents capable of inhibiting oxygen uptake and metabolism generally, do not alter the distribution of 15 μm fluorescent microspheres (Rattigan *et al.* 1997a).

Based on the evidence available from work in this laboratory over the last few years, it would seem likely that Kuznetsova *et al.* (1998) were in fact measuring nutritive flow in muscle using LDF. My intent was thus to explore this notion and to assess the application of LDF for use as a method to measure nutritive and perhaps non-nutritive flow in skeletal muscle.

1.9 Aims of the Present Study

The present study had two main aims:

1. to further explore the use of 1-methylxanthine as a method for measuring changes in nutritive flow in skeletal muscle under normal physiological situations (e.g. exercise), pathophysiological conditions (tumour necrosis factor-α administration) and as a result of sciatic nerve severance).

2. to investigate the use of laser Doppler flowmetry as method of measuring nutritive and non-nutritive flow in hindlimb perfusion and *in vivo*.

CHAPTER 2

Materials and Methods

2.1 Rat Hindlimb Perfusion Studies

2.1.1 Animals

Male hooded Wistar rats (180-205 g) were raised on a commercial diet (Gibsons, Hobart) containing 21.4% protein, 4.6% lipid, 68% carbohydrate, and 6% crude fibre with added vitamins and minerals together with water *ad libitum*. Rats were housed at a constant temperature of 21 ± 1°C in a 12h/12h light-dark cycle. All procedures adopted and experiments undertaken were approved by the University of Tasmania Ethics Committee in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (1990).

2.1.2 General Surgical Procedures

The surgical and perfusion procedures were essentially as described by others (Ruderman *et al.* 1971) with modifications by Colquhoun *et al.* (Colquhoun *et al.* 1988). Briefly, animals were anaesthetized via an intra-peritoneal injection of pentobarbital sodium (60 $mg.kg^{-1}$ body weight) and the tail and tarsus of the perfused legs tied off firmly. An incision was made along the midline of the abdomen and the epigastric vessels and the iliolumbar vessels all ligated. Following this the testicles were ligated and removed. Ligatures were then placed around the duodenum and the rectum, and the gut was excised. For those experiments where only one hindleg was perfused (LDF, sciatic nerve stimulation) flow was directed exclusively to either the right or left limb and prevented from entering the contralateral leg by ligation of the left common iliac artery and vein. Prior to cannulation of the abdominal aorta and vena cava, heparin (1000 $I.U.kg^{-1}$ body weight) was injected into the vena cava. A further ligature was placed around the abdomen (at the level of the L_3-L_4 vertebrae region) to prevent access of the perfusate to the muscles of the back.

The rat was then connected to the perfusion apparatus and killed with a lethal intra-cardiac injection of sodium pentobarbital. A pictorial view of the surgical ligations is shown in Figure 2-1.

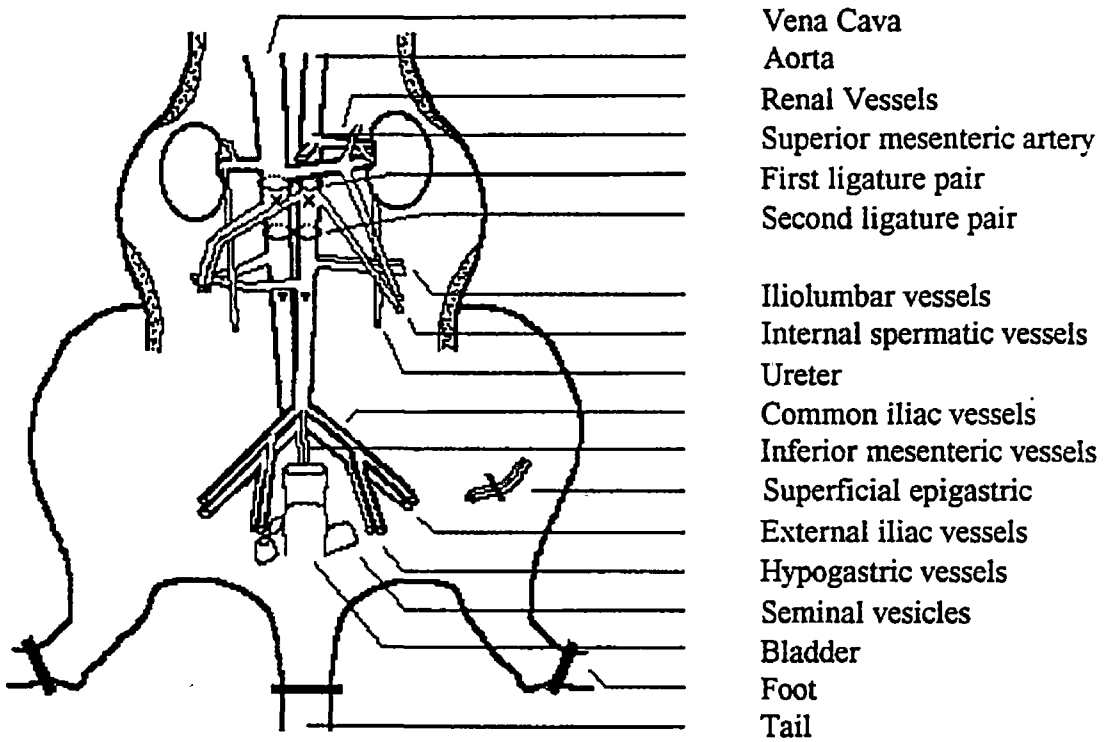


Figure 2-1 Pictorial view of perfused hindlimb surgical ligations.
Modified from Dora (Dora 1993) (see above for further details).

2.1.3 Perfusion Media

2.1.3.1 Erythrocyte-Free Hindlimb Perfusions

The perfusion medium used for the erythrocyte-free hindlimb perfusions was a modified Krebs-Henseleit bicarbonate buffer containing 4% bovine serum albumin

(BSA, fraction V; Boehringer Mannheim, Australia), 8.3 mM glucose, 2.54 mM $CaCl_2$, 118 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, and 25 mM $NaHCO_3$. Once made, the perfusion buffer was filtered using a 0.45 μm pore filter under pressure to ensure that it was free of any particulate matter. The buffer was maintained at a pH of 7.4 by saturation with 95% O_2-5% CO_2

2.1.3.2 Red Blood Cell Perfusions

The perfusion medium consisted of a modified Krebs-Henseleit buffer as described above, containing 5% BSA. This was combined with washed bovine erythrocytes to give a final haematocrit of 38%. Heparin (0.2 $ml.L^{-1}$) and pyruvate (0.1 $g.L^{-1}$) were added to blood mixture. The fresh bovine erythrocytes were washed three times using saline (0.9% NaCl) and filtered through four layers of pre-washed cheesecloth. They were then washed twice more in saline and then stored in Krebs-Henseleit bicarbonate buffer gassed with 95% O_2-5% CO_2 at 4°C until use. Erythrocytes were never more than 3 days old when used. As with previous similar studies ((Rattigan *et al.* 1996), this perfusion medium resulted in minimal oedema.

2.1.4 General Perfusion Procedure

Perfusions conducted on sciatic nerve severed rats were performed at 32°C in a temperature-controlled cabinet with erythrocyte-free medium (as described above) delivered at a constant flow rate of 13 $ml.min^{-1}$ (for two hindlimb perfusions, this is equivalent to 0.43 $ml.min^{-1}.g^{-1}$ muscle which is higher than physiological flow rates *in vivo*) (James *et al.* 1986). The buffer reservoir was kept on ice and continuously stirred, whilst being gassed with 95% O_2-5% CO_2. Perfusate was then pumped at a constant flow rate by a peristaltic pump (Masterflex, Cole-Palmer, USA) to a heat exchanger coil where the temperature was brought to 32°C prior to passing through a silastic lung, also gassed with 95% O_2-5% CO_2. Venous oxygen tension was continuously monitored using a thermostatically controlled (32°C) in-line Clark-type oxygen electrode (0.5 ml capacity). Arterial perfusion pressure was monitored continuously via a pressure transducer located proximally to the aorta. Recording of PO_2 and pressure was performed continually by either a dual pen chart recorder (Omniscribe series D5000) or an IBM compatible PC computer with WINDAQ data acquisition software (DATAQ Instruments). All agent infusions were performed

using a peristaltic pump (LKB Microperpex). A typical perfusion apparatus setup is shown in Figure 2-2.

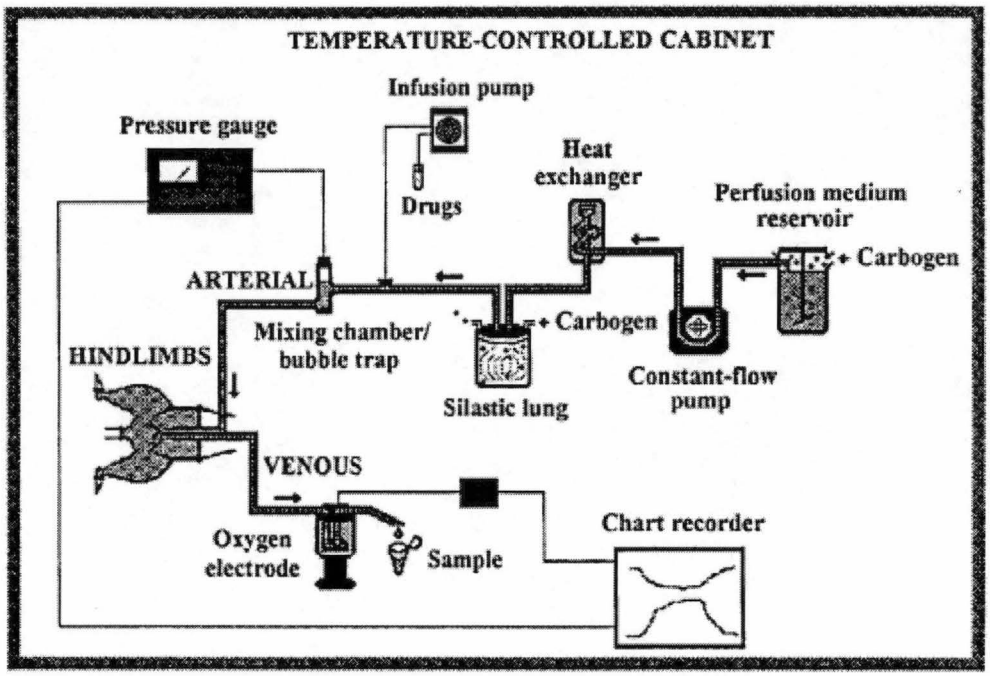


Figure 2-2 Perfusion apparatus for the constant-flow perfusions.
Reproduced from Dora (Dora 1993).

2.1.4.1 Calculation of Oxygen Uptake.

The oxygen electrode was calibrated before and after each perfusion using 100% oxygen and air. Arterial PO_2 (PaO_2) was determined by joining up the arterial and venous cannulae to bypass the perfused tissue, but with the same length of polyethylene tubing so that any loss to the atmosphere was constant. The oxygen uptake (VO_2) of the perfused tissue was calculated from the difference between PaO_2 and venous PO_2 (PvO_2), the flow rate, and the perfused muscle mass, whilst taking into account the Bunsen coefficient at 32°C according to the following equation:

$$VO_2\ (\mu mol.h^{-1}.g^{-1}) = \frac{1.3499 \times (PaO_2\text{-}PvO_2) \times \text{flow rate (ml/min)} \times 60\ \text{(ml/h)}}{1000\ \text{ml} \times \text{perfused tissue (g)}}$$

where 1.3499 ($\mu mol.L^{-1}.mmHg$-1) is the Bunsen coefficient for oxygen solubility in human plasma at 32°C ((Christoforides *et al.* 1969) (Bunsen coefficient = 1.256 at 37°C, 1.508 at 25°C) and perfused muscle mass is assumed to be $1/12^{th}$ of body mass in 180-200 g rats ((Ruderman *et al.* 1971).

2.1.5 Blood Perfusion Procedure

The perfusion medium was equilibrated with 95% air-5% CO_2) at 4°C to enable normal saturation of the erythrocytes with oxygen. Gassed perfusate then entered a temperature -controlled cabinet (37°C) at a constant flow rate. The arterial perfusate was temperature equilibrated (37°C) by passage through an in-line heat exchanger and water-jacketed arterial line. To ensure a constant arterial oxygen concentration, the perfusate was further equilibrated with 95% air-5% CO_2 by passage through a silastic tube oxygenator before entering the hindlimb.

2.1.5.1 Determination of Oxygen Consumption in Blood Perfusions

The total oxygen content of perfusate samples was determined in the sciatic nerve stimulation experiments (Chapter 3) using the TasCon oxygen content analyser (manufactured by the Physiology Department, University of Tasmania). For the LDF experiments (Chapter 4) oxygen content was determined using an in-line A-Vox oxygen analyser (A-Vox Systems Inc, San Antonio, Texas). Oxygen consumption was calculated as described in the respective chapters.

2.2 In Vivo Studies

2.2.1 Animals

Male hooded Wistar rats or male Wistar rats were cared for as above.

2.2.2 General Surgical Procedures

Rats were anaesthetized using pentobarbital sodium (50 $mg.kg^{-1}$ body weight) and had polyethylene cannulas (PE-50, Intramedic®) surgically implanted into the carotid artery, for arterial sampling and measurement of blood pressure (pressure transducer Transpac IV, Abbott Critical Systems) and into both jugular veins continuous infusion of anaesthetic and other intravenous infusions. A tracheotomy tube was inserted, and the animal allowed to spontaneously breathe room air 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 lubricating 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, heart rate and blood pressure using WINDAQ data acquisition software (DATAQ Instruments). The surgical procedure generally lasted approximately 30 min and then the animals were maintained under anaesthesia for the duration of the experiment using a continual infusion of pentobarbital sodium (0.6 $mg.min^{-1}.kg^{-1}$). 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 using a water-jacketed platform and a heating lamp positioned above the rat.

2.2.3 Euglycaemic Hyperinsulinaemic 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. Rats were then allocated into experimental groups, control or euglycaemic hyperinsulinaemic clamp. All infusion volumes in control groups were matched to the volumes of insulin (10 $mU.min^{-1}.kg^{-1}$) (Humulin R, Eli Lilly, Indianapolis, IN) and glucose infused in the clamp animals. Glucose (30% w/v solution) was infused to maintain blood glucose levels at or above basal level (approximately 5 mM) whilst infusing insulin for a period of 120 min (unless otherwise stated).

2.2.4 1-Methylxanthine Infusion during In Vivo Experiments

Since 1-methylxanthine (1-MX, Sigma Aldrich Inc) clearance was very rapid, it was necessary to partially inhibit the activity of xanthine oxidase (Rattigan *et al.* 1997b). To do this, an injection of a specific xanthine oxidase inhibitor, allopurinol (Emmerson *et al.* 1987) (10 $\mu mole.kg^{-1}$) was administered as a bolus dose 5 minutes prior to commencing the 1-MX infusion (0.4 $mg.min^{-1}.kg^{-1}$, dissolved in saline). This allowed constant arterial concentrations of 1-MX to be maintained throughout the experiment.

2.2.5 Glucose Assay

A glucose analyser (Yellow Springs Instruments, Model 2300 Stat plus) 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.

2.2.6 Insulin ELIZA Assay

Human insulin levels at the end of the euglycaemic insulin clamp were determined from arterial plasma samples by ELIZA assay (Dako Diagnostics Ltd, UK), using human insulin standards.

2.2.7 TNF ELIZA Assay

Arterial plasma TNF levels at the end of each experiment were determined by ELIZA assay (OptEIA™ Rat TNF-α Set, Pharmingen, USA) using recombinant rat TNF standards.

2.2.8 Data Analysis

All data is expressed as means ± SE. Mean femoral blood flow, mean heart rate and mean arterial blood 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 blood pressure in millimetres of mercury divided by femoral blood flow in millilitres per minute and expressed as resistance units (RUs). Glucose uptake in the hindlimb was calculated from A-V glucose difference and multiplied by femoral blood flow and expressed as $\mu mol.min^{-1}$. The 1-MX disappearance 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 $nmoles.min^{-1}$.

2.3 Analytical Methods

2.3.1 1-Methylxanthine Analysis by HPLC

2.3.1.1 Treatment of Perfusate Samples

Perfusate samples (1.0 ml) from hindlimb perfusions were mixed with 0.2 ml of 2 M perchloric acid (PCA) to precipitate proteins and left on ice for 5 min. If the samples were collected from a blood perfusion, the sample was immediately centrifuged for 15 sec at 8000 x g to remove the red blood cells before mixing with PCA. PCA treated samples were then centrifuged at 8000 x g for 10 min and the supernatant neutralised using 2.5 M K_2CO_3. Samples were then stored at -20°C till assayed using HPLC.

2.3.1.2 Treatment of In Vivo Blood Samples

Duplicate arterial (A) and venous (V) samples (300 µl) were taken at the end of the experiment and placed on ice. These blood samples were immediately centrifuged and 100 µl of plasma mixed with 20 µl of 2 M PCA. 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, allopurinol and oxypurinol concentrations as described previously ((Rattigan *et al.* 1997b, Rattigan *et al.* 1999).

2.3.1.3 HPLC Analysis of Purines

Analysis of purines was by reverse-phase High Performance Liquid Chromatography (HPLC) essentially as described previously ((Rattigan *et al.* 1997a, Wynants *et al.* 1987).

2.3.2 2-Deoxyglucose Uptake

2.3.2.1 Hindlimb Perfusions

The hindlimb was allowed to equilibrate to a steady state of perfusion pressure and PvO_2. Insulin (0.4 $U.ml^{-1}$ in saline) (Actrapid Insulin, Novo Nordisk) was infused at a rate of 1 in 200 for 25 min into the perfusion line immediately prior to a small continuously mixed bubble trap to give a final concentration of 2 $mU.ml^{-1}$. In addition, 2-deoxy-D-[1-^{3}H]glucose (2DG) (10 $\mu Ci.ml^{-1}$; 15 $Ci.mmol^{-1}$; NEN Research Products) and [U-14C]sucrose (3.14$\mu Ci.ml^{-1}$; 552 $mCi.mmol^{-1}$; NEN Research Products) in 2 mM sucrose/0.9% NaCl were infused at 35-40 µl.min.

Following the conclusion of the perfusion, the soleus, extensor digitorum longus (EDL), plantaris, gastrocnemius red and gastrocnemius white and tibialis anterior were dissected apart, weighed and then freeze dried for approximately 48 hours. The dry samples were then weighed and transferred into larger vials. Next, each sample was rehydrated using 150 µl of distilled water and 1 ml of Soluene-350 tissue solubiliser (0.5 M Quarternary ammonium hydroxide in Toluene, Packard USA) was added to each vial. The samples were then placed in a 50°C water bath to facilitate their solubilisation. Once dissolved, 100 µl of glacial acetic acid was added to each

vial of solubilised muscle to neutralise the basic Soluene. Scintillant (15 ml; Biodegradable Counting Scintillant-BCA, Amersham USA) was added to each vial and radioactive counts (disintegrations per minute, dpm) were determined using a scintillation counter (Beckman LS3801, USA). Perfusate samples (200 µl) were also counted after adding 3 ml of scintiallant to each vial.

Insulin-mediated 2DG uptake, as represented by R'g was calculated using the following equation:

$$R'g\ (umol.h^{-1}.g^{-1}) = \frac{2 \times [^{3}H\ dpm\ in\ muscle - (^{14}C\ dpm\ in\ muscle \times {}^{3}H\ dpm./{}^{14}C\ dpm.\ ratio\ in\ perfusate)]}{dry\ wt\ muscle\ (g) \times (^{3}H\ dpm.\ per\ ml\ perfusate\ /\mu moles\ glucose\ per\ ml\ perfusate)}$$

2.3.2.2 In Vivo Experiments

At 45 min prior to the completion of the experiment, a 50 µCi bolus of 2-deoxy-D-[2,6-^{3}H]glucose (2DG; specific activity = 44.0 Ci.mmol^{-1}, Amersham Life Science) in saline was administered. Plasma samples (20 µl) were collected at 15, 30 and 45 min to determine plasma clearance of the radioactivity. At the conclusion of the experiment, the soleus and plantaris muscles were removed, clamp frozen in liquid nitrogen and stored at -80°C until assayed for 2DG uptake.

The frozen soleus and plantaris muscles were ground under liquid nitrogen and homogenised using an Ultra Turrax™. Free and phosphorylated 2DG were separated by ion exchange chromatography using an anion exchange resin (AG1-X8) (James *et al.* 1985, Kraegen *et al.* 1985). Scintillant (15 ml) was added to each radioactive sample and radioactivity determined as above. From this measurement and a knowledge of plasma glucose and the time course of plasma 2DG disappearance, R'g, which reflects glucose uptake into the muscle, was calculated as previously described in detail by others (James *et al.* 1985, Kraegen *et al.* 1985).

2.3.3 Measurement of Total Flow by Microsphere Entrapment

2.3.3.1 Hindlimb Perfusion

Microsphere entrapment can be used to determine total flow to the hindleg during the hindlimb perfusion technique. This technique was used both in the sciatic nerve severance work and in the sciatic nerve stimulated rats to ascertain the distribution of microspheres in the hindlimb.

These experiments were usually performed as a separate study, but under the same conditions, in order to compare experimental (sciatic nerve severed and sciatic nerve stimulated rats) and control animals (sham-operated or non-sciatic nerve stimulated rats).

Ten minutes before the end of the respective experiment, a bolus dose of 400,000 (200,000 in the case of the sciatic nerve stimulation work since it only involves a single hindlimb) yellow-green 15 μM FluoSpheres® (Fluorescent Microsphere Resource Centre, University of Washington, Seattle) was injected immediately prior to the arterial cannula. This injection was performed over a period of 10 seconds to minimise any interruptions to the normal perfusate flow and pressure. During this final 10 min of the experiment, the venous perfusate was collected in order to ascertain the number of microspheres which passed through the hindlimb.

2.3.3.2 Microsphere Distribution Assay

Following the conclusion of the experiment, all hindlimb muscles and remaining muscle and tissue below the abdominal ligation were dissected free from the rat (where only a single hindlimb was perfused, tissues were removed from the contralateral unperfused leg to confirm that flow had not moved past the ligatures). All tissues were briefly blotted and weighed, transferred to 50 ml centrifuge tubes, and digested using the method of Van Oosterhout et al ((Van Oosterhout *et al.* 1995) with modifications as described in Rattigan *et al.* (Rattigan *et al.* 1997a). Each muscle was digested by heating at 58-60°C for approximately 3 h in 5-10 volumes of 2 M ethanolic KOH (2 M KOH in 95% ethanol) containing 0.5% Tween-80 (Sigma). Once dissolved, the homogenate was centrifuged for 20 min at 2000 x g (IEC centrifuge) to collect the microspheres at the base of the tube. The pellet was then

washed in 10 ml of 0.25% Tween-80 solution, followed by distilled water alone. After the final wash, as much of the supernatant as possible was removed without disturbing the pellet. The microsphere pellet in each tube was then resuspended in 5 ml of Cellosolve® (2-ethoxyethyl acetate, Aldrich Chemical Co Inc) and left overnight at 4°C to allow complete dissolution of the microspheres, releasing the lipophilic fluorescent dye. The samples were vortexed, centrifuged (2000 x g, 20 min) and the supernatant carefully removed for fluorescence determination. The fluorescence intensity of the organic phase was measured against a solvent blank using an Aminco-Bowman Spectrophotofluorimeter with excitation and emission settings at 495 nm and 510 nm, respectively. In order to calculate the microsphere numbers per sample, the fluorescence reading was referenced to a standard curve, which was produced by diluting a known amount of microspheres in the solvent.

CHAPTER 3

The Effect of Exercise from Sciatic Nerve Stimulation on 1-MX Metabolism in the Perfused Rat Hindlimb.

3.1 Introduction

As described in the first chapter, there are two methods currently being developed in this laboratory for measurement of nutritive flow within skeletal muscle, laser Doppler flowmetry and 1-MX metabolism. The 1-MX method involves infusion of an endogenous substrate for capillary endothelial xanthine oxidase, 1-MX, and measurement of its metabolism by HPLC.

It is generally accepted that there is a marked increase in functional capillary surface area as a result of capillary recruitment within skeletal muscle during exercise (Segal 1992). In the isolated perfused rat hindlimb, sciatic nerve stimulation leads to vasodilation with increased VO_2, particularly if the hindlimb has pre-existing vascular tone caused by the presence of a vasoconstrictor (Colquhoun *et al.* 1990). Any change in capillary flow (recruitment) should be reflected by changes in 1-MX metabolism. Thus, the aim of this chapter was to investigate the effect of exercise (in the form of sciatic nerve stimulation), which is known to increase nutritive flow in the constant-flow perfused rat hindlimb, on 1-MX metabolism. A positive outcome lends further support to the use of 1-MX as an indicator of changes in capillary surface area.

3.2 Methods

3.2.1 Sciatic Nerve Exposure for Stimulation

Following an equilibration period of 30 min, the skin was removed from the inner and outer thigh of the right hindlimb to expose the femoral vein and sciatic nerve in the flank. The sciatic nerve was cut to allow positioning of the distal cut end in a suction

electrode. The knee was secured at the level of the tibiopatellar ligament, and the Achilles tendon was attached to a Harvard Apparatus isometric transducer, allowing transmission of tension development from the calf muscle groups. Voltage across the electrodes was monitored on a cathode ray oscilloscope (Telequipment DM64) and tension development was recorded during contraction on a Yokagawa 3056 chart recorder.

3.2.2 Hindlimb Perfusions

Specific details for hindlimb perfusions are given in Section 2.1. Perfusion pressure was monitored. Following 30 min equilibration at 4 $ml.min^{-1}$, the perfusate flow rate was increased to 15.0 ± 0.1 $ml.min^{-1}$ (equivalent to 0.95 ± 0.01 $ml.min^{-1}.g^{-1}$ muscle) for the remainder of the experiment (see Figure 3-1 for experimental protocol). At the same time, 5 mM 1-MX was infused into the perfusion line (at 1 in 200 of the perfusate flow rate) proximal to a small stirred bubble trap and the arterial cannula to give a final concentration of 25 μM.

Prior to contraction, the resting length of the muscle was adjusted to attain maximal active tension on stimulation. Sciatic nerve stimulation was then commenced using 200 ms trains of 0.1 ms pulses at 100 Hz every 2 seconds at 6 V to attain full fibre recruitment (Rattigan *et al.* 1996) for the last 15 min of the perfusion.

Arterial samples were taken at the beginning (5 min) and on completion of the experiment (55 min) for analysis of lactate and purines. Venous samples were also collected at 5, 15, 25, 35, 45, 50 and 55 min from the vena cava for determination of lactate and purines. At 55 min, an additional venous sample was obtained from the femoral vein of the working hindlimb while still being stimulated. This was done using a syringe fitted with a 26 G needle. Duplicates of all samples were taken in airtight glass syringes for analysis of oxygen content.

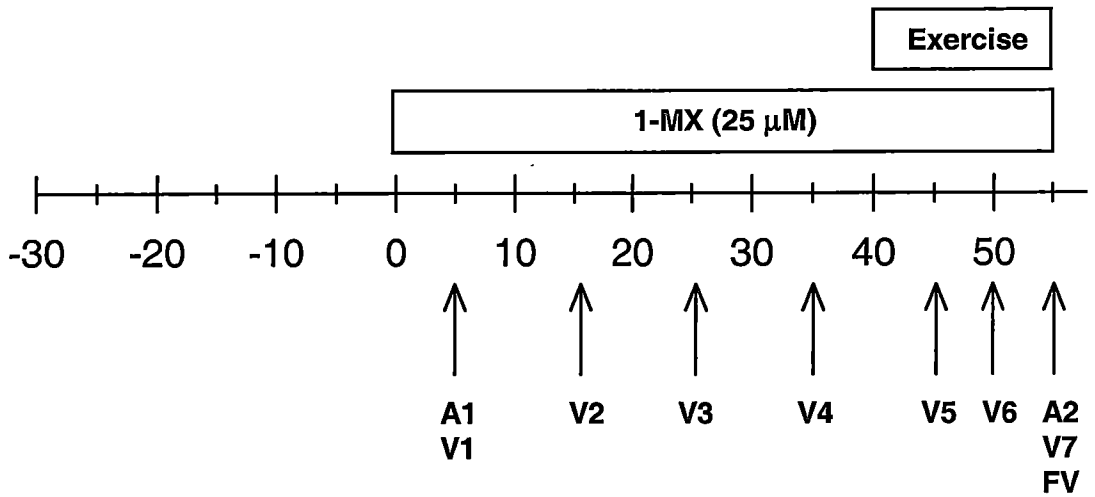


Figure 3-1 Experimental protocol for sciatic nerve stimulation experiments. Flow rate in the first 30 min (equilibration period) was 4 $ml.min^{-1}$. From time = 0 onwards, the flow rate was 15 $ml.min^{-1}$.

3.2.3 Measurement and Calculation of Oxygen Uptake

The total oxygen content of perfusate samples was determined using a galvanic cell oxygen analyser (TasCon oxygen content analyser manufactured by the Physiology Department, University of Tasmania). The rates of oxygen uptake were calculated from arterio-venous difference and flow rate, and were expressed per gram of perfused muscle, as estimated previously (Dora *et al.* 1992).

3.2.4 Muscle Incubations

To assess whether contraction directly affected xanthine oxidase activity, soleus (30 ± 1.5 mg) and extensor digitorum longus (31.7 ± 0.7 mg) muscles were dissected from 10 rats weighing 65-70 g and incubated at 37°C in Krebs-Henseleit buffer containing 5 mM HEPES, pH 7.4, 5 mM pyruvate, 35 mM mannitol and 1.27 mM CaCl2. Animals no greater than this size were essential so that muscles did not exceed the size where diffusion of oxygen became limiting (Bonen *et al.* 1994). After pre-incubation for 30 min, 5 soleus and 5 EDL muscles were subject to field stimulation

using the identical conditions as for the hindlimb calf muscle group above. Stimulation was continued for 15 min. As controls, 5 soleus and 5 EDL muscles were incubated without stimulation for a total of 45 min. Muscles were stored at -80°C until assays could be conducted.

3.2.5 Xanthine Oxidase Activity

Xanthine oxidase activity was measured at 293 nm and 37°C in 50 mM phosphate buffer, pH 7.4, containing 0.1 mM EDTA essentially as described by others (Wajner and Harkness 1989)s. Commercially available (Sigma Chemical Co.) buttermilk xanthine oxidase (2.0 units.mg^{-1}) was used to construct direct linear plots for the determination on Km and Vmax for xanthine (XAN) and 1-MX.

3.2.6 Statistical Analysis

Unpaired Student's *t*-test was used to test the hypothesis that there was no difference between sets of data. For the time courses, one way repeated measures analysis of variance (ANOVA) was performed and when a significant difference was found, multiple comparisons (Dunnett's method) were made by comparing with the time point just prior to exercise (35 min). Significant differences were recognised at $P < 0.05$.

3.3 Results

3.3.1 Changes to Total Blood Flow

Figure 3-2 shows the effect of contraction of the calf muscle group on the distribution of flow to various muscles of the calf and thigh of the perfused leg. As a result of exercise there was a significant increase in flow to several muscles of the group, including soleus, plantaris, and the gastrocnemius red and white. In addition, total flow to the calf was increased as a result of exercise from 5.20 ± 0.78% (n = 5+ to 12.11 ± 1.38% (n = 7), representing a 2.3-fold increase ($P < 0.01$). This was balanced by a decrease in the flow to the thigh muscles: 24.01 ± 1.79% (n = 5) without exercise and 18.01 ± 2.75% (n = 7) with exercise. This decrease was not significantly different for the whole thigh, but there was a significant decrease in the vastus and quadriceps/adductor muscle groups with exercise.

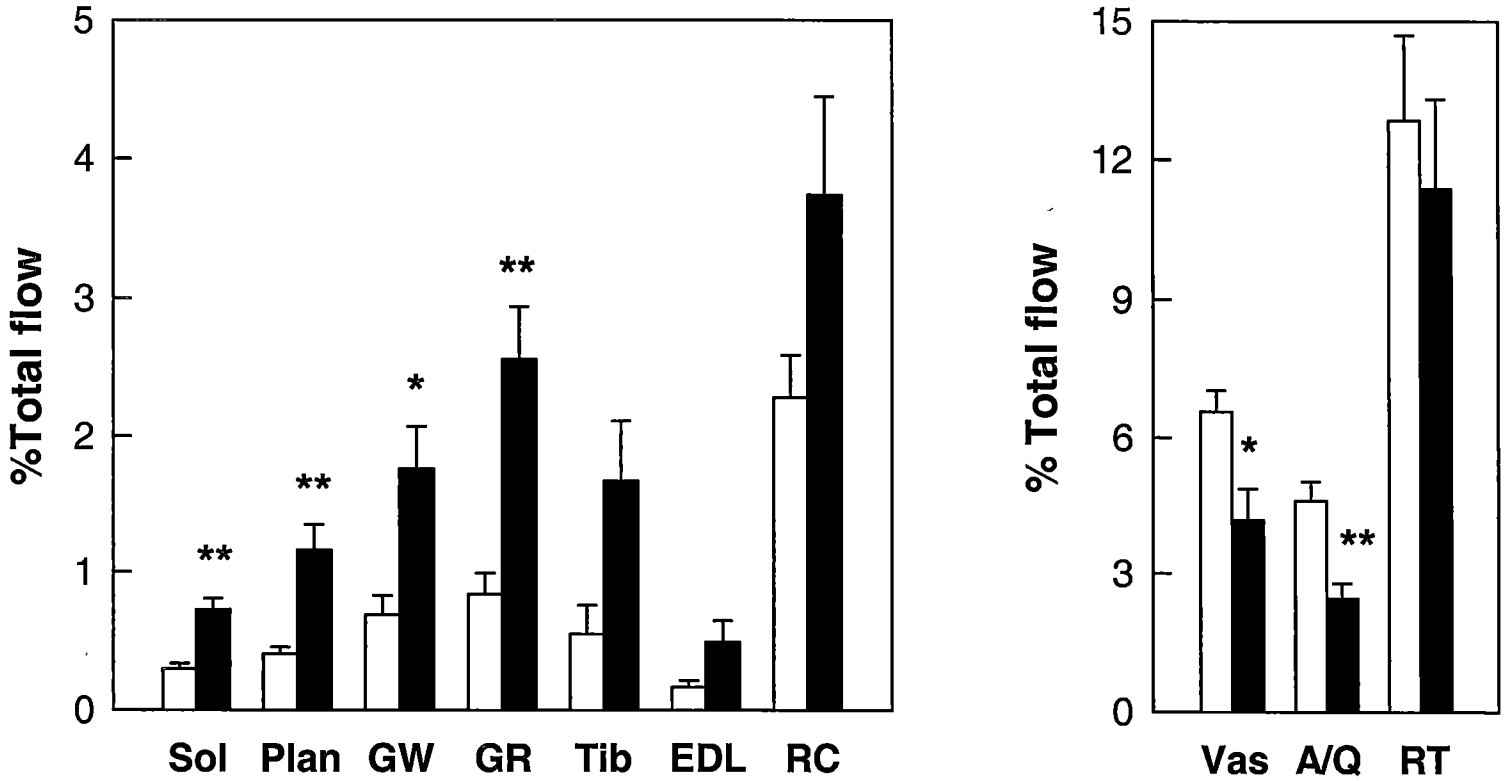


Figure 3-2 Effect of exercise on the distribution of fluorescent microspheres to the calf and thigh muscles in the perfused rat hindlimb. Conditions of rest (open bars) or during contraction of the calf muscle group (filled bars) are shown. Microspheres were recovered from the soleus (Sol), plantaris (Plan), gastrocnemius red (GR), gastrocnemius white (GW), tibialis (Tib), extensor digitorum longus (EDL), remaining calf muscles (RC), vastus group (Vas), adductor/quadriceps group (A/Q) and remaining thigh muscles (RT). Values are mean ± SE (n = 5 for rest and n = 7 for contracting). * $P < 0.05$, ** $P < 0.01$, compared to resting blood flow.

Flow to all the muscle and other tissues of the hindlimb were also calculated and these are shown in Table 3-1. There was no significant difference in any of the tissues studied between rest and exercise. As total flow to the hindlimb was 15 $ml.min^{-1}$, muscle received an average total flow of 0.49 $ml.min^{-1}.g^{-1}$ and this did not change as a result of exercise.

Table 3-1. Distribution of fluorescent microspheres in the perfused rat hindlimb to various tissues as percentage of total recovered.

Tissue	Rest	Exercise
Total muscle	48.5 ±1.9	44.9 ± 1.5
Bone	27.5 ± 2.7	30.8 ± 1.3
WAT	3.3 ± 0.6	2.4 ± 0.4
Skin	7.1 ± 0.9	4.8 ± 1.0
Viscera	10.7 ± 1.2	11.8 ± 1.3
Remainder	0.7 ± 0.2	0.7 ±0.2
Contralateral leg	1.7 ± 0.6	4.0 ± 1.3
Perfusate	0.6 ± 0.1	0.7 ± 0.1

A bolus of 200 000 yellow-green 15μm FluoSheresValues® was injected at rest or during exercise. Perfusate and various tissues were sampled for microsphere content. Values are expressed as a percentage of the total number injected and are means ± SE; n = 5 rest and n = 7 for exercise.

3.3.2 Perfusion Pressure, Oxygen Uptake and Lactate Release

Perfusion pressure was monitored continuously throughout the experiment, however, discrete points were taken to correspond with samples collected for analysis of oxygen, lactate and purine content. Figure 3-3 shows data for perfusion pressure,

VO_2 and lactate release before and during exercise. Statistical analysis by one-way repeated measures ANOVA (with comparison to the 35 min time point using Dunnett's method) showed that exercise causes a significant rise in VO_2 and lactate release, but not pressure.

3.3.3 1-MX Metabolism

Figure 3-4 shows the time course for the metabolism of 1-MX by the perfused hindlimb. Fifteen minutes was required for equilibration of the 1-MX metabolism during exercise. After this time the recovery of 1-MX + 1-MU was quantitative (~100%). Contraction of the calf muscle group (representing ~15% of the total hindlimb muscles) had no significant effect on the conversion of 1-MX. Indeed, using the same statistical test as for the previous figure, exercise caused no change in any of the three parameters.

3.3.4 Metabolism of the Working Muscles

Figure 3-5 shows data for oxygen uptake, lactate release and 1-MX metabolism particular to the working muscles in which flow had increased as a result of sciatic nerve-mediated contraction. Data for resting hindlimb and non-working muscles of the stimulated hindlimb are included for comparison. The data for non-working muscles during exercise were calculated by the difference between the total rate of release (in $\mu mol.h^{-1}$) from the working hindlimb and working muscles and then relating this back to the mass of the non-working muscles. In this study, the average mass for the total hindlimb muscles and working muscles were 17.14 and 2.63g, respectively. Hence, the average non-working muscle mass was 14.51g. VO_2 and lactate release both showed an increase across the whole hindlimb during exercise, whereas the increased conversion rate of 1-MX to 1-MU was only observed in the working muscle when the perfusate was sampled from the vein draining the working muscle and the increase in flow to those muscles was taken into account. These metabolic changes were only occurring in the working muscles as there was no difference between the data for the resting hindlimb and the non-working muscles. Also samples taken from the femoral vein of non-stimulated hindlimbs showed the same conversion of 1-MX to 1-MU as resting hindlimb.

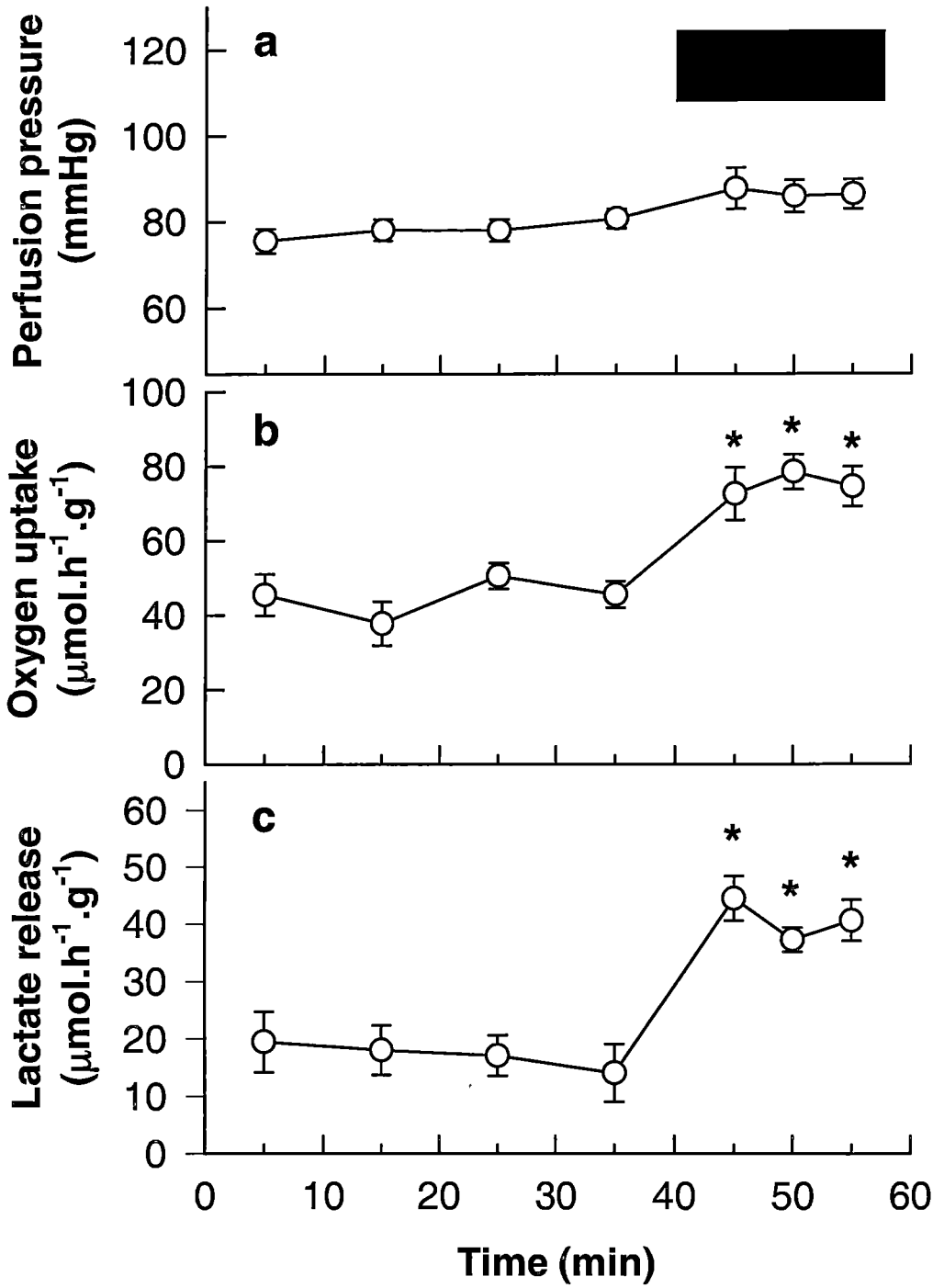


Figure 3-3 Effect of exercise on perfusion pressure (a), oxygen uptake (b) and lactate release (c) in the perfused rat hindlimb.

Period of contraction of the calf muscle group is indicated by the filled bar. Values are mean ± SE (n = 6). Comparisons were made to the 35 min (pre-exercise) time point using Dunnett's method after one-way repeated measures analysis of variance. * $P < 0.05$.

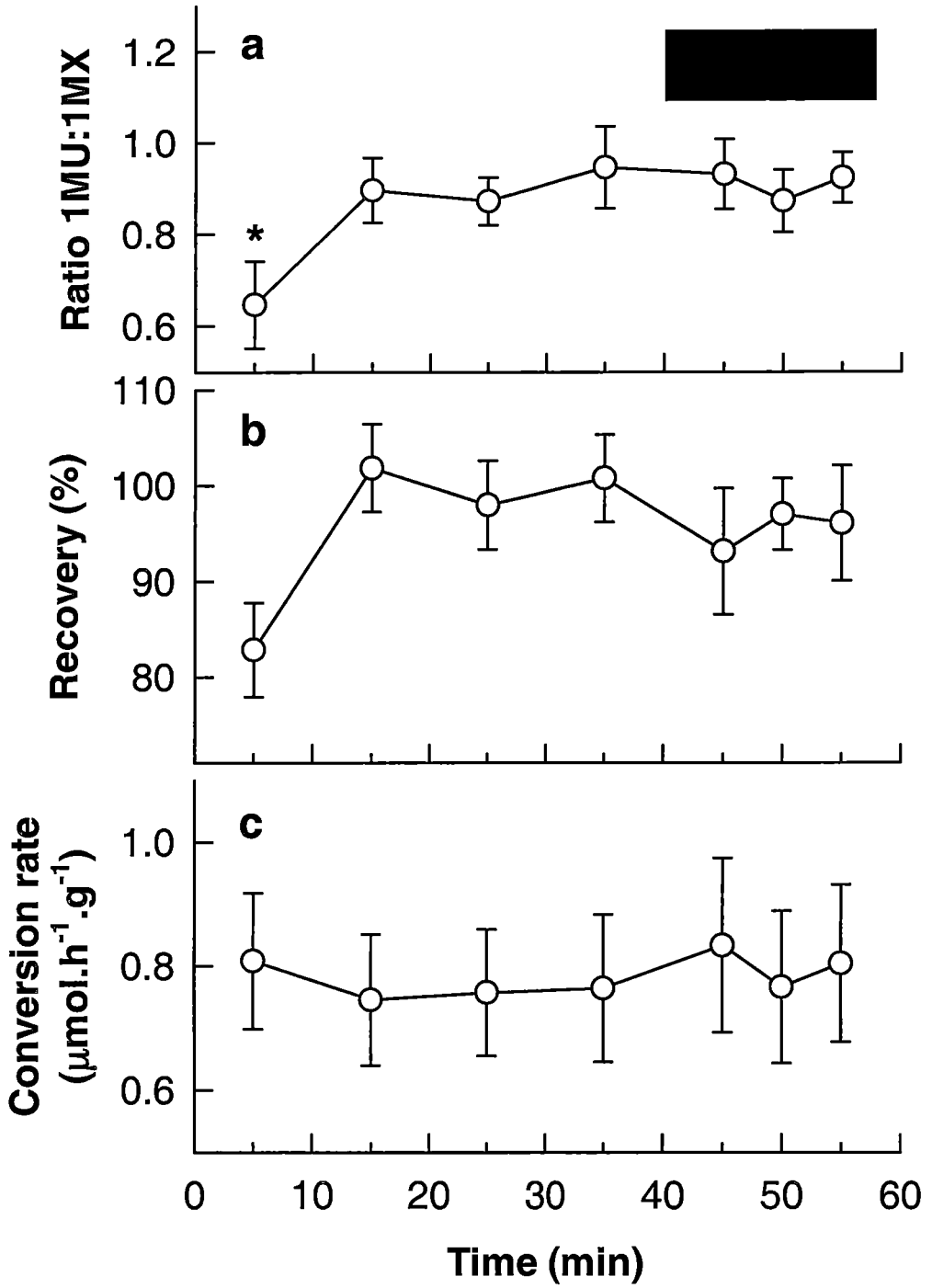


Figure 3-4 Effect of exercise on the ratio of 1-MU:1-MX (a), recovery of 1-MU + 1-MX (b) and conversion rate of 1-MX to 1-MU (c) in the perfused rat hindlimb. Period of contraction of the calf muscle group is indicated by the filled bar. Values are mean ± SE (n = 6). Comparisons were made to the 35 min time point using Dunnett's method after one-way repeated measures analysis of variance. * $P < 0.05$.

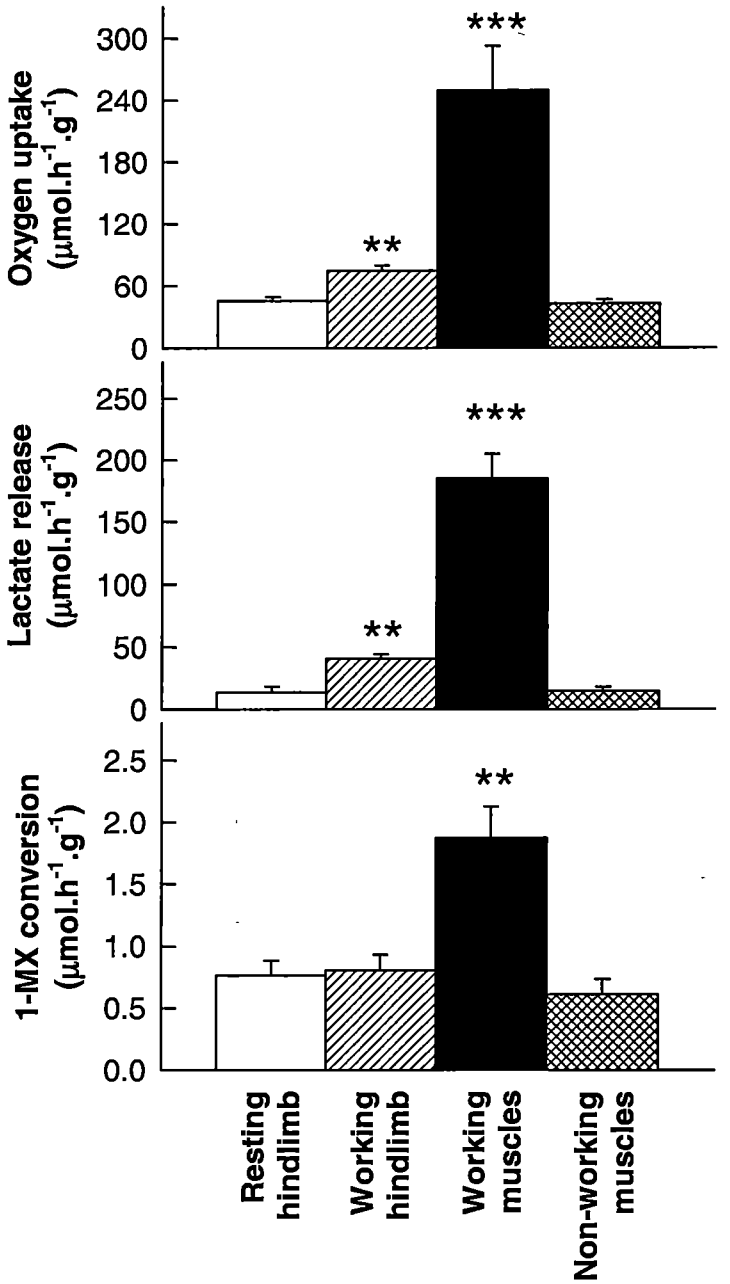


Figure 3-5 Effect of exercise on metabolism in the perfused rat hindlimb.

Values were determined from Figures 3-3 and 3-4 at 35 min and 55 min as well as the femoral vein samples taken at 55 min and regional blood flow estimates from Figure 3-2 as detailed in the text. Values were calculated from arterio-venous differences across the whole hindlimb during rest conditions (Resting hindlimb), across the whole hindlimb during contraction of the calf muscle group (Working hindlimb), across the calf muscle group as it was contracting (Working muscles) and across the non-working muscles perfused muscles of the same leg (Non-working muscles). Values are mean ± SE (n = 6). ** $P < 0.01$, *** $P < 0.001$, compared to resting hindlimb.

The concentration of purines in the venous sample from the working hindlimb, the resting hindlimb and from the working muscles, is shown in Table 3-2. At rest, adenosine and inosine levels were below the detectable level of 0.05 μM. During exercise, inosine release from the whole hindlimb increased into the detectable range and samples from the working muscle showed increased concentrations of inosine and the other measurable purines. In addition, adenosine levels became detectable.

Table 3-2. Venous concentration (μM) of purines in the perfused rat hindlimb.

Purine	Resting hindlimb	Working hindlimb	Working muscle
Adenosine	N.D.	N.D.	1.02±0.23
Inosine	N.D.	0.61±0.10	2.25±0.41
Hypoxanthine	0.50±0.17	0.76±0.13	2.63±0.6**
Xanthine	0.29±0.10	0.50±0.07	1.78±0.51*
Uric acid	5.63±0.91	7.55±0.58	14.71±1.14***

For the resting and working hindlimb values, venous samples were taken from the vena cava at times 35 and 55 min respectively. For the working muscle values (calf muscle group), venous samples were taken from the femoral vein after time 55 min. Values are means ± SE; n = 6, except for adenosine (n = 4). Working hindlimb and working muscle values were compared to resting hindlimb values by unpaired Student's t-test * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. N.D. not detectable.

3.3.5 Xanthine Oxidase Activity

Values for xanthine oxidase activity in unstimulated soleus and EDL muscles were 0.032 ± 0.04 and 0.040 ± 0.002 units.g(wet wt)$^{-1}$, respectively. Stimulation for 15 min did not significantly alter these values (soleus: 0.039 ± 0.009 and EDL: 0.039 ±

0.007 units.g(wet wt)$^{-1}$. For 20 milliunits of the purified enzyme, the V_{max} for xanthine (V_{max}^{XAN}) and 1-MX ($V_{max}^{1\text{-}MX}$) were 17.8 and 35.0 nmol.min-1 and the K_m for xanthine (K_m^{XAN}) and 1-MX ($K_m^{1\text{-}MX}$) were 2.6 and 7.6 μM respectively (direct linear plots; data not shown).

3.4 Discussion

This study appears to be the first report where the metabolism of an exogenously applied non-physiological substrate is increased in association with muscle contraction. Perhaps most importantly, since the recovery of both substrate and product is quantitative, it is clear that the substrate does not become involved in the energy metabolism of the muscle itself and thus the increased conversion reflects a change external to the muscle. As xanthine oxidase is predominantly located in the endothelial cells that constitute the capillaries (Jarasch *et al.* 1986), it seems likely the increased conversion of 1-MX to 1-MU results from increased exposure to the enzyme, in turn, resulting from the increase in functional capillary surface area owing to increased muscle contractile activity (Segal 1992). Thus, the present findings further support the use of 1-MX for assessing the change in capillary recruitment in muscle and the general principle that capillary endothelial enzymes may be targeted for such studies.

1-MX metabolism has been previously shown in this laboratory to parallel VO_2 in the perfused hindlimb. In this study, 1-MX metabolism was decreased by the vasoconstrictor, serotonin (Rattigan *et al.* 1997a). Serotonin is thought to decrease functional capillary surface area and thus nutritive (capillary) flow in muscle as part of its action to inhibit oxygen uptake, lactate output and metabolism generally (Clark *et al.* 1995, Dora *et al.* 1994). In that study (Rattigan *et al.* 1997a), total hindlimb flow was constant and there was no change in flow to individual muscles, nor to the distribution of flow between muscle and non-muscle tissue. As such, this suggests that both nutritive and non-nutritive vascular routes are within each muscle where non-nutritive vessels may involve closely associated connective tissue (Borgstrom *et al.* 1988, Myrhage and Eriksson 1980).

Whereas there are other possibilities to explain the presently observed increase in 1-MX conversion that may involve processes other than increased exposure to xanthine oxidase, these seem unlikely. Thus, an increase in 1-MX conversion to 1-MU could result from an increase in total flow to the calf muscle if the concentration gradient from artery to vein was lowered. Equally if blood flow velocity was able to influence the conversion of 1-MX to 1-MU a change in total flow rate to the resting hindlimb should alter the rate of conversion. To check these possibilities, control experiments were conducted where total flow was increased from 4 to 15 $ml.min^{-1}$ and blood samples were taken before and after the flow change to analyse arteriovenous differences in 1-MX and 1-MU. Results showed that 1-MX metabolism was not affected by total flow change.

A second possibility is that exercise directly activated xanthine oxidase leading to an increase in 1-MX conversion. However, the activity of xanthine oxidase was not affected by prior contraction of incubated soleus and EDL muscles. Therefore it is unlikely that activation of the enzyme has occurred in the perfused muscle during exercise. The data therefore support the notion that the conversion of 1-MX is a valid indicator of exposure to xanthine oxidase and hence an indicator of increased functional capillary surface area.

It should be noted that the perfused rat hindlimb preparation has no significant functional sympathetic nerve activity input to regulate flow during exercise. In addition, the isolated perfused rat hindlimb is considered to have low vascular tone, at least at rest (Folkow *et al.* 1974). However, the observation that exercise increased flow to working muscles and increased the metabolism of 1-MX suggests that either some basal tone controlling access to muscle capillaries is present or the intrinsic resistance is higher for the nutritive capillary network than the non-nutritive functional shunts. Clearly, the resistance owing to either process is overcome by locally released vasodilators (Ballard *et al.* 1987, Joyner and Dietz 1997, Lash 1996), and the vascular pumping action (Lash 1996, Laughlin 1987, Tschakovsky *et al.* 1996) of working muscle. For a system of constant total flow this would mean that flow increases in working muscle at the expense of flow from non-working regions, particularly as the perfusion pressure remained constant (Figure 3-3). Decreased flow to non-working muscles during exercise has been reported to occur *in vivo* (Asanoi *et*

al. 1992, Maeda *et al.* 1997, Musch and Poole 1996), although not necessarily in all non-working muscles (Ahlborg *et al.* 1975, Maeda *et al.* 1997). The mechanism for this is not known, but may involve direct vasoconstriction mediated by the endothelium (Jarasch *et al.* 1986, Maeda *et al.* 1997) or sympathetic and neural factors (Jacobsen *et al.* 1994).

We have recently postulated that the non-nutritive flow present in resting muscle may provide an opportunity for flow amplification to nutritive capillaries of muscle during exercise (see Chapter 1) (Clark *et al.* 1998). Thus if non-nutritive flow was 80% at rest and nutritive flow 20%, switching the entire flow to nutritive by increased sympathetic outflow, would lead to a 5-fold amplification without any change in total blood flow to muscle. Further increases in total flow owing to increased cardiac output would further add to the amplification.

Table 3-2 shows that adenosine, a putative endogenous dilator from working muscle (Ballard *et al.* 1987) was elevated in perfusate sampled from the femoral vein. As adenosine is rapidly converted by the perfused hindlimb to inosine, hypoxanthine, xanthine and uric acid (Richards 1993), it is possible that the large release of purine breakdown products found in the perfusate from working muscle have originated from locally released adenosine. If this is the case then the increase in endogenous purine breakdown products in perfusate of working muscle origin are likely to have had an inhibitory influence on the metabolism of 1-MX. Previous studies (Rattigan *et al.* 1997a) have shown that xanthine (XAN) inhibited the conversion of 1-MX to 1-MU by the perfused rat hindlimb. The potential inhibition of 1-MX conversion by endogenously released XAN can be calculated using the determined values for K_m and V_{max} at 37°C for XAN and 1-MX in the following equation for competitive inhibition (Cornish-Bowden 1979):

$$V_{1\text{-}MX} = \frac{V_{max}^{1\text{-}MX}[1\text{-}MX]}{K_m^{1\text{-}MX}\{1 + ([XAN]/K_m^{XAN})\} + [1\text{-}MX]}$$

where [1-MX] and [XAN] are the concentrations of 1-MX and xanthine, respectively. If the values for [XAN] (0.29 μM) and [1-MX] (25 μM) during resting conditions are used in this equation, then the rate of conversion is 26.2 $nmol.min^{-1}$. This rate falls to 23.1 $nmol.min^{-1}$ for working muscle conditions ([XAN] = 1.78 μM), a decrease of 12%. Thus, a change in XAN concentration from 0.29 to 1.78 μM depresses the conversion of 1-MX to 1-MU and the observed value for 1-MU:1-MX of 0.8 would correspond to 1.05 had the concentrations of XAN remained unchanged. These calculations are conservative, as values for XAN are those of the venous perfusate concentrations. The actual concentrations of XAN exposed to the enzyme could be higher as most of the XAN has been converted to uric acid by the time the blood was sampled at the venous outflow (Table 3-2). Also, both xanthine and hypoxanthine are naturally-occurring substrates for xanthine oxidase and would inhibit exogenously added 1-MX metabolism in a competitive manner. Indeed in a previous study, we have shown that constant infusion of 16 μM xanthine inhibited the conversion of 23 μM 1-MX by 16%. Therefore, the increase in 1-MX conversion of 2.5-fold may be an underestimate of the actual extent of capillary recruitment. Application of more direct methods such as laser Doppler flowmetry or contrast enhanced ultrasound with micro-bubbles may reveal the extent of this underestimation.

Finally, it is important to note that the stimulation protocol used in this study results in an early fatiguing of Type II fibres leaving a plateau of sustained aerobic tension. Thus, the pattern of fibre recruitment differs from that occurring with voluntary activity with muscles rich in Type I and IIA fibres initially recruited at low levels of activity. Consequently, venous blood samples taken from the working muscles cannot discriminate between effects occurring in fibres of different types, between fibres that fatigue at different rates, or indeed fibres that have different dependencies on purine metabolism. Thus, the values for 1-MX conversion reflect the end result of all processes.

In summary, 1-MX metabolism was found to increase in association with increased VO_2 and lactate production in working muscle. This, together with previous findings where 1-MX metabolism decreased in association with decreased VO_2 owing to

vascular shunting by serotonin (Rattigan *et al.* 1997a), suggests that 1-MX may be a useful indicator of changes in functional capillary surface area when changes in VO_2 are difficult to determine or result from non-vascular events (e.g. uncoupling of oxidative phosphorylation).

CHAPTER 4

The Effect of NE and 5HT on Laser Doppler Flowmetry in Blood Perfusion

4.1 Introduction

As described in previous chapters, research from our laboratory using the constant-flow perfused rat hindlimb has shown that skeletal muscle metabolism may be controlled by vasomodulators that act to alter flow distribution within muscle (Clark *et al.* 1995). These vasomodulators can be classified into two main groups, Type A and Type B vasoconstrictors. Type A vasoconstrictors act to increase metabolism (e.g. oxygen uptake and lactate release and contractile performance) by redirecting flow from a non-nutritive route to nutritive capillaries within muscle (Newman *et al.* 1996). Conversely, Type B vasoconstrictors, have the opposite effect on muscle metabolism. These findings suggest that vasoconstrictors are capable of controlling skeletal muscle metabolism by altering the proportion of nutritive to non-nutritive flow without altering total flow to the muscle. Nutritive vessels are considered to be those in direct contact with skeletal muscle cells (Hudlicka 1973). However, the nature of the non-nutritive route is unclear and there is only limited evidence available as to their anatomical nature. There appears to be two possibilities. One theory (as discussed in Chapter 1) is that the non-nutritive vessels are located in connective tissue and septa closely associated with muscle tissue. This is supported by recent data showing that vessel flow measured in the tibial tendon of the biceps femoris was inversely related to resting muscle metabolism (Newman *et al.* 1997). The other possibility is the non-nutritive route may be made up of relatively short capillaries which are located throughout the muscle, but due to the nature of their capillary walls only allow minimal nutrient exchange.

So far the only method we have available for measuring changes in nutritive flow within muscle under different conditions is 1-MX metabolism. Due to the limitations with the 1-MX method, we decided to develop a non-invasive method for measurement of nutritive and non-nutritive flow within skeletal muscle. Laser

Doppler flowmetry is thought to measure microvascular perfusion but whether it can selectively detect nutritive flow in the presence of non-nutritive flow in a constant total flow preparation is unknown thus far. Thus, in this study we have compared muscle LDF signal changes, using macro surface and implantable micro probes positioned either on or within the hindlimb skeletal muscle. The responses to changes in total flow and to vasoconstrictors that are known to alter hindlimb skeletal muscle by altering the proportion of nutritive to non-nutritive flow were characterised.

4.2 Methods

4.2.1 Hindlimb Perfusions

Hindlimb perfusions were conducted essentially as described previously in Chapter 2 using hooded Wistar rats (205 + 5 g body weight). Modifications included two heat exchangers (as opposed to one), and a small magnetically stirred bubble trap (0.5 ml capacity, with an injection port) immediately prior to the arterial cannula. Perfusion medium contained washed bovine red blood cells (38% haematocrit) and is gassed with 95% air, 5% CO_2. The arterial perfusate temperature was maintained at 37°C. In addition the rat was placed on a water-jacketed platform heated to 37°C, so that the hindlimb temperature could be maintained at 35-37°C, and the entire apparatus including the rat was contained within a heat controlled cabinet at 37°C. Only one hindlimb was perfused. All perfusions were conducted at a constant flow rate of 4.0 ± 0.1 $ml.min^{-1}$ (or 0.27 ± 0.01 $ml.min^{-1}.g^{-1}$ muscle) when either norepinephrine (NE) or serotonin (5-HT) was injected. However, to hasten complete removal of preceding doses, flow was momentarily increased to 8 $ml.min^{-1}$ for 2 min. This also improved the reproducibility of subsequent identical doses.

4.2.2 Laser Doppler Flowmetry

4.2.2.1 Surface Measurements

Previous studies using fluorescent microspheres for determining regional flow (Rattigan *et al.* 1997a) have suggested that the muscles of the thigh and hip, of which the biceps femoris constitutes approximately 17% (Delp and Duan 1996), receive

flow at approximately 76% of the total hindlimb rate (or 0.20 ± 0.02 $ml.min^{-1}.g^{-1}$). A small hole (approximately 4 mm diameter) was made in the middle of the biceps femoris corresponding to the point "bf" in Figure 4-1 and the exposed area covered by thin plastic wrap (e.g. Saran Wrap®) to prevent drying. The hindlimb was clamped by the foot so that the laser Doppler flow probe (Perimed PF 2, operating wavelength of 632 nm) could be positioned over the centre of the hole in the skin. For the measurement of red cell flux, the probe was placed vertically above and approximately 1 mm from the surface of the muscle. The probe comprised three fibres, each 800 μm diameter with one for illumination and two for detection. Settings on the detector unit were 4 kHz (gain setting 10) with a time constant of 3 sec, unless otherwise indicated. The signal (0-5 volts) was continuously recorded on an IBM compatible PC using a DI-190 I/O module and WINDAQ software. The data are expressed as volts. For each perfusion "biological zero" was determined by switching off the perfusion pump for 5 min and waiting until venous perfusate flow ceased, with the LDF probe still in position.

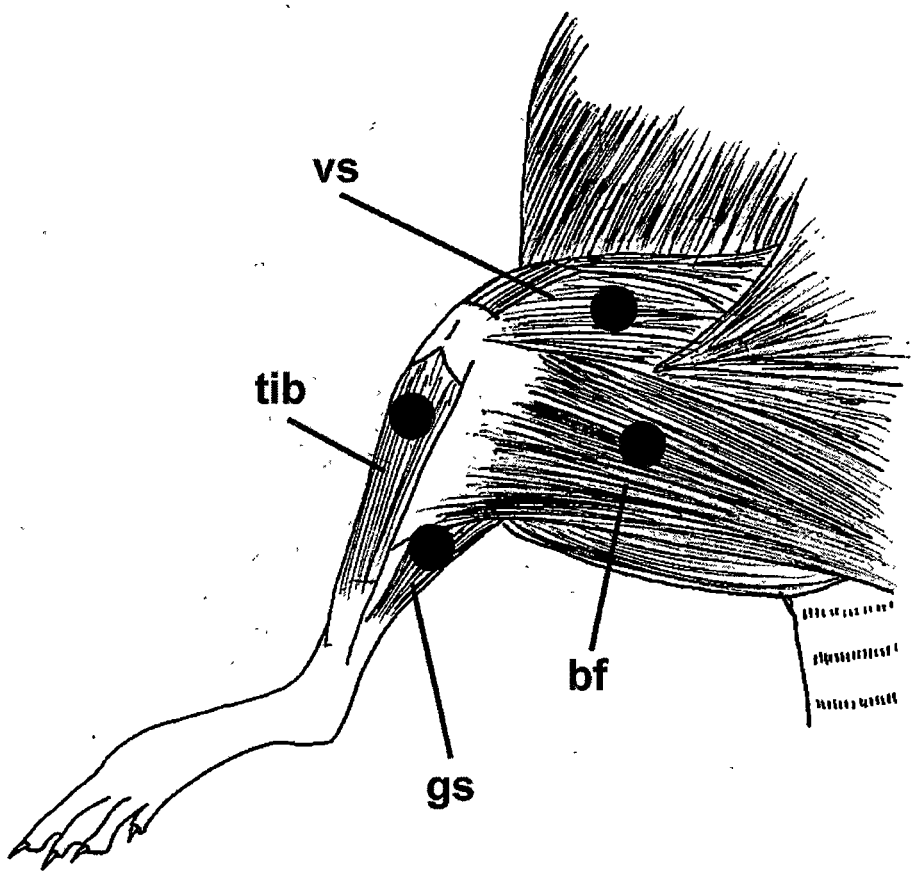


Figure 4-1 General areas chosen for positioning of LDF probe either on or impaled into muscles of the perfused rat hindlimb.

The macro LDF probe (3 x 0.8 mm) was always positioned over the biceps femoris (bf) through a hole in the skin. The micro optic fibre probe (0.26 mm diameter) was inserted through a small incision in the skin in the approximate positions shown on either the tibialis (tib), vastus (vs), biceps femoris (bf), or gastrocnemius (gs) muscles.

4.2.2.2 Within Muscle Measurements

A Moor Instruments Lab Server and Lab Satellite fitted with two P10M master probes was used with two P10s TCG 260 μm slave probes each fitted with TCG fixed

fibres. The operational wavelength of the laser light source was 780 nm. The fibre consisted of a single core of optic fibre (200 μm diameter) surrounded by a protective flexible outer sheath. The fibre was found to be sufficiently robust as to be able to be inserted into the muscle unaided by prior needle puncture. Measurements were made using the PO setting (moorLAB V36 embedded software). This setting scales the raw LD flux output by a factor of 10-fold and was used to improve the quality of the recorded signal. Subsequently, LD values were scaled to express the results in perfusion units (PU) by a factor from measurements made on the manufacturer's calibration fluid for each probe. Small incisions were made into the skin covering the mid-region of any two of the tibialis, vastus, gastrocnemius or biceps femoris (Figure 4-1) of the perfused leg and the two probes inserted. The procedure for insertion of each probe involved initial puncturing of the epimysium and muscle body to a depth of approximately 2 mm with the probe at right angles to the muscle surface, followed by rotation of the probe through 90° to be parallel to the longitudinal direction of the muscle fibres. The probe was then inserted a further 6 mm and taped in place. This procedure avoided wounding and only 4 of the 97 sites were considered corrupted due to bleeding. Indeed, as pointed out by Oberg (Oberg 1990), by using very thin optical fibres (50-200 μm), the trauma can be minimal with little disturbance to blood flow. After completion of the perfusion the final placements were confirmed by surgical examination. In some animals the probes were repositioned in other muscles up to two additional times. This allowed assessment of as many as six different sites per hindlimb. The LDF signal (0-5 volts) was continuously recorded on an IBM compatible PC using a DI-190 I/O module and WINDAQ® software. The data are expressed as perfusion units (PU), to be consistent with the manufacturer's recommendations and to be distinguished from those of the surface probe which differed in size and operating wavelength. Biological zero was not subtracted from any of the data shown.

4.2.3 Vasoconstrictor Infusions

To determine the linearity of LDF response, perfusion flow rate was varied from 4.0 through 6.0 to 8.0 $ml.min^{-1}$ and LDF signal recorded. With flow set at 4 $ml.min^{-1}$, administration of norepinephrine (NE, 0 - 0.3 nmol), angiotensin II (0.3 nmol), vasopressin (0.03 nmol), or serotonin (5-HT, 0 - 3 nmol) was made as a 12.5 or 25 μl

bolus (over 2 sec) into the stirred injection port. In some experiments constant infusions of NE (85 nM) or 5-HT (850 nM) were made by infusing into the injection port a concentrated stock solution of each agent at 1.7% of the perfusate flow rate.

4.2.4 Measurement of Oxygen Uptake

Perfusate entering and leaving the hindlimb was passed through an in-line A-Vox Analyser (A-Vox Systems Inc, San Antonio, Texas) and the signal continuously recorded using WINDAQ software. VO_2 was then calculated using the following equation:

$$VO_2\ (\mu mol.h^{-1}.g^{-1}) = \frac{\text{A-V } O_2\ (\text{ml } O_2/100\text{ml}) \times \text{flow rate (ml/min)} \times 321.43}{\text{rat weight (g)}}$$

4.2.5 Microscopy

In some experiments, the skinned leg of an anaesthetised rat was positioned on the stage of an inverted microscope (Nikon Diaphot) so that the vessels under the probe could be confirmed as muscle capillary network, devoid of any major vessels.

4.2.6 Statistical Analysis

Data were analysed using Sigma Stat™ (Jandel Scientific). For comparison of basal signal strength for nutritive, non-nutritive, and mixed sites, unpaired analysis (Student's t-test) was used. For effects of vasoconstrictors and flow, paired analysis (Student's t-test) was used.

4.3 Results

4.3.1 Surface Measurements

Since the macro laser Doppler flow probe was designed primarily for measurement of human skin blood flow, it was necessary to establish the suitability of the method for perfused rat hindlimb studies. Others have shown that capillary red cell velocity in resting muscle does not exceed 1 $mm.s^{-1}$ for a blood flow of 0.05 $ml.min^{-1}.g^{-1}$ (Tyml

1987), thus at a flow rate of 0.27 $ml.min^{-1}.g^{-1}$ as used in the present perfusions, cell velocities should be less than the critical limit of 8 $mm.s^{-1}$. Secondly, the flux signal remained constant when the probe to tissue distance was varied from 0 to 1.5 mm and thus slight movements of the perfused hindlimb in relation to the probe would not be expected to affect the signal. Thirdly, positioning of the probe was important and it was placed in the same location in the centre of the anterior end of the biceps femoris in each experiment. Without exception this site always behaved the same, displaying increased signal response to NE and decreased signal response to 5-HT injection. Other sites responded differently. Thus, from a total of 28 sites involving 11 in the centre of the biceps femoris (all responding as above), 8 on the tibialis anterior and 9 on the tibialis tendon of the biceps femoris, 20 responded as above, 3 responded in an opposite manner (NE inhibitory and 5-HT stimulatory) and 5 appeared as intermediate with no response to either NE or 5-HT. Fourthly, the LDF signal was linear for flow rates between 1 and 10 $ml.min^{-1}$ ($r = 0.833$; $P < 0.001$, $n = 59$). At flow rates above this, the critical red cell velocity of 8 $mm.s^{-1}$ is likely to have been exceeded.

A time course from a typical experiment is shown in Figure 4-2 where the LDF signal of the surface of the biceps femoris muscle vessels was measured as well as perfusion pressure and oxygen uptake (VO_2) for the entire hindlimb during successive injections (25 µl) of 300 pmol NE and 3 nmol 5-HT. NE (300 pmol), equivalent to a peak concentration of 15-50 nM (estimated from injection and perfusion flow rates), increased LDF signal in association with increased perfusion pressure and a stimulation of oxygen uptake. Values for LDF and oxygen uptake returned to basal approximately 3 min following NE injection. 5-HT (3 nmol), equivalent to a peak concentration of 150-500 nM, decreased LDF signal in association with increased perfusion pressure and an inhibition of hindlimb oxygen uptake.

Figure 4-3 shows a positive correlation ($r = 0.909$ and $P < 0.001$) for change in oxygen uptake as a function of change in LDF signal from a number of experiments where the dose of injected norepinephrine ranged from 50 to 300 pmol, and of serotonin from 1 to 3 nmol.

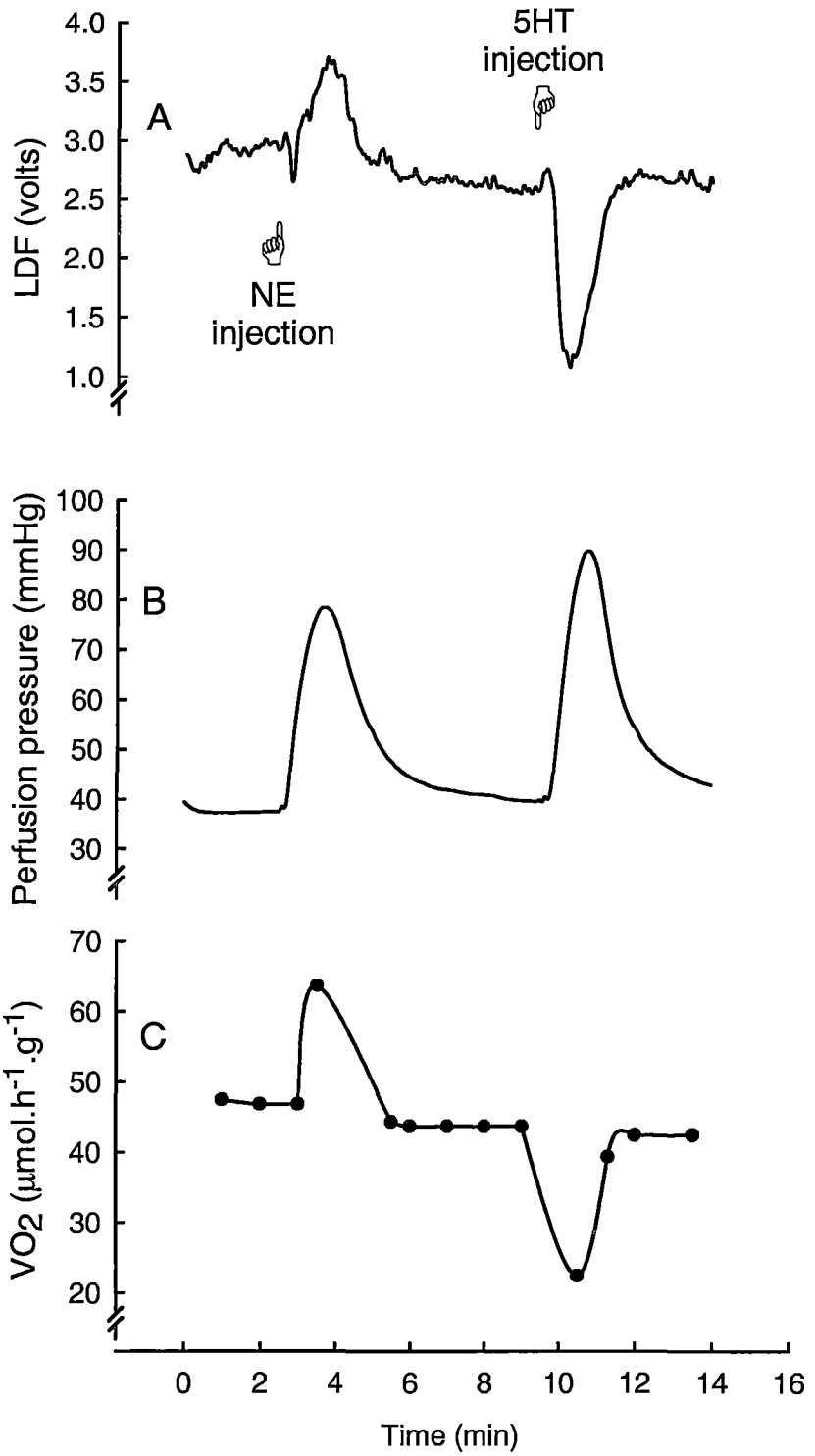


Figure 4-2 Typical trace recorded using macro surface probe.

Figure shows LDF signal from muscle capillaries at the centre of the anterior end of the biceps femoris (A), afterial perfusion pressure (B) and oxygen uptake (C) following injection of 300 pmol NE and 3 nmol 5-HT into the constant-flow perfused rat hindlimb. Biological zero has not been subtracted.

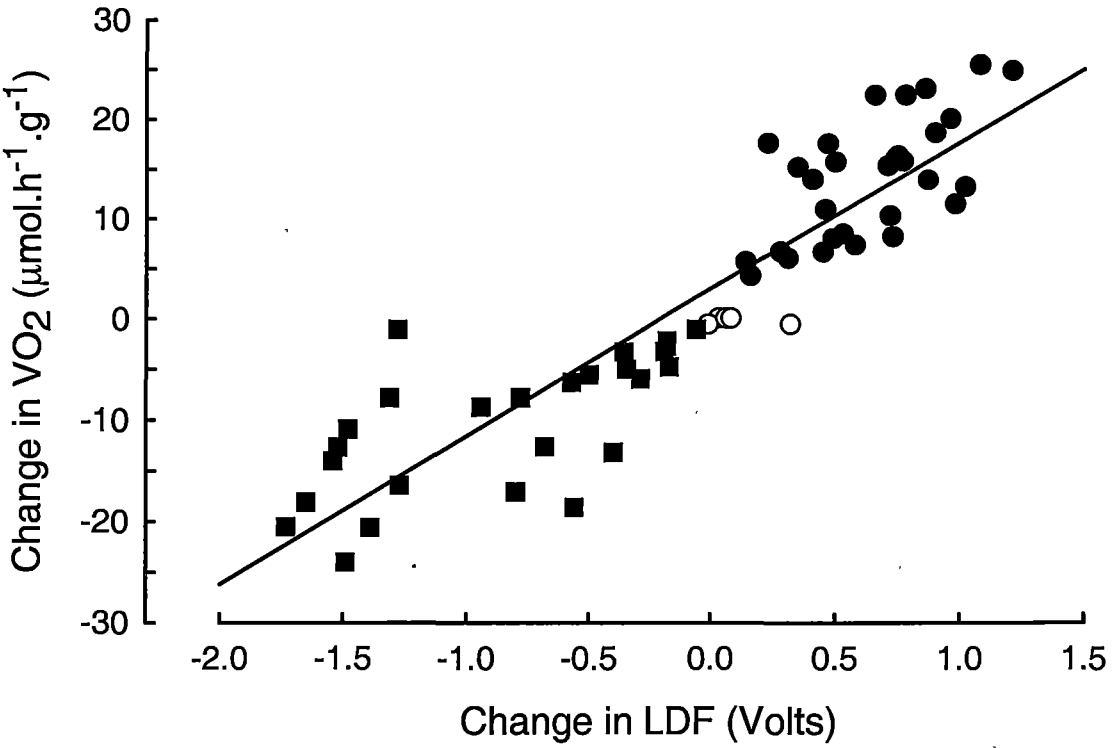


Figure 4-3 Relationship between peak change in oxygen uptake and LDF signal from the surface macro probe for the constant-flow perfused rat hindlimb.

Symbols: (●) NE, (○) vehicle, (■) 5-HT.

4.3.2 Implantable Probes

The macro probe (total diameter including housing was 6 mm) could not be impaled into the muscle without serious tissue disruption and as indicated above, provided the probe was positioned over muscle, only NE-positive sites were observed. Thus to investigate signal changes at higher spatial resolution micro implanable probes were used. Figure 4-4 shows the properties of LDF signal from randomly positioned micro probes placed in either the tibialis, vastus, biceps femoris, or gastrocnemius muscles. The majority of sites responded positively to norepinephrine (Figure 4-4A) with an

increase in LDF signal of greater than 5 % of basal, but some clearly responded negatively with a decrease in LDF signal (decreasing by more than 5% of basal). In addition, there was a group that did not respond (i.e. less than ± 5% of basal signal). Figure 4-4B shows the responses of these sites to serotonin. Again, three types of response were discernable with the majority responding negatively to serotonin and two other groups where the response was positive (signal increasing) or showing no change. Invariably the sites that responded positively to norepinephrine were those that responded negatively to serotonin. These sites have been designated as "NE-positive". The other two types of site are designated "NE-negative" or "mixed" depending upon whether the LDF signal decreased in response to NE and increased in response to 5-HT, or showed less than 5% change to either vasoconstrictor. It is important to note that although norepinephrine and serotonin each cause a pressure rise (vasoconstriction), they have opposite effects on metabolism, reflected by a stimulation or inhibition of oxygen uptake, respectively.

Characteristic traces of NE-positive, NE-negative sites and mixed are shown in Figure 4-5. NE positive sites showed LDF signal changes in parallel to oxygen uptake, i.e. increases and decreases with NE and 5-HT, respectively. NE-negative showed LDF signal changes opposite to oxygen uptake. For the total of 97 sites examined 56.7% were found to be NE-positive, 16.5% NE-negative and 24.7% mixed. Only 2.1% were corrupted due to bleeding. Table 4-1 also shows that basal LDF signal strength was greater at NE-negative sites than either NE-positive or mixed sites and all three sites responded significantly to a doubling of flow rate from 4 to 8 ml.min-1. Although not shown by the data of Table 4-1, a break down of the data revealed there were differences between the muscle types with basal signal from NE-positive sites in tibialis (24.7 ± 2.2; n = 28) similar to gastrocnemius (21.7 ± 4.5; n = 12) but greater than either biceps femoris (12.9 ± 2.3, $P < 0.01$) or vastus (12.4 ± 2.8 PU; n = 9, $P < 0.02$).

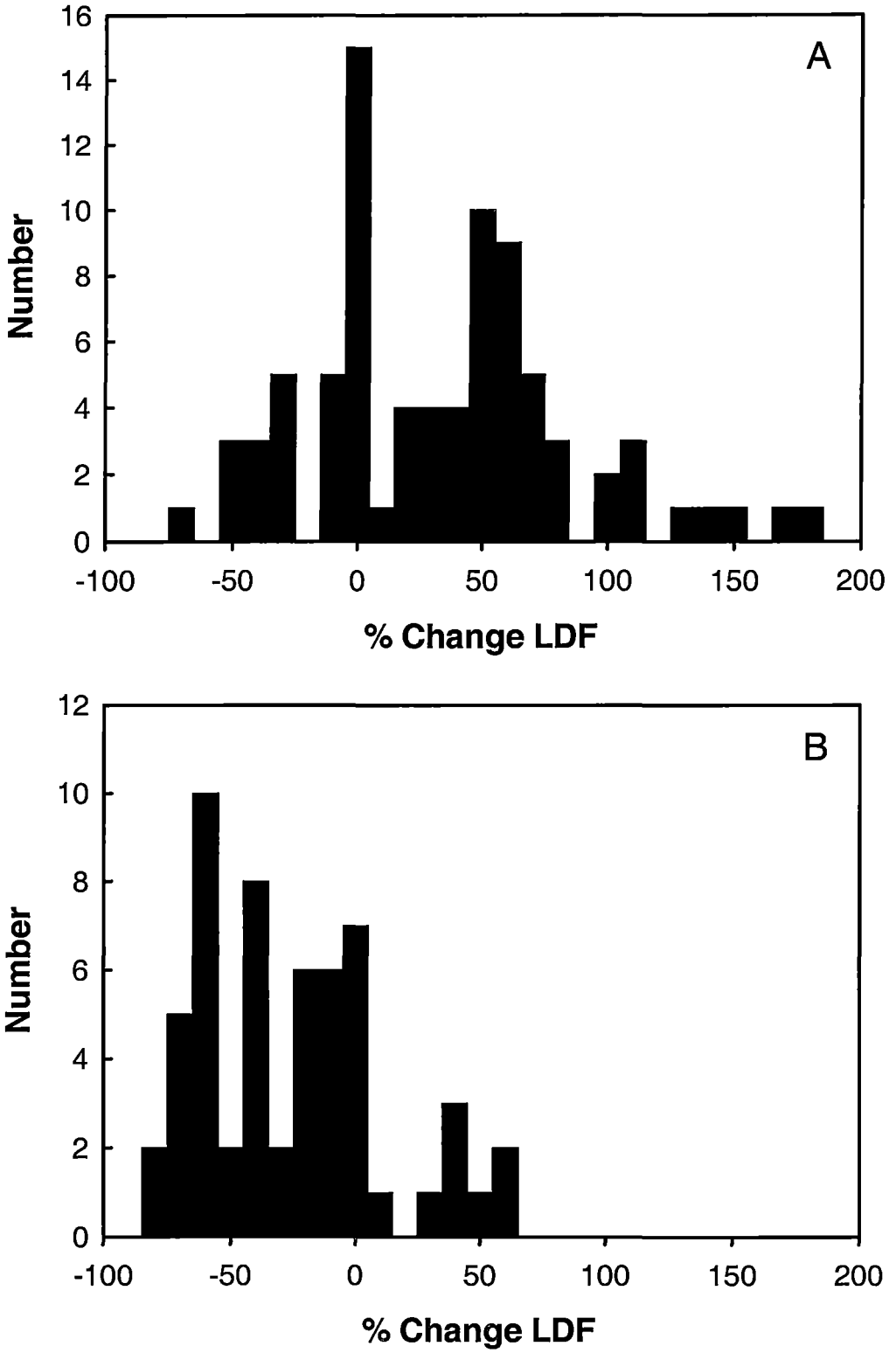


Figure 4-4 Distribution of LDF signal changes from implanted micro probes. Values obtained after NE (A) or 5-HT (B) were expressed as a percentage of the basal signal and grouped in intervals of 10% starting from + 5% and - 5%.

Table 4-1 Properties of LDF signal from randomly positioned muscle micro probes

Site identity	Fraction of Total, %	Basal Signal at 4ml/min, PU	Range, PU	Signal at 8 ml/min, PU	n
NE-positive	56.7	20.7 ± 1.8	3.4 - 61.9	35.8 ± 3.4*	47
NE-negative	16.5	33.2 ± 5.4#	10.4 - 87.4	53.7 ± 10.5*	16
Mixed	24.7	22.3 ± 2.7	5.0 - 60.1	35.7 ± 6.1*	22
Corrupted	2.1				

Sites were classified as NE-positive, NE-negative or mixed depending on responses to the vasoconstrictors NE and 5-HT as shown in Figure. 4-4. Those deemed as "corrupted" resulted from bleeding at the point of probe insertion. A total of 97 sites were assessed from 35 hindlimb perfusions involving sites on each of the tibialis, vastus, biceps femoris, and gastrocnemius muscles. Response to flow was assessed when flow was increased from 4 to 8 $ml.min^{-1}$. Otherwise, flow was maintained constant at $4ml.min^{-1}$. Values are expressed in perfusion units and are means ± SE; *, $P< 0.05$ relative to basal. #, $P< 0.05$ relative to 'NE-positive'. Biological zero has not been subtracted.

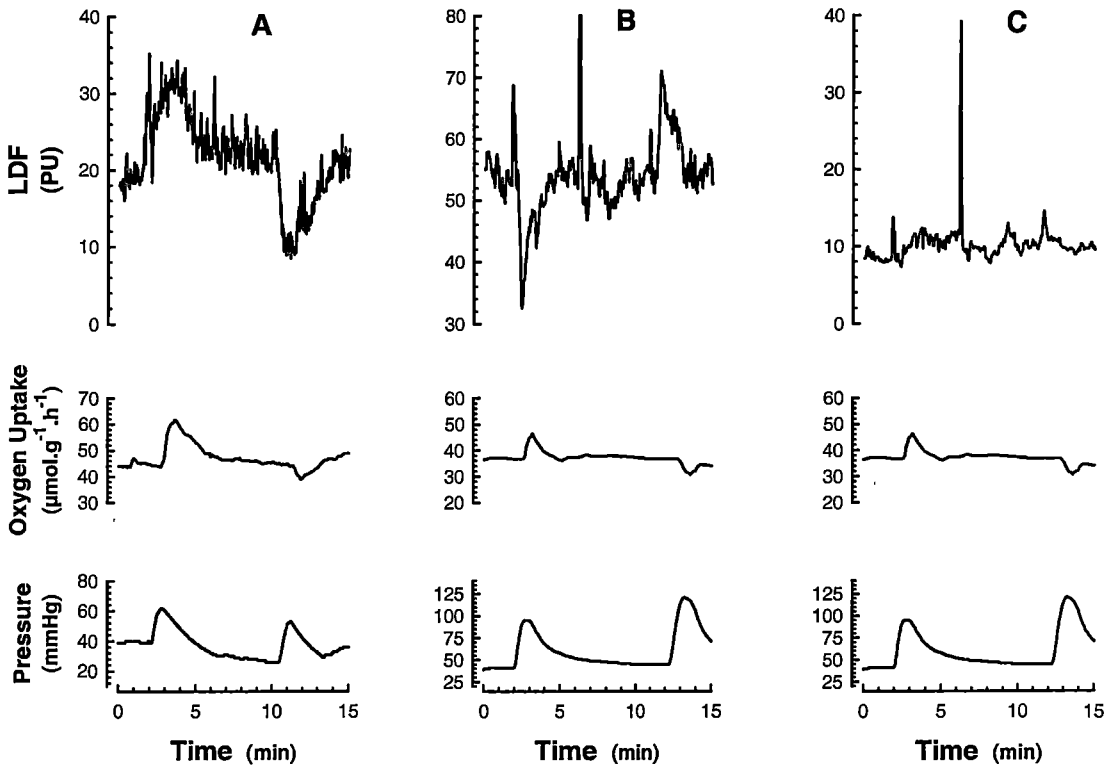


Figure 4-5 LDF tracings for NE-positive (A), NE-negative (B) and mixed (C) sites from impaled micro probes.

Bolus injections of 3 nmol of NE or 5-HT were made as shown during perfusion of the rat hindlimb at constant total flow and sites classified as described in the text. Perfusion pressure as well as arterio-venous oxygen difference for the whole hindlimb were also recorded. Tracings are representative of the groups whose 'n' values are given in Table 4-1.

Table 4-2 summarises the response of NE-positive sites to flow and vasoconstrictors, including 5-HT, NE, vasopressin and angiotensin II. Whole body oxygen uptake as well as perfusion pressure are also shown. Increasing the pump flow rate progressively from 4, through 6 to 8 $ml.min^{-1}$ increased the LDF signal from 14.2 ± 1.0 to 22.8 ± 1.4 PU. Allowing for a biological zero of approximately 7.4, the increase due to flow represents a 2-fold increase. Whole hindlimb oxygen uptake and

perfusion pressure increased by 29.4% and 65.3%, respectively. Three of the vasoconstrictors, NE, vasopressin, and angiotensin II each increased LDF signal parallel to their effects on oxygen uptake. 5-HT inhibited both and in many cases the signal strength due to 5-HT was indistinguishable from biological zero for that site.

Table 4-2 Characteristics of the NE-positive sites.

Flow, ml/min	Additions	Peak LDF, PU	Peak VO2 µmol/h/g	Peak pressure, mmHg
4	none	14.2 ± 1.0 (8)	43.5 ± 1.4 (8)	32.6 ± 1.8 (9)
6	none	18.8 ± 1.2 (8)*	51.6 ± 1.9 (8)*	43.7 ± 1.7 (9)*
8	none	22.8 ± 1.4 (8)*	54.6 ± 2.4 (8)*	53.9 ± 1.9 (9)*
4	none	15.2 ± 1.2 (9)	44.1 ± 1.3 (9)	32.6 ± 2.0 (9)
4	5-HT	6.5 ± 0.7 (9)*	31.8 ± 1.7 (9)*	104.8 ± 15.5 (9)*
4	none	15.3 ± 1.0 (9)	44.1 ± 1.5 (9)	33.7 ± 1.8 (9)
4	NE	31.0 ± 5.3 (9)*	56.4 ± 2.6 (9)*	59 ± 5.1 (9)*
4	none	15.5 ± 0.8 (8)	43.5 ± 1.6 (9)	35.3 ± 2.2 (8)
4	AVP	40.3 ± 10.3 (8)*	53.7 ± 2.9 (9)*	69.9 ± 5.3 (8)*
4	none	13.9 ± 1.0 (9)	43.4 ± 2.0 (7)	34.2 ± 2.3 (8)
4	AII	35.5 ± 4.0 (9)	51.8 ± 2.3 (7)*	47.9 ± 7.2 (8)*

Sites identified as NE-positive in initial assessment were further assessed in response to flow change and to bolus injections of serotonin (5-HT, 3 nmol), norepinephrine (NE, 3 nmol), arginine vasopressin (AVP, 30 pmol) or angiotensin II (AII, 300 pmol). Values are mean ± SE; *, $P < 0.05$ relative to 4ml.min^{-1} "none". LDF values have not been corrected for biological zero.

4.3.3 Sustained Infusions

Figures 4-6 and 4-7 show the effects of sustained infusions of NE or 5-HT on LDF signal from NE-positive and NE-negative sites. Traces for oxygen uptake and perfusion pressure are also shown. As with bolus injections, the changes in LDF signal at NE-positive sites closely paralleled changes in oxygen uptake. Thus, for NE there was an increase LDF signal soon after the rise in pressure and accompanying the rise in oxygen uptake. The initial transient increase in LDF signal at NE-positive sites was not always present but was always followed by a plateau until the NE was withdrawn and then all three, pressure, oxygen uptake and LDF signal reversed to return to baseline values within 5 min. The LDF signal at the NE-negative sites followed much the same pattern but in the opposite direction.

4.3.4 Biological Zero

LDF signal was recorded after the pump had been stopped and venous flow had ceased completely. This occurred within 5 min and was conducted wherever possible for each of the two probes at the end of the perfusion. The mean value ± SE for 38 sites was 7.64 ± 0.66 (range 3.92 - 17.87) PU. When expressed as a percentage of the basal signal this represented 40.6 ± 2.8%, indicating that the biological zero in these experiments more closely represented a constant fraction of the basal signal than a constant absolute value. As argued recently, residual movement of the arrested blood cells constitutes a major component of the biological zero along with movements of other aggregates in the tissue matrix (Leahy *et al.* 1999). For these reasons biological zero has not been subtracted from any value obtained.

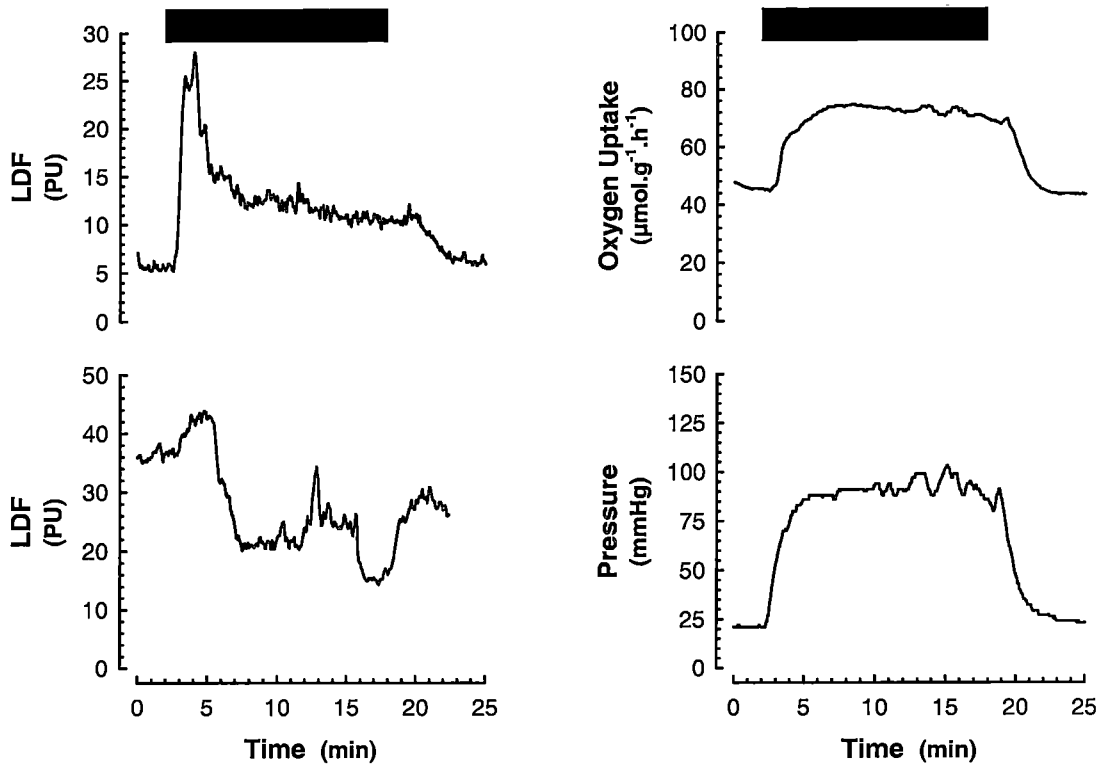


Figure 4-6 LDF tracing from NE-positive (top, left) and NE-negative (bottom, left) sites using micro impaled probes following the constant infusion of 83 nM norepinephrine (filled bar).

Hindlimbs were perfused at constant total flow and an infusion of a stock solution of 5 µM NE introduced for the period shown. Representative tracings for perfusion pressure, as well as oxygen uptake for the whole hindlimb are also shown.

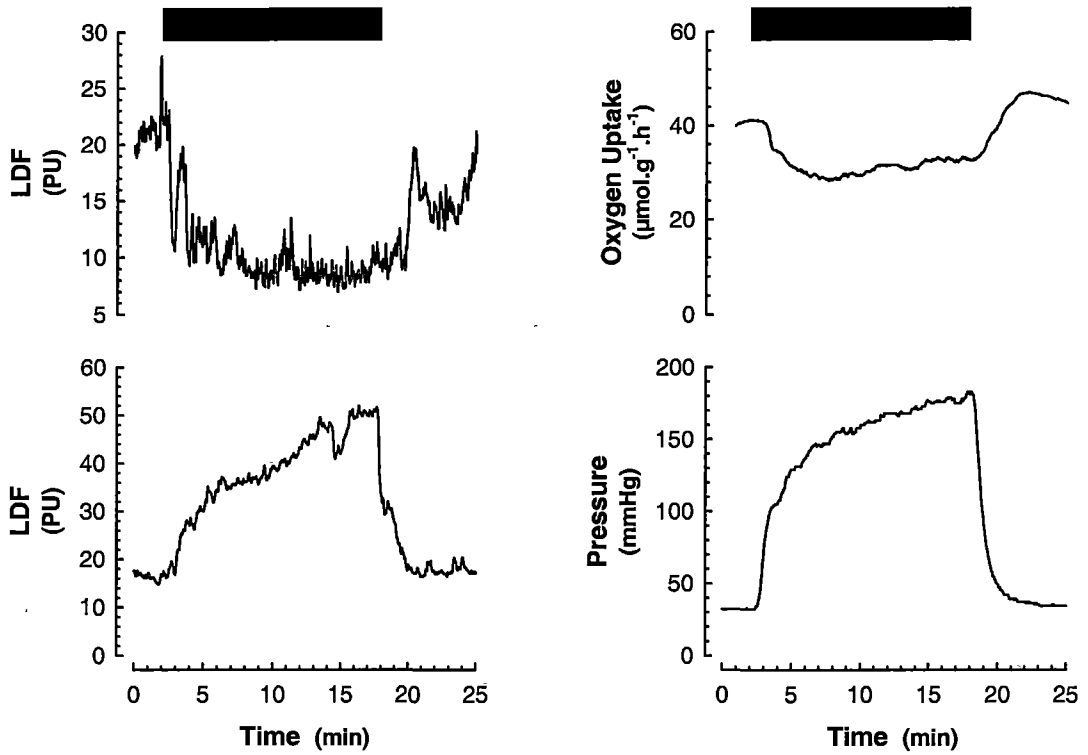


Figure 4-7 LDF tracing from NE-positive (top, left) and NE-negative (bottom, left) sites using micro implanted probes following the constant infusion of 750 nM serotonin (filled bar).

Hindlimbs were perfused at constant total flow and an infusion of a stock solution of 50 µM 5-HT introduced for the period shown. Representative tracings for perfusion pressure, as well as oxygen uptake for the whole hindlimb are shown.

4.4 Discussion

The main finding emerging from this section work was the heterogeneity of sites identified from micro LDF probes placed at random in various muscles in the perfused rat hindlimb. This heterogeneity was not detected with the much larger surface probe unless the probe was moved to regions where tendon vessels were apparent. Using the micro probes, three characteristic types of site were seen when the hindlimb was injected with bolus amounts of the vasoconstrictors NE or 5-HT.

Some sites responded with increased LDF signal from NE and decreased LDF signal from 5-HT and were designated as 'NE-positive'. Those responding with decreased LDF signal from NE and increased LDF signal from 5-HT were designated as 'NE-negative'. Other sites failing to respond to either NE or 5-HT were termed 'mixed'.

Since the vasoconstrictors, NE and 5-HT have been previously described as increasing or decreasing muscle nutritive flow based on their respective effects to stimulate or decrease muscle metabolism (Clark *et al.* 1995), it is proposed that the NE-positive sites are located in the nutritive vascular route. Similarly, NE and 5-HT have previously been shown to decrease or increase, respectively, putative non-nutritive flow in tibial tendon vessels of the biceps femoris (Newman *et al.* 1997), the NE-negative sites reflect the positioning of the probe in the non-nutritive vascular route. A 'mixed' site could represent a location where nutritive and non-nutritive sites are both present so that a positive response from one is obscured by a negative response of similar magnitude from the other. Indeed, close inspection of mixed traces (e.g. Figure 4-5C). shows LDF signal fluctuation at the points where NE and 5-HT were injected. It is unlikely that mixed sites were where there was no blood flow as increasing the flow from 4 to 8 $ml.min^{-1}$ significantly increased LDF signal at these sites (Table 4-1) and the basal LDF signal was greater than biological zero.

In addition to their differing character with respect to responses to the vasoconstrictors NE and 5-HT, two other differences were detected. Firstly, the proportion of sites assessed favoured NE-positive over NE-negative by a factor of 56.7% to 16.5%, or approximately 3.5:1. The ratio may be closer to 2.4:1 if it is assumed that mixed sites comprise equal proportions of NE-positive (nutritive) and NE-negative (non-nutritive) components. Secondly, NE-negative sites generally showed a higher basal LDF signal than NE-positive sites (Table 4-1). Together these additional properties suggest that non-nutritive vessels are relatively fewer than nutritive, and are lower resistance with higher capacitance.

Anatomical models of the muscle microvasculature depicted by Lindbom and Arfors and their colleagues (Borgstrom *et al.* 1988) as well as those of Myrhage and Eriksson (Myrhage and Eriksson 1980) provide a possible explanation for the close co-existence of nutritive and non-nutritive sites within the muscle body. Detailed

drawings of the microvasculature of the rabbit tenuissimus muscle (Borgstrom *et al.* 1988) show feed arteries that branch to supply transverse arterioles which in turn cross the muscle body to firstly supply terminal arterioles and capillaries of the muscle and then to end in vessels supplying the connective tissue (N.B. in this depiction the muscle is flat). Thus, two vascular networks operate in parallel. A number of studies using intravital microscopy have shown that relative flow in the two networks can be influenced by various physiologically relevant agents and conditions (Borgstrom *et al.* 1988, Lindbom 1986, Lindbom and Arfors 1985) that redistribute flow consistent with the two regions representing the nutritive and non-nutritive routes of muscle (Clark *et al.* 1998). For the tenuissimus muscle the region containing nutritive capillaries is approximately 3000 μm wide and somewhat less for the non-nutritive (Lindbom and Arfors 1984). Thus a LDF probe of 200 μm diameter could be placed to detect exclusively one or the other route. For muscles other than the tenuissimus that are cylindrical, the same vascular arrangement is observed (Myrhage and Eriksson 1980), but now the transverse arteriole radiates out from the centre of the muscle fibril and the connective tissue vessels are contained in the perimysium. The dimensions are similar but because of the cylindrical nature of the perimysium the probability of positioning a 200 μm probe to measure non-nutritive flow exclusively would be less than for the tenuissimus. In addition, the probability of receiving a mixed signal would be greater. The data presented in this chapter are not inconsistent with this vascular arrangement.

When studying this data, it must be kept in mind that the geometrical arrangement of the vasculature may influence the LDF signal. Work has been done in this laboratory using polymer tubes of 250, 100 and 50 μm internal diameter (Clark *et al.* 2000). By investigating the LDF signal seen with various arrangements of the polymer tubing, the effect of vessel arrangement within muscle can be assessed. Signal strength was greatest from the smallest tube and least from the largest tube. Furthermore, if a single tube (100 μm) was doubled back on itself to cross the field of measurement three times, the LDF signal at any flow rate was approximately 3-fold greater than that for the same tube crossing the field of measurement only once. If a constant amount of flow was switched from flowing through five tubes in the field of measurement to one tube in a manifold of five tubes, there was a progressive increase

in signal. Thus it can be deemed that LDF signal derives predominantly from non-vectorial cell speed and less from cell number. So even when total flow remains constant, LDF signal strength increases as tube diameter decreases, as the flow is switched from a single to a multiple pass tube that crosses the field of measurement more than once, or as the number of tubes being perfused decreases (Clark *et al.* 2000). This data may explain the effects of vasoconstrictors in muscle in that an increase in LDF signal induced by NE may derive from switching of flow from larger to smaller vessels within the field of measurement, rather than switching flow to vessels outside the field of measurement such as those in connective tissue. On balance however, the data would favour the latter of these two possibilities, and certainly rule out the likelihood of NE recruiting capillary flow from vessels already carrying flow within the field of measurement. In addition, the existence of a heterogeneity of sites with opposite responses to NE would support the notion that NE is mediating the movement of flow from one site to the other. 5-HT would be mediating the opposite.

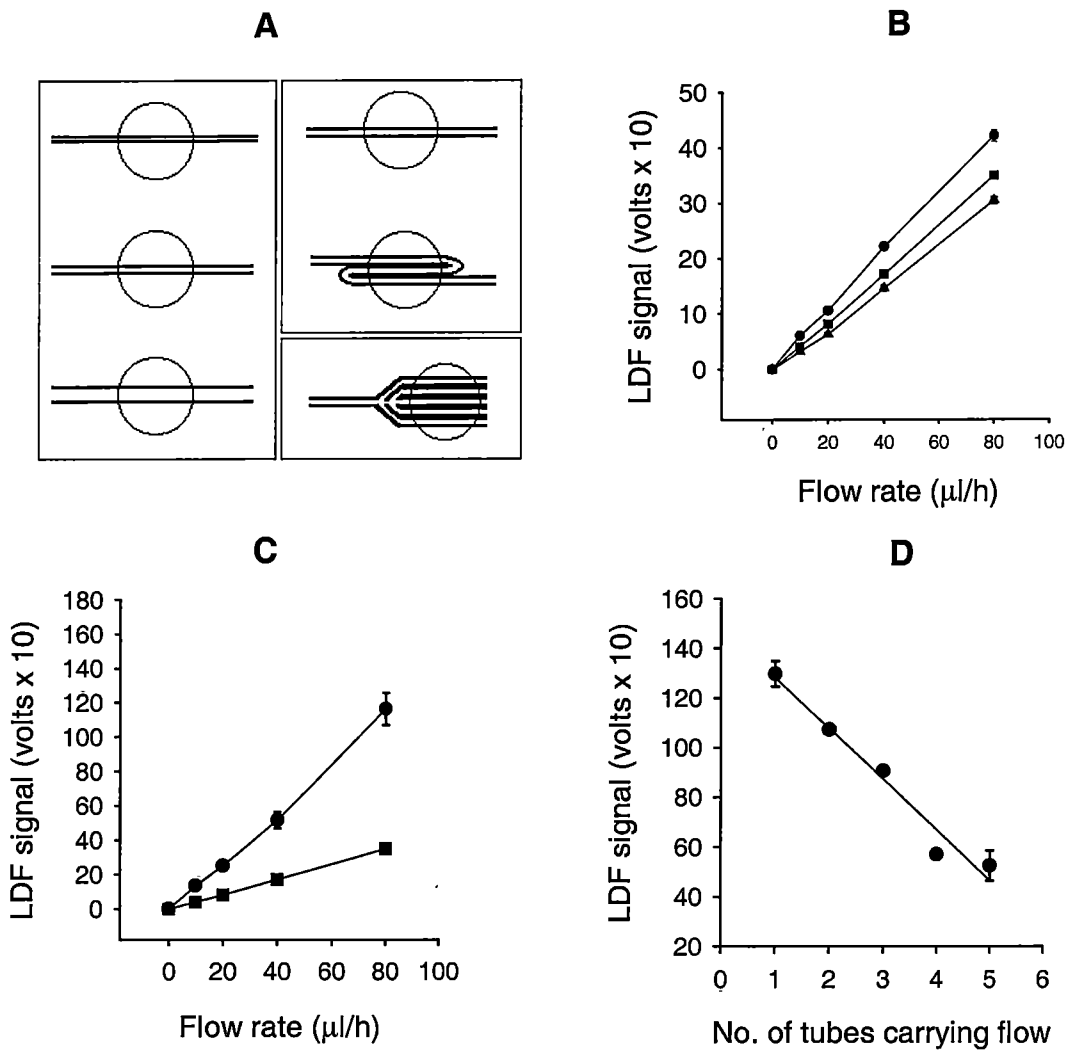


Figure 4-8 Polymer tube studies *in vitro*

A, models used were (*Left hand panel*) three tubes of differing diameter, 50, 100, and 250 µm; (*Upper right hand panel*) 100µm tube in single pass or triple pass array; (*Lower right hand panel*) five tube manifold of 100µm tubes. The circle indicates the position of the LDF probe (not to scale). **B**, Effect of flow rate on LDF signal for three different tube sizes of 50 (●), 100 (■) and 250 µm (▲) as shown in A (*Left hand panel*). The hematocrit was 3%. **C**, Effect of flow rate on LDF signal for single pass (■) or triple pass (●) 100µm tubes as shown in A (*Upper right hand panel*). The hematocrit was 3%. **D**, Effect of changing the number of tubes of a five tube manifold carrying flow on LDF signal. Red cell containing perfusate (9% hematocrit) was delivered at constant-flow (120µl.h^{-1}) to a manifold of five equal 100µm (ID) tubes that could be blocked or opened by fitted taps. LDF signal was recorded

simultaneously for all five tubes using the model as shown in Panel A (*Lower right hand panel*). Taps were opened or closed at random to direct flow to 1,2,3,4, or 5 of the capillaries. Mean± SE values are shown for n = 6 determinations for each of B, C, and D. Error bars, when not visible, are within the symbols. Reproduced from Clark *et al.* (Clark *et al.* 2000).

There are relatively few studies deploying intramuscular micro LDF probes (Oberg 1990) and no one has addressed the issue of heterogeneity of response to vasoconstrictors. There are, however, a number of studies were larger LDF probes have been used on the surface of skinned muscle similar to the one we report herein. The larger surface probe records frequency changes from much larger volumes of tissue, in many cases approaching 1 mm^3 and therefore penetrates considerably below the surface. Of particular interest are muscle blood flow studies where total and regional flow have been manipulated by pharmacological agents. The findings suggest that the surface LDF probes detect predominantly flow we would characterize as nutritive. In one such study (Gustafsson *et al.* 1993), adenosine was infused into anaesthetised rabbits . Mean arterial blood pressure decreased, there was an increase in flow heterogeneity, a decrease in local oxygen consumption (vastus medialis) and a decrease in LDF signal in the same region on the contralateral leg. The authors concluded that adenosine had caused a marked reduction in capillary flow with increased tissue oxygenation. Our studies using the constant-flow perfused rat hindlimb show that vasodilators, such as adenosine, strongly oppose the effect of Type A (cf. Type B) vasoconstrictors such as NE and redirect flow from the nutritive to the non-nutritive route (Clark *et al.* 1994).

In a study by (Kuznetsova *et al.* 1998), muscle surface LDF signal was recorded from the biceps femoris muscle of anaesthetised rats that had been injected with chlorisondamine chloride, an autonomic blocking agent that blocks nicotinic ganglionic transmission, as well as the β_1-blocker, atenolol. Total muscle blood flow was assessed with radioactive microspheres. Infusion of angiotensin II or phenylephrine increased LDF signal without affecting total muscle blood flow. In a separate series of experiments, isoproterenol decreased LDF signal despite a large

increase in total blood flow. These findings are consistent with our observations in the constant-flow perfused rat hindlimb where angiotensin II and phenylephrine are both Type A vasoconstrictors and increase oxygen uptake, increasing nutritive flow. Furthermore, isoproterenol has been found to be a vasodilator which opposes Type A vasoconstriction and redirects flow from the nutritive to the non-nutritive route. Thus oxygen uptake is inhibited (Clark *et al.* 1994).

In skeletal muscle of the rat, LDF signal has been shown to correlate well with other measurements of flow, such as radioactive microspheres or electromagnetic flowmetry, each of which measure volume flow (Smits *et al.* 1986). However, as pointed out by Kuznetsova *et al.* (Kuznetsova *et al.* 1998), the linear correlation may not apply if tissue perfusion, estimated by LDF, changes without any significant change in volume flow to an organ. In fact the principal made by these authors was that certain types of vasoconstrictors, as outlined above, were able to cause a dissociation between volume blood flow and LDF signal. Kuznetsova *et al.* (Kuznetsova *et al.* 1998) were of the view that changes in LDF signal accompanying changes in vascular tone were best explained by changes in red cell velocity. Thus agents such as isoproterenol, that decrease LDF signal in the light of increased total blood flow do so by decreasing red cell velocity, the result of decreased arteriolar-venular pressure gradient.

In our perfused hindlimb system, total flow was maintained constant and we have previously shown, using microspheres, that agents such as 5-HT do not alter total flow between muscles of high and low oxidative capacity, or between muscle and non-muscle tissue (Newman *et al.* 1997). The fact that 5-HT infusion results in a marked decrease in oxygen uptake, a change in flow pattern ((Newman *et al.* 1996) and an increase in flow in connective tissue and tendon vessels (Newman *et al.* 1997), suggests that 5-HT acts to redirect flow from nutritive capillaries to non-nutritive vessels, which may include tendon vessels. Takemiya and Maeda (Takemiya and Maeda 1988) have reported that at rest blood flow in the tendon of tibialis anterior, gastrocnemius, and soleus exceeds that of the same muscles by a factor of approx. 2-fold. In addition, they showed that norepinephrine, or exercise, decreased tendon vessel flow and that exercise-mediated decrease in tendon vessel flow occurred in conjunction with increased muscle flow. Together their findings also imply that

blood flow can be switched from either of the two routes to match demand. However, while the changes in LDF signal at nutritive sites correlate with changes in putative nutritive flow deduced from changes in metabolism, there may be other explanations to account for the change in LDF signal that we have observed in perfused muscle. One possibility concerns the redistribution of flow within each muscle. For example, during exercise blood flow to the high oxidative portion of gastrocnemius muscle is substantially increased and flow to the low oxidative portion of the same muscle is decreased (Laughlin and Armstrong 1982, Laughlin and Armstrong 1983). This serves to illustrate that flow within the same muscle can be differentially controlled by exercise and this phenomenon may extend to vasoconstrictors. Thus, since the cranial portion of the biceps femoris is less oxidative than the caudal portion (as indicated by citrate synthase activity) and is composed of a greater portion of Type IIB fibers (Delp and Duan 1996), it is possible that NE could increase flow to the caudal portion of the biceps femoris, resulting in higher perfusion of oxidative fibers and greater oxygen consumption. Conversely, 5-HT could increase perfusion of the cranial portion of the biceps femoris, resulting in increased flow to low oxidative fibers and correspondingly, diminished oxygen consumption. Alternatively, and as suggested from previous studies (Newman *et al.* 1996), it is possible that flow redistribution between vessels of a different geometry within the same region may have occurred.

The proportion by which vasoconstrictors were able to alter muscle metabolism during constant infusion (oxygen uptake was stimulated by 69.5% by NE and inhibited by 29.5% by 5-HT) was similar to the amount that they changed flow indicated by LDF signal at the NE-positive sites (NE= +51%; 5-HT= –61%). It would therefore seem likely that redistribution of flow between the 'nutritive'(NE-positive) route and nearby connective tissue (such as the perimysium, epimysium or tendon), albeit the 'non-nutritive' (NE-negative) route, could account for the observed changes in metabolism as argued previously (Clark *et al.* 1995, Clark *et al.* 1998).
Finally, LDF probe dimensions may be important in determining the nature of the signal received. The surface LDF probe used in this study had a detector surface area of approx. 1 mm^2 (two optical fibers of 800 μm diameter each) and when placed on the surface detected signal from a volume of approx. $1mm^3$ of tissue. When

positioned on the center of the anterior end of the biceps femoris the signal appeared to be only of one kind, responding positively to NE and negatively to 5-HT. This contrasts with measurements using a much smaller probe (0.03 mm^2) inserted in the muscle, where LDF signals were heterogeneous with some responding as above (i.e. NE-positive), some responding in an opposite manner to the above (i.e. NE-negative), and some failing to respond (mixed). With the smaller probe it was also noted that the NE-positive sites outnumbered the other two by approx. 3:1. Thus it appears likely that the larger probe receives signal from a mixture of sites that convey a character that is predominantly NE-positive. Alternatively, NE-negative sites may not located near the surface of the muscle, although for various reasons alluded to above this is unlikely.

In summary, micro LDF probes when positioned randomly in the body of a number of hindlimb muscles identify sites that differ in their response to vasoconstrictors. This heterogeneity is not visible to larger probes on the muscle surface. Over half of the sites show properties consistent with a nutritive role for muscle metabolism. Non-nutritive sites are present but at a lower proportion and nearly a quarter of the sites are likely to represent a mixture of both nutritive and non-nutritive. Non-nutritive sites show a higher basal signal.

CHAPTER 5

Insulin Stimulates Laser Doppler Flow Signal *In Vivo* Consistent with Nutritive Flow Recruitment

5.1 Introduction

Skeletal muscle accounts for 80-90% of insulin-mediated glucose disposal and an impairment of this appears to be pathogenetically involved in the insulin resistance of obesity, hypertension and type II diabetes. There has been considerable interest lately in reports that insulin has a haemodynamic vasodilatory effect as part of its action to increase glucose uptake by skeletal muscle. This vascular effect of insulin appears to be impaired in obesity and type II diabetes. There is ongoing controversy as to whether or not glucose and insulin delivery, or skeletal muscle blood flow, plays an important role in determining overall rates of insulin-mediated glucose disposal (Baron *et al.* 1991, Yki-Jarvinen and Utriainen 1998).

In addition to insulin's putative vasodilatory action in skeletal muscle, animal studies have shown that insulin also mediates an increase in capillary recruitment, that may be independent of the changes in total blood flow (Rattigan *et al.* 1997b). Moreover, this laboratory has recently shown that when capillary recruitment is prevented *in vivo*, an acute insulin resistant state is induced (Rattigan *et al.* 1999). However, since the method used for assessing capillary recruitment in both of those studies (i.e. 1-MX metabolism) was indirect, laser Doppler flowmetry has been used in the present study to measure capillary perfusion *in vivo*. Chapter 4 explored the use of laser Doppler flowmetry in the perfused rat hindlimb under conditions of constant flow. This knowledge is now applied to measurement of changes in LDF signal during the hyperinsulinaemic euglycaemic clamp *in vivo*. Epinephrine was also included as it is known to increase total blood flow to the leg without increasing nutritive (capillary) flow (Rattigan *et al.* 1997b).

5.2 Methods

5.2.1 Laser Doppler Studies In Vivo

Male Wistar rats weighing approximately 300 g were used for all experiments described in this chapter. The hyperinsulinaemic euglycaemic clamp was used as described in detail in Chapter 2. Insulin (10 $mU.min^{-1}.kg^{-1}$), epinephrine (0.125 $\mu g.min^{-1}.kg^{-1}$) or saline, as well as glucose (to maintain blood glucose at 5 mM) were infused via a jugular cannula.

The rat hindlimb was skinned and covered with thin plastic wrap to avoid drying out. Hindlimb muscle LDF signal was determined using a scanning LDF (Lisca Li PIM 1.0, Laser Doppler Perfusion Imager) at baseline before saline, insulin or epinephrine and at one hour after the commencement of infusion of saline or insulin and 15 min after epinephrine. Positioning of the scanner was made using the knee as a reference. This ensured that the area and orientation were identical between animals. Triplicate scans of the hindlimb were made, each taking 5 min to complete and timed to occur 5 min before, on and 5 min after the times designated above. Each scan was viewed on screen and analysed using the manufacturer's operational software to give average perfusion units (volts) of the area analysed. To assess muscle perfusion a square of 225 mm^2 (15 mm x 15 mm) in the top left hand corner of each scan and covering mostly muscle (biceps femoris) was analysed. Means from triplicate analyses before and after addition of saline (control), insulin, or epinephrine were used for comparison. The scanning probe covered a total area of approximately 900 mm^2 (30mm x 30 mm) of skinned thigh muscles.

A stationary LDF probe (Perimed Periflux PF 4001 with Master Probe 418) was also used and signals were recorded continuously throughout the experiment. The stationary probe was positioned over a 4 mm^2 area of skinned biceps femoris, in an identical position to that used in the perfused hindlimb studies (see Chapter 4).

5.3 Results

5.3.1 Glucose Metabolism During the Insulin Clamps

As seen with similar experiments by this research group (Rattigan *et al.* 1997b, Rattigan *et al.* 1999) there was no significant difference in arterial glucose concentrations between saline and insulin animals either before commencement of infusions or at the end of the experiment (data not shown). Insulin increased glucose infusion rate from zero to a maximum of 130 ± 7 $\mu mol.min^{-1}.kg^{-1}$ (23.42 $mg.min^{-1}.kg^{-1}$) (n = 11; 5 scanning and 6 stationary LDF probe) at the end of the clamp (2 hours). The time course was similar to previously (Rattigan *et al.* 1997b), so that following 1 hour of insulin, glucose infusion had already reached the maximum.

5.3.2 Clamp Experiments for LDF Signal by Scanning Probe

Figure 5-1 shows that insulin tended to increase femoral blood flow (29 ± 6%) but at 1 hour after commencement this was not yet a significant change (as opposed to the 2 hour insulin-mediated increase in femoral blood flow seen in Rattigan *et al.* (Rattigan *et al.* 1997b). However, at this same time point insulin had markedly increased the scanning LDF signal over the biceps femoris (top left hand square within the field of measurement (see Figure 5-2) by 62 ± 8% ($P < 0.05$; n = 5). Saline infusion controls showed no increase in either femoral blood flow or scanning LDF signal over the biceps femoris at 1 hour ($P > 0.05$; n = 4) (Figure 5-1).

Epinephrine is a faster acting agent than insulin and as noted previously (Rattigan *et al.* 1997b) increases femoral blood flow. In the present studies epinephrine (0.125 $\mu g.min^{-1}.kg^{-1}$) increased femoral blood flow by 37 ± 5% ($P < 0.05$; n = 3) at 15 min. Despite this effect, scanning LDF signal was unchanged (4 ± 8%; $P > 0.05$) (Figure 5-1). The rise in LDF signal was greater with insulin than either epinephrine or saline ($P < 0.05$; ANOVA).

Representative scans for a saline control and an insulin clamp at 1 hour are shown in Figure 5-2. Settings on the scanner were high resolution, threshold voltage 5.30 and 64 x 64 pixels. Since the average area scanned was 900 mm^2 each pixel represented approximately 0.2 mm^2. Analysis of the total scanned area and of the connective tissue region around the knee failed to reveal an effect of insulin on those scans where

a significant increase in muscle signal due to insulin had occurred. Thus values before and after insulin were 1.13 ± 0.25 and 1.26 ± 0.22 volts ($P > 0.05$; total area) and 2.51 ± 0.35 and 2.69 ± 0.37 volts ($P > 0.05$; connective tissue).

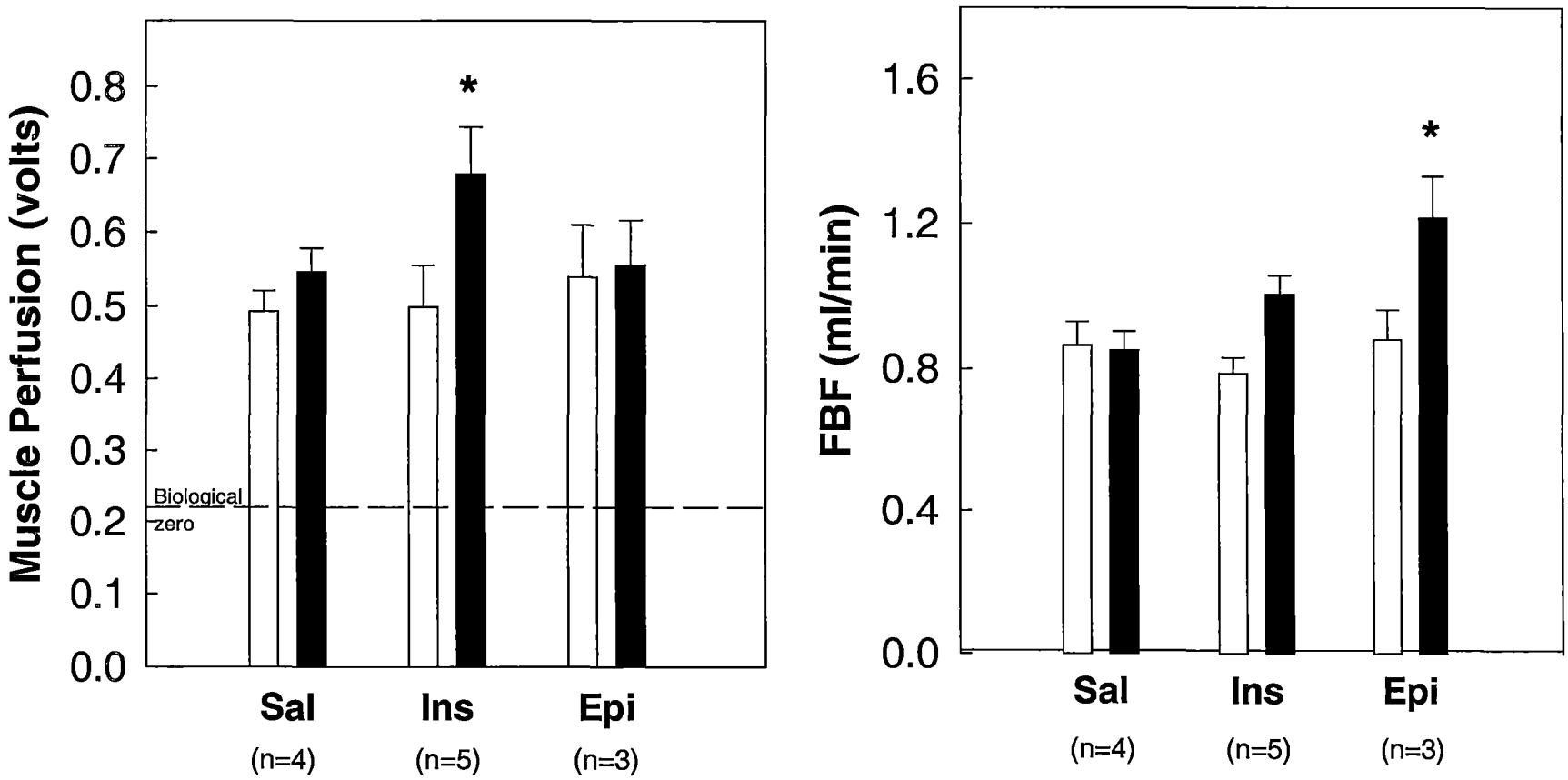
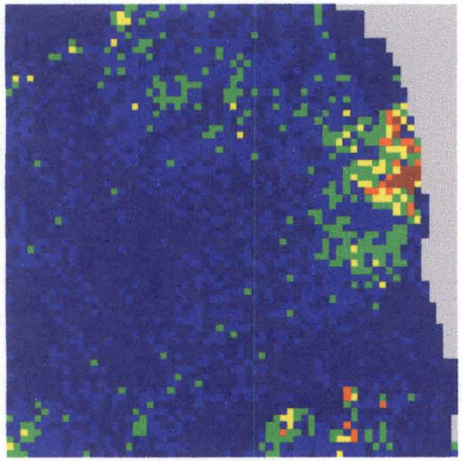
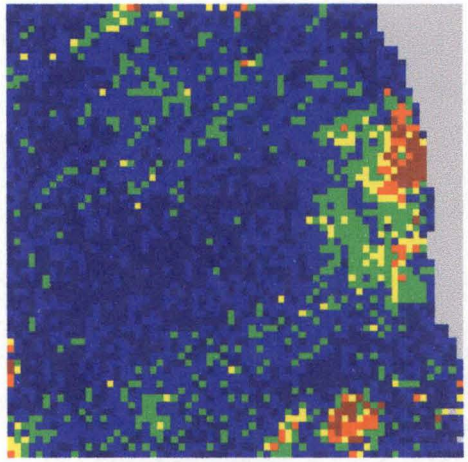


Figure 5-1 Total femoral blood flow and scanning LDF measurement of lateral surface of thigh muscles of anaesthetised rats following insulin/glucose, epinephrine or saline infusions.

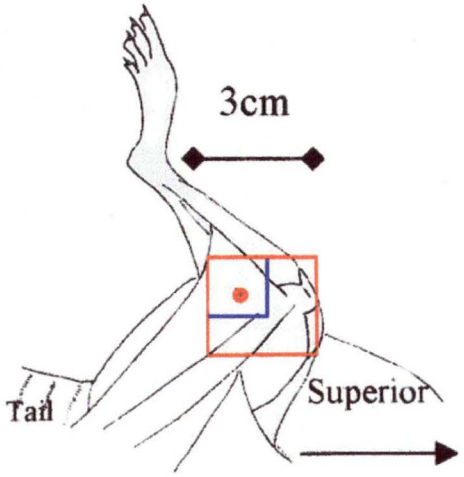
Reproduced from Clark *et al.* (Clark *et al.* 2000).



A



B



C

Figure 5-2 (previous page) Representative scans of LDF before (A) and after (B) insulin in the same animal.

The area analysed by the scanning LDF is demonstrated by the red square in (C). The position of the stationary probe is shown as: (●), in (C). Colour coding to indicate relative perfusion is as follows:

(■) > 1.59; (■), 1.59; (■), 1.27; (■), 0.95; (■), 0.64; (■) < 0.32 volts.

Reproduced from Clark *et al.* (Clark *et al.* 2000).

5.3.3 Stationary LDF Probe

The stationary probe was positioned over the centre of the anterior end of the biceps femoris muscle as in the perfused hindlimb studies in the previous chapter (Chapter 4). The signal was continuously recorded and mean values are shown in Figure 5-3 for the 0 - 80 min period following commencement of insulin infusion. For the stationary LDF probe experiments, insulin increased femoral blood flow (66%, $n = 6$; $P < 0.05$) and LDF signal 47 ± 12% ($P < 0.05$) relative to saline controls ($n = 5$) which did not affect FBF. Epinephrine also increases femoral blood flow (39%) but LDF signal did not change significantly (-0.3%; Figure 5-3). Figure 5-3 shows the time courses for insulin-mediated changes in femoral blood flow (upper panel) and LDF signal (lower panel). The change in LDF signal was significant at 20 min and preceded the increase in femoral blood flow that occurred at 60 min.

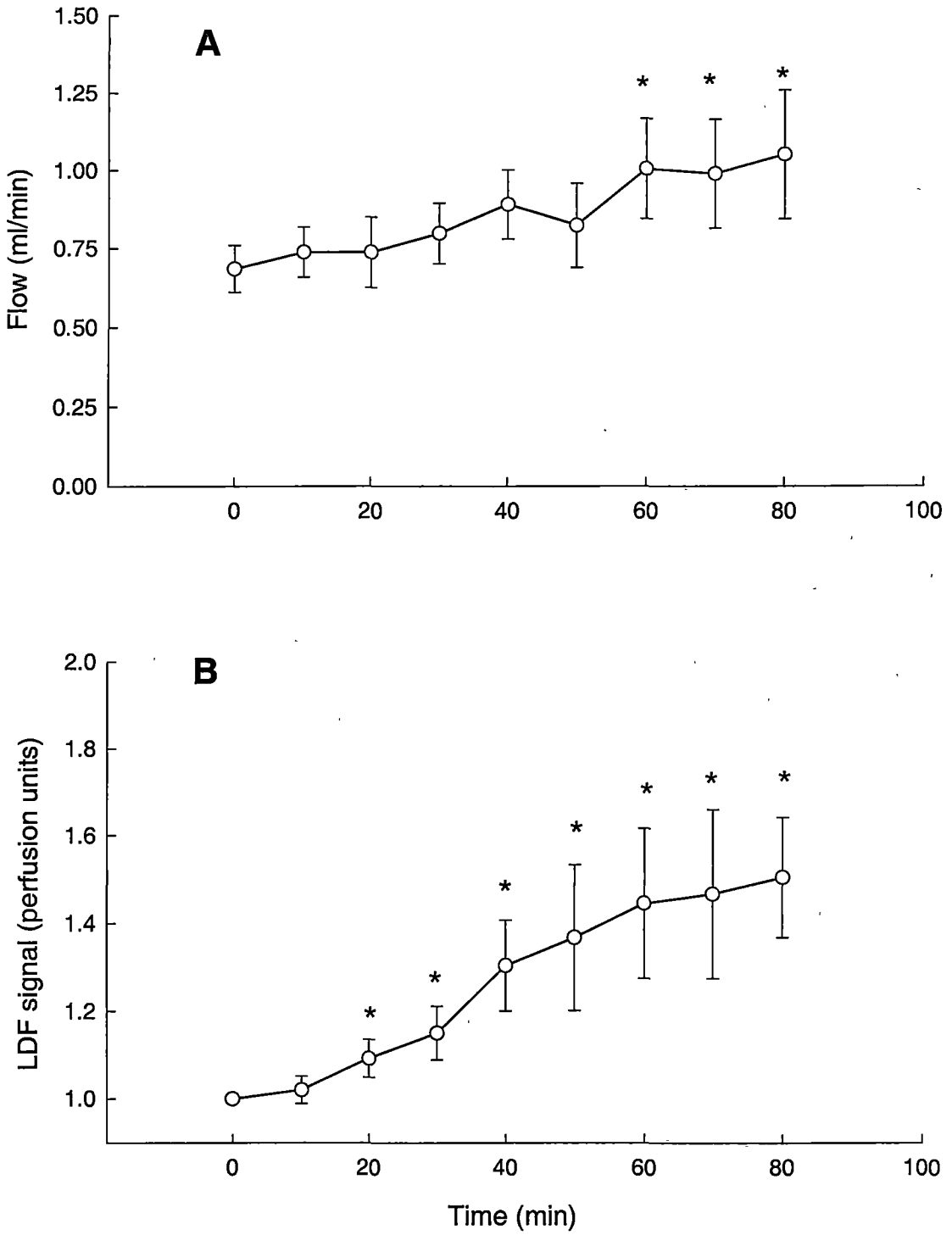


Figure 5-3 Time course for changes in femoral blood flow (A) and for stationary probe LDF signal (B) during a hyperinsulinaemic euglycaemic clamp.

Reproduced from Clark *et al.* (Clark *et al.* 2000).

5.4 Discussion

The main finding emerging from this study was the increase in muscle surface LDF signal mediated by insulin. This would appear to reflect an increase in nutritive flow in muscle consistent with the previously reported increase in capillary recruitment determined by increased metabolism of the marker substrate 1-methylxanthine (Rattigan *et al.* 1997b). The notion that insulin has led to an increase in nutritive flow, or muscle capillary flow, is based on the strong correlation observed between LDF signal from similarly placed probes and oxygen uptake as altered by two different vasconstrictors, NE and 5-HT in the constant flow perfused rat hindlimb as discussed in Chapter 4.

The increase in nutritive flow by insulin is independent of changes in total leg blood flow as detected by the flow probe positioned around the femoral artery. Thus, epinephrine which increased total flow had no effect on the LDF signal. In addition, time course studies using the stationary probe indicated that the LDF signal had increased at least 30 min before there was a significant increase in femoral flow. An increase in nutritive flow due to insulin without an increase in total flow implies that flow has been redistributed from another route that may not be visible to the LDF probe. This may be because of the position or size of the probe. Comparison with the perfused rat hindlimb work in Chapter 4 is helpful in considering these issues. Although almost fully dilated, the isolated perfused hindlimb preparation responds to vasoconstrictors by either increasing or decreasing metabolism. Vasoconstrictors that increase metabolism in the constant flow preparation do so by redirecting flow from a non-nutritive route located in the closely associated connective tissue of the perimysium and related sheaths. Some of the vessels are visible and relatively free from a background of muscle nutritive capillaries. One study has shown that the vasoconstrictor, NE, that increased overall hindlimb metabolism, redirected flow from these vessels to the muscle nutritive route (Newman *et al.* 1997). Accordingly, it would seem likely from the present study that insulin has acted similarly to redirect flow from the non-nutritive route to the nutritive route. Since insulin does not affect blood pressure within this time frame (Rattigan *et al.* 1997b), recruitment of nutritive flow at the expense of non-nutritive flow would appear to involve a combination of

vasodilatory and vasoconstrictor activity. There are reports of both of these activities of insulin in association with glucose *in vivo* (Renaudin *et al.* 1998).

The present findings in the rat *in vivo* show that insulin increased femoral blood flow and increased LDF signal and epinephrine, despite similar changes in femoral blood flow, did not increase LDF signal. These results are similar to our previously reported increase in capillary recruitment as measured by 1-MX metabolism *in vivo* in the presence of insulin (Rattigan *et al.* 1997b). In that study, 1-MX metabolism was increased as a result of insulin action to increase capillary recruitment or nutritive flow. A large part of this conclusion rested on prior knowledge that 1-MX metabolism in the perfused rat hindlimb closely parallels changes in nutritive flow (see Chapter 3) (Rattigan *et al.* 1997a, Youd *et al.* 1999). Similarly Chapter 4 clearly shows that LDF signal changes in parallel to changes in nutritive flow. Thus, an increase in muscle LDF signal due to insulin and independent of changes in total flow support the contention that insulin increases capillary recruitment in human skeletal muscle (Baron *et al.* 1993a) and the observation that insulin increases blood volume in human muscle (Raitakari *et al.* 1995). In conclusion, insulin acts *in vivo* to stimulate LDF signal consistent with capillary recruitment as part of its effect to increase muscle glucose uptake. The recruitment may be independent of changes in total blood flow as epinephrine, which also increased femoral blood flow, did not increase LDF signal. The present findings support previous observations by our group of increases in capillary recruitment in muscle as measured by 1-MX metabolism.

This work has implications for clinical diagnostics. Use of LDF probes will be developed for measurement of muscle blood flow in humans. It will be necessary to use implantable intramuscular probes (as described in Chapter 4). Unfortunately, current implantable probes are not suited to this application, since these probes are not strong enough in intensity to sample from a large enough area within the skeletal muscle test area to obtain a true indication of the muscle perfusion. Such applications will have to wait upon the design of more suitable implantable probes.

CHAPTER 6

Sciatic Nerve Severance-Induced Insulin Resistance in Muscle Without Loss of Capillary Exposure.

6.1 Introduction

Increased capillary recruitment due to insulin action may play a key role in increasing both insulin and glucose access to muscle and therefore glucose uptake by this tissue. As mentioned previously in this thesis, use of the 1-methylxanthine method has shown that insulin acts *in vivo* to increase capillary recruitment (Rattigan *et al.* 1997b). In addition, the increase in capillary recruitment may be independent of changes in total blood flow as the latter can be manipulated by agents such as epinephrine, without changes in capillary recruitment (Rattigan *et al.* 1997b). Moreover, if capillary recruitment is prevented by pharmacological intervention (Rattigan *et al.* 1999) or by TNF (see Chapter 7), insulin-mediated glucose uptake is also markedly blocked, with up to 60% of the latter possibly attributed to accessing. Similarly, in the perfused rat hindlimb pharmacological intervention to reduce nutritive flow and increase non-nutritive flow in a constant-flow preparation, gave rise to a marked reduction in insulin-mediated glucose uptake (Rattigan *et al.* 1993).

Characteristics of the sciatic nerve severed model are that the onset of insulin resistance is rapid and reproducible. Muscles originally innervated by the sciatic nerve (including the gastrocnemius red and white, tibialis anterior, plantaris, extensor digitorum longus, soleus and parts of the thigh muscle group, i.e. the semitendinosus, biceps femoris, semimembranosus and adductor magorus (Snell 1992)) each become insulin resistant to varying degrees. Since the sciatic nerve contains vasomotor fibres in association with the motor neurons (Schmalbruch 1986), it is likely that severance could lead to rapid blood flow changes to, and within, the previously innervated muscles. Accordingly, in the present study we have tested the hypothesis that sciatic nerve severance leads to a rapid change in regional blood flow in formerly innervated muscles, which by denying access for insulin and glucose contributes to the insulin resistance. The isolated perfused hindquarter was used where the denervated leg

could be directly compared to the contra lateral control leg (nerve intact) in terms of insulin-mediated glucose uptake as well as alterations in capillary exposure (metabolism of 1-MX) and total blood flow (microspheres).

6.2 Methods

6.2.1 Sciatic Nerve Severance Surgical Procedure

Rats were anaesthetized for sciatic nerve severance with an intra-peritoneal injection of ketamine ($10mg.100g^{-1}$ body weight) mixed with xylazine ($1mg.100g^{-1}$ body weight) in 0.9% saline. A small incision of approximately 0.5 cm was made in the thigh of the left leg and the muscles were separated to expose the sciatic nerve, as described in Burant *et al.* (1984). A section (approximately 3 mm) of the sciatic nerve was then removed, the skin sutured over and the wound dusted with topical antibiotic powder (Apex Laboratories, Australia). Sham-operated animals underwent the same operative procedure, except the sciatic nerve was only visualized (without severance) before closing the wound. Denervated animals were sham operated on their contra-lateral legs for all two hindlimb perfusions and control animals were sham operated on both legs. Animals were then allowed to recover for the chosen period of time before the hindlimb perfusions were performed (i.e. 3 h or 24 h).

6.2.2 Hindlimb Perfusions

Generally, all hindlimb perfusions were conducted essentially as described in Chapter 2 using 180-200 g hooded Wistar rats. Both hindlimbs were perfused in all experiments. Experiments to determine insulin-mediated 2DG uptake (see Section 2.3.2) were performed in separate animals from perfusions to determine total flow distribution using fluorescent microspheres and measurement of 1-MX metabolism (capillary exposure).

Experiments to determine microsphere distribution and 1-MX metabolism in the presence of 2 $mU.ml^{-1}$ insulin were performed in the same animal. Commencing immediately after equilibration (t = 0 min), a solution of 1-MX was infused for the remainder of the experiment (65 min) to give a final concentration of 23 μM. Insulin was infused at t = 40 min to give a final concentration of 2 $mU.ml^{-1}$. Fluorescent

microspheres were injected as a bolus over a period of 10 sec (as described in Section 2.3.3.1) starting at t = 60 min. Vena cava samples were taken at t = 20, 30, 40, 50, 60 and 65 min as well as femoral vein samples at 65 min. This latter sampling was done using a syringe fitted with a 26 G needle. At the end of the perfusion the muscles were removed and processed to determine microsphere content as described in Section 2.3.3.2.

6.2.3 Statistical Analysis

The significance of difference between treatments was tested by one-way analysis of variance and least-significant difference analysis. Values of $P < 0.05$ were taken as significant.

6.3 Results

6.3.1 2-Deoxyglucose Uptake

Figure 6-1 shows data for 2-deoxyglucose (2DG) uptake conducted 24 hours after denervation. The EDL, soleus, plantaris, gastrocnemius white and tibialis muscles of the denervated leg were found to be insulin resistant when compared to the corresponding muscles of the contra-lateral sham operated leg. Although a trend was also apparent for the gastrocnemius red muscle, this was not significant. Data from the right and left hindlimbs of control animals in which both legs had undergone sham operations are also shown. 2DG uptake for corresponding muscles from either leg was the same and no different from the contra-lateral (CL) leg. These experiments were conducted in case denervating one leg of the test animals restricted mobility resulting in changes in the contra-lateral leg from under-use, or alternatively, load-bearing was shifted to the CL leg so that in either case the CL leg was no longer a true control. For the majority of the muscles a trend was apparent suggesting that the CL leg may have been affected to be intermediate between denervation and true controls. This trend, although not statistically significant, was most apparent for all of the muscles sampled except the gastrocnemius white.

Combining the results for all six muscles showed that denervation significantly reduced insulin-mediated glucose uptake from 530 ± 70 to 362 ± 40 $\mu mol.h^{-1}.g^{-1}$ ($P <$

0.05; n = 5). Uptake by sham operated legs in control animals did not differ between legs and was approximately 680 ± 80 $\mu mol.h^{-1}.g^{-1}$. This did not differ from uptake by CL legs

Assessment of insulin-mediated uptake of 2DG into individual muscles as soon as 3 hours after denervation suggested rapid onset of the change. R'g for the plantaris muscle of DL was 30 ± 2 compared to 39 ± 3 $\mu mol.h^{-1}.g^{-1}$ ($P < 0.05$, n = 4) and other muscles were close to significance.

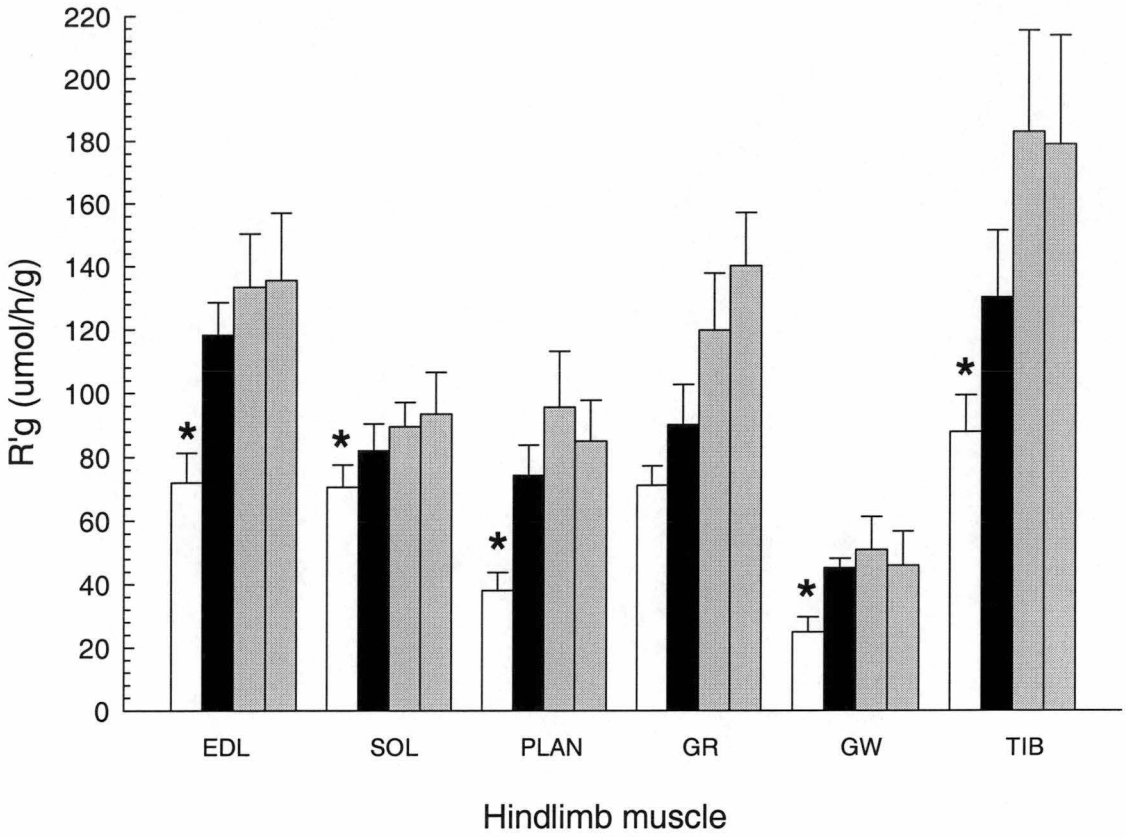


Figure 6-1 Effect of 24 hour sciatic nerve severance on 2-deoxyglucose uptake by individual muscles of the perfused hindlimb.

Denervation of one leg (open bar) was conducted 24 hours prior to perfusing both legs. The contra-lateral leg (solid bar) underwent a sham operation. In separate control animals each leg, left and right (grey bars) underwent sham operations. Values are means ± SE for n = 5. *, $P < 0.05$ when compared to contra-lateral leg

6.3.2 1-Methylxanthine Metabolism and Fluorescent Microsphere Distribution

Figure 6-2 shows the time course for a representative experiment involving 1-MX and insulin infusions and the injection of a bolus of microspheres. The hindquarter was perfused at constant flow throughout (0.43 $ml.min^{-1}.g^{-1}$ muscle; 13 $ml.min^{-1}$) and the perfusion pressure remained constant at 27 ± 2 mmHg for the period 0 to 40 min.

Oxygen uptake was also constant throughout this period at 9.0 ± 0.6 $\mu mol.h^{-1}.g^{-1}$ muscle. Since the isolated hindquarter is essentially fully dilated (Lindinger and Hawke 1999) the vasodilatory action of insulin (or indeed any vasodilator) is not evident under these conditions and the perfusion pressure and hence vascular resistance did not change. Thus pressure remained constant throughout the insulin infusion (40-65 min). Oxygen uptake was stimulated to a small degree by insulin, increasing from 9.0 ± 0.6 to 10.7 ± 0.8 $\mu mol.h^{-1}.g^{-1}$ muscle by t = 60 min. Perfusion pressure underwent a transient drop during microsphere injection, but then returned to the original value. Oxygen uptake was slightly stimulated due to the injection of microspheres.

The perfused rat hindlimb, that was denervated 24 hours earlier, was clearly insulin resistant to uptake of glucose analogues (Figure 6-1). However, the same hindlimbs did not show any large changes with regard to total flow (Figure 6-3). Denervation also had no effect on blood flow to intact, innervated tissues (including white adipose tissue, skin, the foot, bone), or indeed the entire leg, when compared to the CL (non-denervated) leg (Figure 6-3).

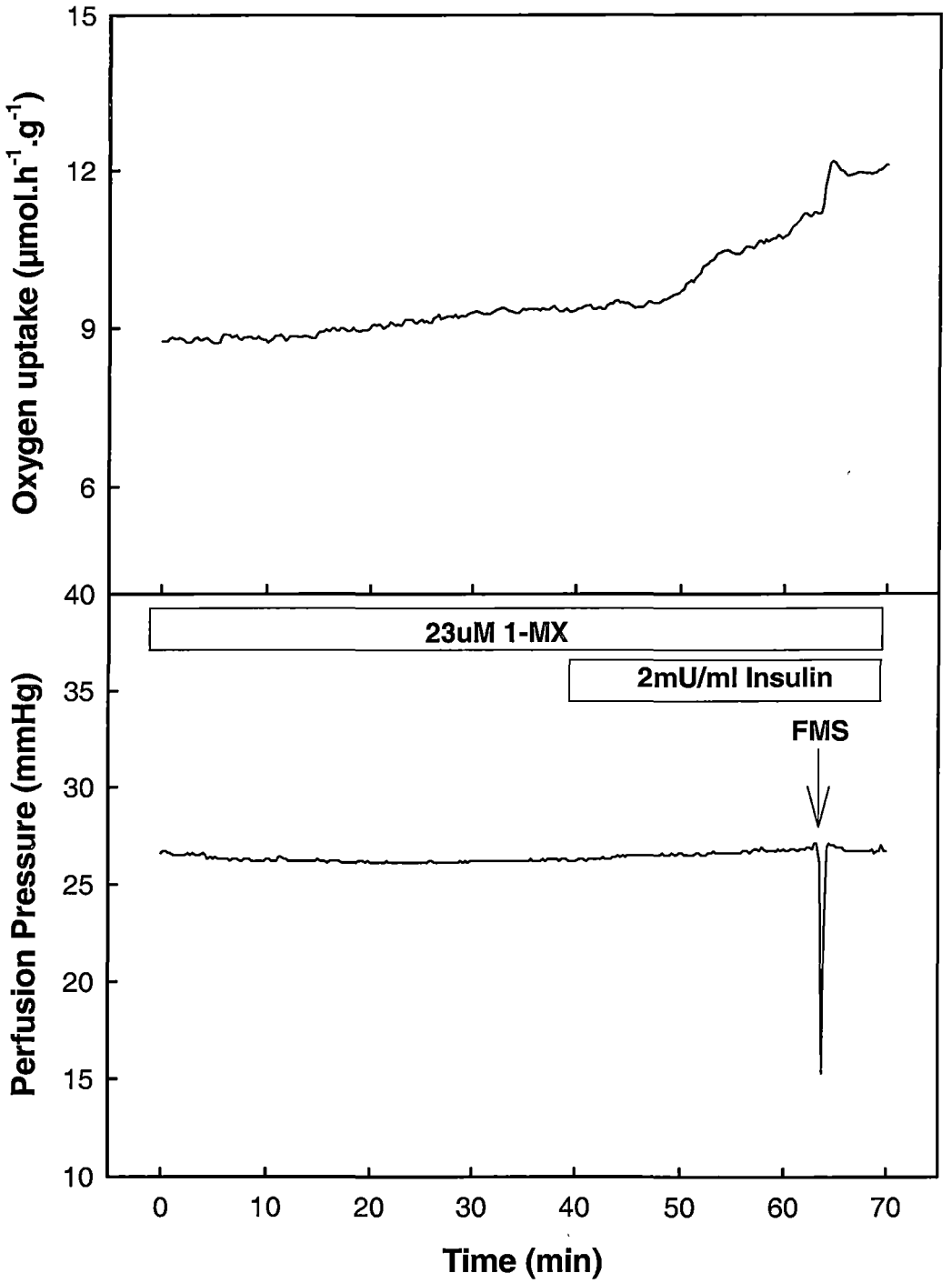


Figure 6-2 Representative trace for time course changes in oxygen uptake and perfusion pressure for hindlimb perfusions.

Infusions of 1-MX and insulin and an injection of a bolus of fluorescent microspheres were made at the times shown (n = 5).

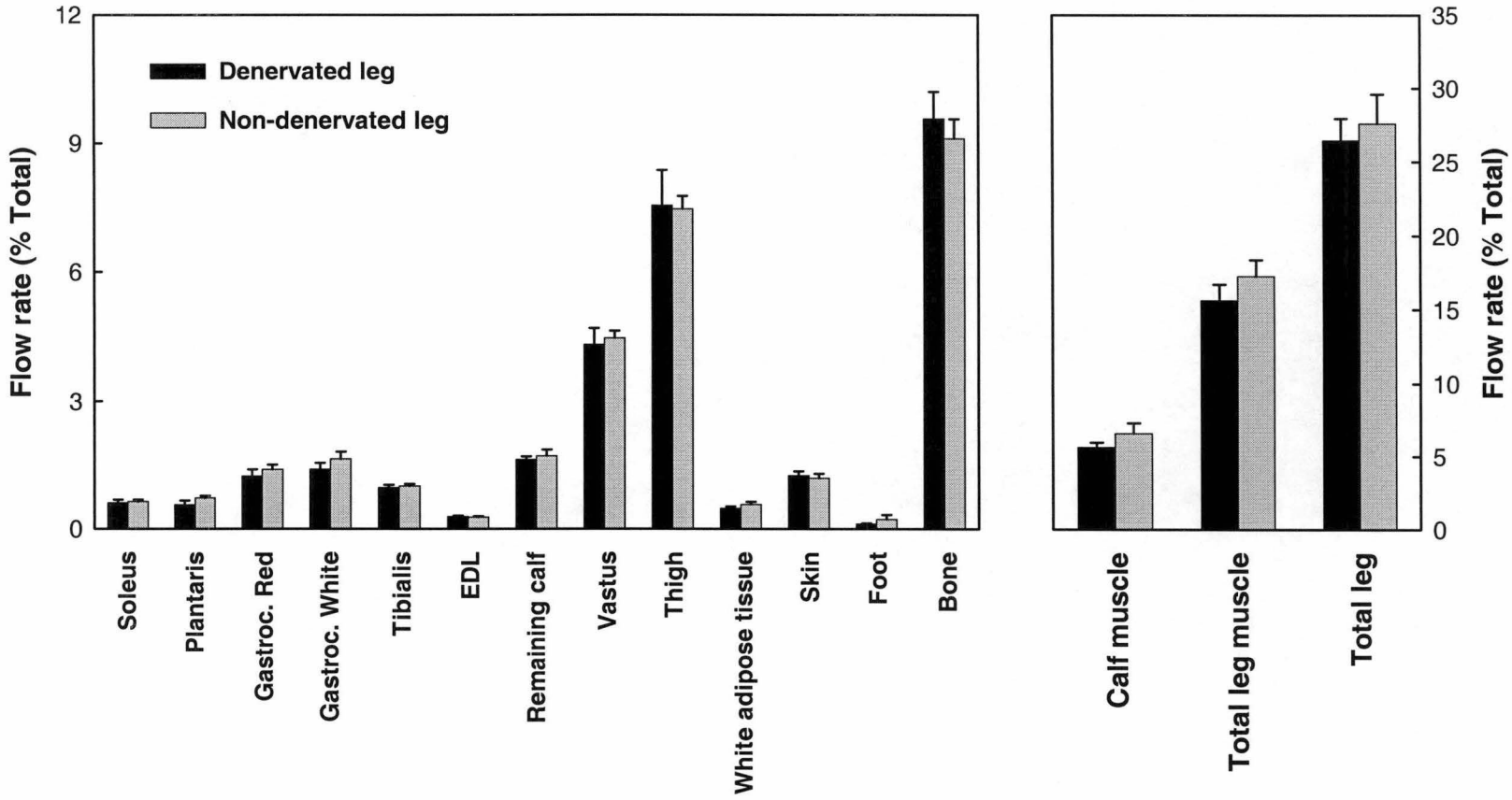


Figure 6-3 Perfusate flow in the denervated and contra-lateral control leg of the perfused hindquarter from microsphere entrapment. Legs underwent denervation (solid bars), sham operation 24 hours prior to being perfused. Insulin was present at 2 $mU.ml^{-1}$. Microsphere (15 μm) content of each muscle was assessed at the end of each perfusion as shown in Figure 6-2. Summated values are shown in the right hand panel. Values are means ± SE for n = 5.

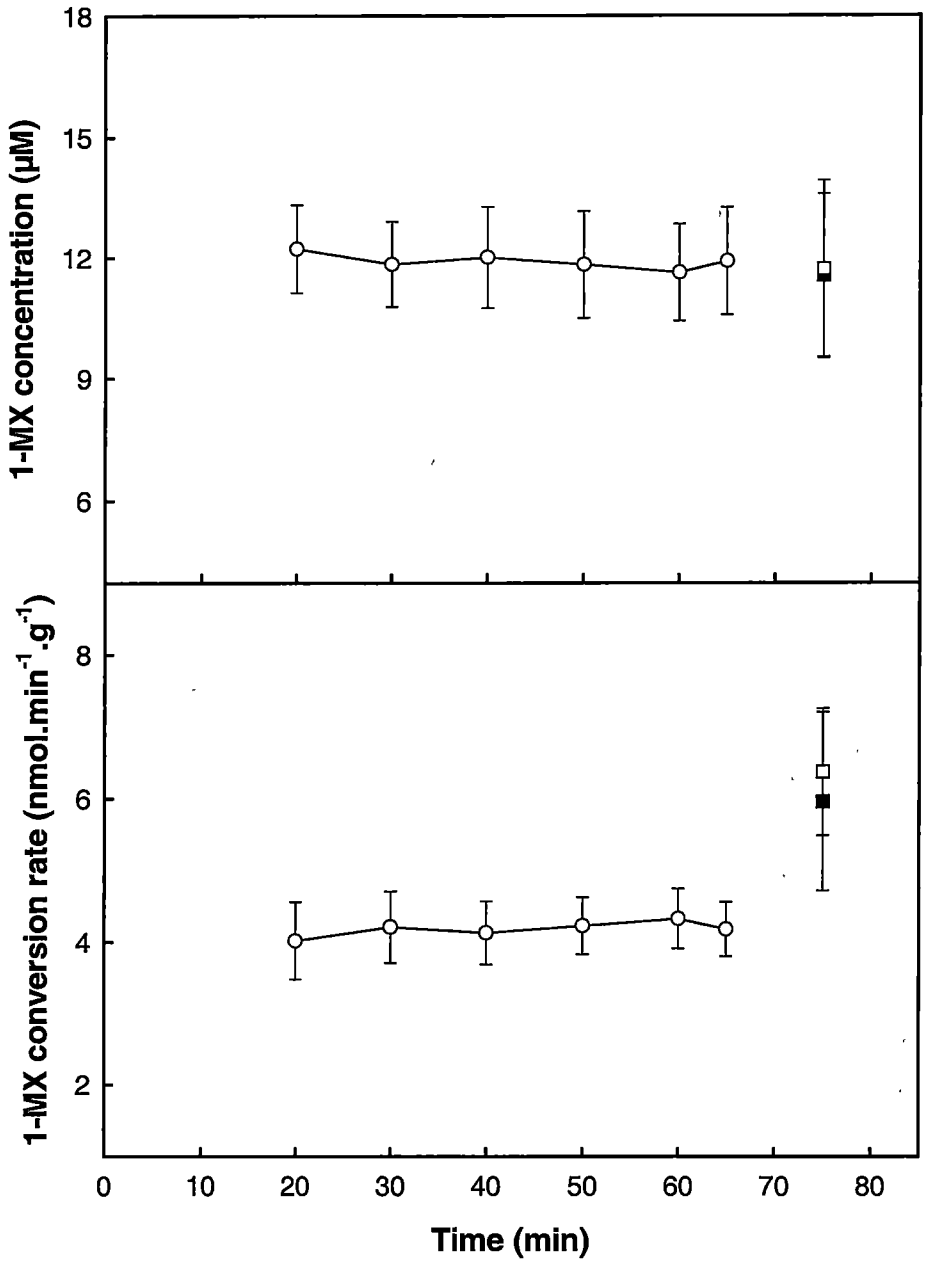


Figure 6-4 Time course for the metabolism of infused 1-MX.

1-MX was infused during hindlimb perfusion to reach a final concentration of 23 μM starting at t = 0 min. Insulin (2 $mU.ml^{-1}$) infusion was commenced at 40 min. Samples of perfusate were taken from the vena cava (○) (draining both legs) until the end of the experiment (t = 65 min), at which time perfusate was sampled from the femoral vein close to the exit point from the calf muscle group. (■) = denervated leg and ( ) = non-denervated leg. 1-MX conversion rates were calculated from vena cava 1-MU concentration and the total hindlimb flow rate (pump rate) or from femoral vein 1-MU concentration and flow deduced from microsphere entrapment for the calf muscle group. Values are means ± SE for n = 5 experiments.

Figure 6-4 shows data for 1-MX metabolism as an index of capillary exposure. Approximately 20 min after commencement of infusion of 23 μM 1-MX, a steady state rate of metabolism was reached, corresponding to a venous perfusate concentration of 12 μM 1-MX (Note: venous perfusate samples are from the vena cava which drains both the DL and CL). This was similar to the equilibrium value reported previously for perfusions conducted at the lower temperature of 25°C (Rattigan et al 1997). Insulin infusion for 40 min did not change the perfusate concentration of 1-MX and thus was concluded not to have altered the total capillary exposure of 1-MX. At the end of the perfusion a sample was taken from the venous blood draining the denervated muscles of the DL and compared with comparable samples from CL. Figure 6-4 shows that the 1-MX concentration was the same for each (DL and CL) and did not differ from that of the entire hindlimb. Although calculated rates of 1-MX metabolism appeared somewhat higher (not significant) from the calf muscle groups (DL and CL) than for the whole hindquarter, this may have been due to higher muscle flow when compared to the hindquarter as a whole.

6.4 Discussion

Using the two hindlimb (hindquarter) perfusion technique we have shown that prior severance of the sciatic nerve of one hindlimb gave rise to a marked insulin resistance in formerly innervated muscles. The onset was rapid and was initially detected in the plantaris at 3 hours and in most other muscles, including the EDL, SOL, GW, TIB as well as the PLAN at 24 hours after surgery. In addition, there was a trend, although not significant, for insulin sensitivity to be reduced in muscles of the contra-lateral leg when compared to animals where both legs had undergone sham operations, possibly reflecting an effect of less use over the 24 hour period. Most importantly, the insulin resistance occurred without any detectable change in haemodynamic parameters. Thus, 1-MX metabolism, a surrogate indicator of capillary exposure, did not differ between denervated and contra-lateral legs. Furthermore, microsphere distribution, as an index of total flow, was similar in all muscles, white adipose tissue, skin, foot and bone of the denervated leg when compared to the contra-lateral leg. Overall these findings suggest that prior denervation did not affect the distribution of perfusate flow either between muscles, between muscle and non-muscle tissue, or within muscles. Thus insulin resistance from denervation is likely to be of cellular origin.

Insulin resistance following sciatic nerve severance has been confirmed at three levels, *in vivo*, in perfusion and with isolated incubated muscles. At the first level, Turinsky (Turinsky 1987a, Turinsky 1987b) and Turinsky *et al.* (Turinsky *et al.* 1990) demonstrated insulin resistance *in vivo* by intravenous infusions of 2-deoxyglucose and insulin into rats under anaesthesia. The studies showed that insulin stimulated 2-DG uptake by soleus and plantaris muscles of the denervated leg were decreased by as much as 80%, as early as 3-6 h post-denervation when compared to the contra-lateral sham muscles. At the second level, perfusion studies by Megeney *et al.* (Megeney *et al.* 1995) using the two hindlimb perfused preparation showed that insulin-mediated 3-O-methylglucose transport was decreased by approximately 35% in muscles of the denervated leg when compared to companion muscles of the contra-lateral control leg 3 days after surgery. At the third level, isolated soleus muscles were shown to be insulin resistant in incubation by a number of research groups including Burant *et al.* (Burant *et al.* 1984), Sowell *et al.* (Sowell *et al.* 1991, Sowell *et al.* 1988) and Henriksen *et al.* (Henriksen *et al.* 1991). From insulin dose response curves for 2DG uptake or 3-O-methylglucose uptake, insulin resistance characterised by a decreased responsiveness to maximum insulin, was observed in muscles removed from the animal as soon as 24 h after denervation. At two of these levels (*in vivo* and in perfusion) changes to either total blood flow to, or within, muscle could explain the insulin resistance by affecting the access of insulin and glucose to the muscle fibres. In this respect a number of early papers (including Ederstrom *et al* and references therein) had reported major changes in peripheral blood flow following denervation (Ederstrom *et al.* 1956). These changes were characterised by increased flow through the affected part, seen particularly in the larger vessels followed by a progressive decrease as the vessels (smaller) regained tone (Wiedeman 1968). Some workers suggested that the increase in blood volume was attributable to reduced sympathetic vasoconstrictor tone that initially becomes evident 2 or so days after damage (Herbert and Hood 1997, Midrio *et al.* 1992). Wiedeman (1968) argued that these changes caused a redistribution of the site of regulation of flow through capillary nets, in turn implying an effect on access for insulin and nutrients. There is also indirect evidence from denervated skeletal muscle of quadriplegic patients where whole body insulin-mediated glucose utilization is reduced by 43%, yet basal and insulin-mediated uptake of 3-O-methylglucose uptake in isolated vastus lateralis strips is normal (Aksnes *et al.* 1996). Morphological assessment of those same

muscle samples indicated a decreased ratio of capillaries: muscle fibres when compared to age-matched healthy controls. Arguments against a role of blood flow contributing to the insulin resistance by affecting insulin access would come from studies with isolated incubated muscles. Insulin resistance is marked in isolated muscles (Burant *et al.* 1984) when incubated with insulin and analogues of glucose. Since incubated muscle depends on access for hormones and substrates by diffusion and not by the normal vascular route, changes in micro- or macro-vascular flow patterns, that might be present *in vivo* or during hindlimb perfusion, can have little bearing. The only way in which an initial haemodynamic change could possibly impinge is through inducing rapid changes in gene expression in the interval between surgery and experimentation (e.g. depressed GLUT4 at 3 days after denervation, Henriksen *et al.* 1991). However, it is unlikely that major changes in protein level of expression could have occurred within 24 h, or even 3 h, after surgery.

In view of the present findings it would now seem likely that the insulin resistance is of muscle cell origin. Indeed a number of workers have examined insulin receptor status post-denervation and are convinced that resistance is attributable to a post-receptor defect (Sowell *et al.* 1989). These workers reasoned that the defect lies in either the signal transmission mechanism between receptor and the glucose transporter system, or at the level of the glucose transporter. At the time and because multiple insulin-sensitive pathways, including glucose transport (Burant *et al.* 1984, Forsayeth and Gould 1982, Sowell *et al.* 1989, Turinsky 1987a), amino acid transport (Forsayeth and Gould 1982, Turinsky 1987b), and glucose-independent activation of glycogen synthase (Burant *et al.* 1984, Smith *et al.* 1988) were affected by denervation, they argued that the simplest mechanism to account for the insulin resistance was one involving a defect at an early common step in insulin's intracellular cascade.

To date, haemodynamic changes occurring with denervation have been restricted to changes in total flow where measurements were conducted *in vivo* using microspheres (Turinsky *et al.* 1998). In that work (Turinsky *et al.* 1998) blood flow 24 h after denervation was increased in soleus, plantaris, and gastrocnemius muscles by 63, 323, and 304% when compared to corresponding sham muscles, respectively. Insulin response in terms of 2DG uptake was decreased by 80, 71 and 50%, in the

same muscles. The authors concluded that changes in blood flow did not contribute to the development of insulin resistance in denervated muscles.

In summary, this study shows that despite the rapid onset of insulin resistance in terms of 2DG uptake into individual muscles, this does not appear to be due to denervation affecting haemodynamic parameters within the skeletal muscle. Alternatively, insulin in vivo could stimulate sympathetic nerve activity in intact animals in contrast to *in vitro*.

CHAPTER 7

Acute Impairment of Insulin-Mediated Capillary Recruitment and Glucose Uptake in Rat Skeletal Muscle *In Vivo* by TNFα

7.1 Introduction

The inflammatory cytokine, tumour necrosis factor α (TNF) is expressed in both adipose tissue and skeletal muscle, and many animal models of obesity and insulin resistance are associated with significantly higher levels of TNF mRNA and protein compared to their lean counterparts (Hotamisligil *et al.* 1993). Similar data has been seen recently in humans with obesity and insulin resistance (Hotamisligil *et al.* 1995, Saghizadeh *et al.* 1996). There is also some evidence for causality. For example, infusion of a TNF receptor (TNFR) IgG fusion protein was found to neutralise TNF *in vivo* and improve insulin action in genetically obese and insulin resistant zucker rats (Hotamisligil *et al.* 1993). Also, infusion of insulin resistant animals with a soluble TNF-binding protein improved *in vivo* insulin action (Hotamisligil and Spiegelman 1994). Furthermore, genetically obese mice lacking either or both of the TNF receptors, p55 and p75, are more insulin sensitive than those still possessing them (Hotamisligil 1999). In humans, TNF is also thought to be strongly linked to the development of the insulin resistance in obesity and type II diabetes. In particular, TNF has been implicated as the cause of the insulin resistance observed in septic, cancer and surgical patients (Michie *et al.* 1988, Offner *et al.* 1990).

Attempts to induce muscle insulin resistance by TNF administration have led to mixed success and the mechanism by which TNF may cause insulin resistance is not clear. Administration of TNF to anaesthetised rats over 3 hour under clamp conditions markedly reduced insulin-mediated uptake of 2-deoxyglucose by muscle (Ling *et al.* 1994). At the isolated cellular level, it has been shown that 3-5 days exposure of 3T3-L1 or 3T3-F442A adipocytes to TNF causes reductions in insulin receptor and insulin receptor substrate-1 tyrosine phosphorylation in response to a

maximal dose of insulin (Guo and Donner 1996, Hotamisligil *et al.* 1994). Yet other researchers have shown that 3-4 days of exposure of 3T3-L1 adipocytes to TNF gives rise to large decreases in GLUT4, insulin receptor and insulin receptor substrate-1 mRNA and protein (Stephens *et al.* 1997, Stephens and Pekala 1991). Shorter exposure times have also been claimed to decrease insulin-stimulated tyrosine phosphorylation of the insulin receptor and insulin receptor substrate-1 in Fao hepatoma cells (Feinstein *et al.* 1993, Kanety *et al.* 1995) and NIH3T3 fibroblasts (Kroder *et al.* 1996) as well as a decrease in insulin-stimulated glucose transport in L6 myocytes (Begum and Ragolia 1996). However, direct effects of TNF on muscle rather than cell lines are less certain. Recently Nolte et al ((Nolte *et al.* 1998) showed that exposure of isolated soleus muscles to 6 $nmol.L^{-1}$ TNF for 45 min had no effect on insulin-stimulated tyrosine phosphorylation of the insulin receptor or insulin receptor substrate-1 or on phosphatidylinositol 3-kinase association with the insulin receptor substrate-1. More importantly, incubation of epitrochlearis and soleus muscles with 6 $nmol.L^{-1}$ TNF for 45 min or 4 hours, or epitrochlearis muscles with 2 $nmol.L^{-1}$ for 8 hours had no effect on insulin-stimulated 2-deoxyglucose uptake.

Studies in this laboratory, as well as others, have shown that in addition to its many direct metabolic actions on skeletal muscle, insulin also has haemodynamic effects that may increase access of insulin and glucose to muscle (Baron and Clark 1997, Rattigan *et al.* 1997b, Yki-Jarvinen and Utriainen 1998). Insulin's haemodynamic effects comprise two components. One is concerned with increasing the total blood flow to skeletal muscle via a nitric oxide (NO)-dependent vasodilation (Steinberg *et al.* 1996). Thus if a nitric oxide synthase inhibitor was present, insulin-mediated glucose uptake was blocked by about 30% (Baron and Clark 1997). The second involves an increase in capillary recruitment (or nutritive flow) within skeletal muscle (Rattigan *et al.* 1997b). Measurement of capillary exposure (or nutritive flow) was assessed using 1-MX metabolism. Metabolism of this exogenously added substrate for capillary endothelial xanthine oxidase was shown to increase in the presence of insulin (Rattigan *et al.* 1997b). In addition, if α-methyl serotonin (α met5HT), an agent that prevented capillary recruitment was administered, the ability of insulin to increase either total blood flow or capillary recruitment was markedly impaired and insulin-mediated glucose uptake was blocked by 60% (Rattigan *et al.* 1999). Thus,

on the one hand mindful of the strong association of TNF with insulin resistance *in vivo*, and on the other with the failure of TNF to cause insulin resistance when incubated with isolated muscles, we undertook the present study to assess whether TNF could induce insulin resistance *in vivo* by influencing haemodynamic parameters.

7.2 Methods

7.2.1 Experimental Procedures

Male rats (245 ± 3 g) were anaesthetised and underwent surgery as described in Chapter 2. Once the surgery was complete and the rat had equilibrated, animals were allocated into either control (saline or TNF alone) as shown in Protocol A (Figure 7-1), or euglycaemic insulin clamp (insulin alone or TNF + insulin) group (Protocol B, Figure 7-1) (n = 6-10 in each group). Saline and TNF infusions were matched to the volumes of insulin and glucose infused during the clamp. TNF (mouse recombinant, Sigma Aldrich Inc) was dissolved in saline and 0.1% bovine serum albumin. 1-MX infusion (0.4 $mg.min^{-1}.kg^{-1}$) was commenced at 60 min prior to the end of the experiment and a bolus dose of [^{3}H]2DG administered for measurement of 2DG uptake as described in Chapter 2.

7.2.2 Statistical Analysis

In order to ascertain differences between treatment groups at the end of the experiment (120 min), one way analysis of variance (ANOVA) was used. When a significant difference ($P < 0.05$) was found, Dunnett's test was used to determine which times were significantly different from saline control (for femoral blood flow, arterial blood pressure, femoral vascular resistance, arterial glucose and 1-MX, hindleg glucose extraction and uptake and hindleg 1-MX extraction and disappearance). Pairwise comparisons were made using the Student-Newman-Keuls Method. An unpaired student's *t*-test was used to determine whether there was a significant difference ($P < 0.05$) between the glucose infusion rates at the conclusion of the experiments. The t-test was also used to test whether the arterial plasma TNF concentrations were significantly different between the treatments. All tests were performed using the SigmaStat™ statistical program (Jandel Software Corp.).

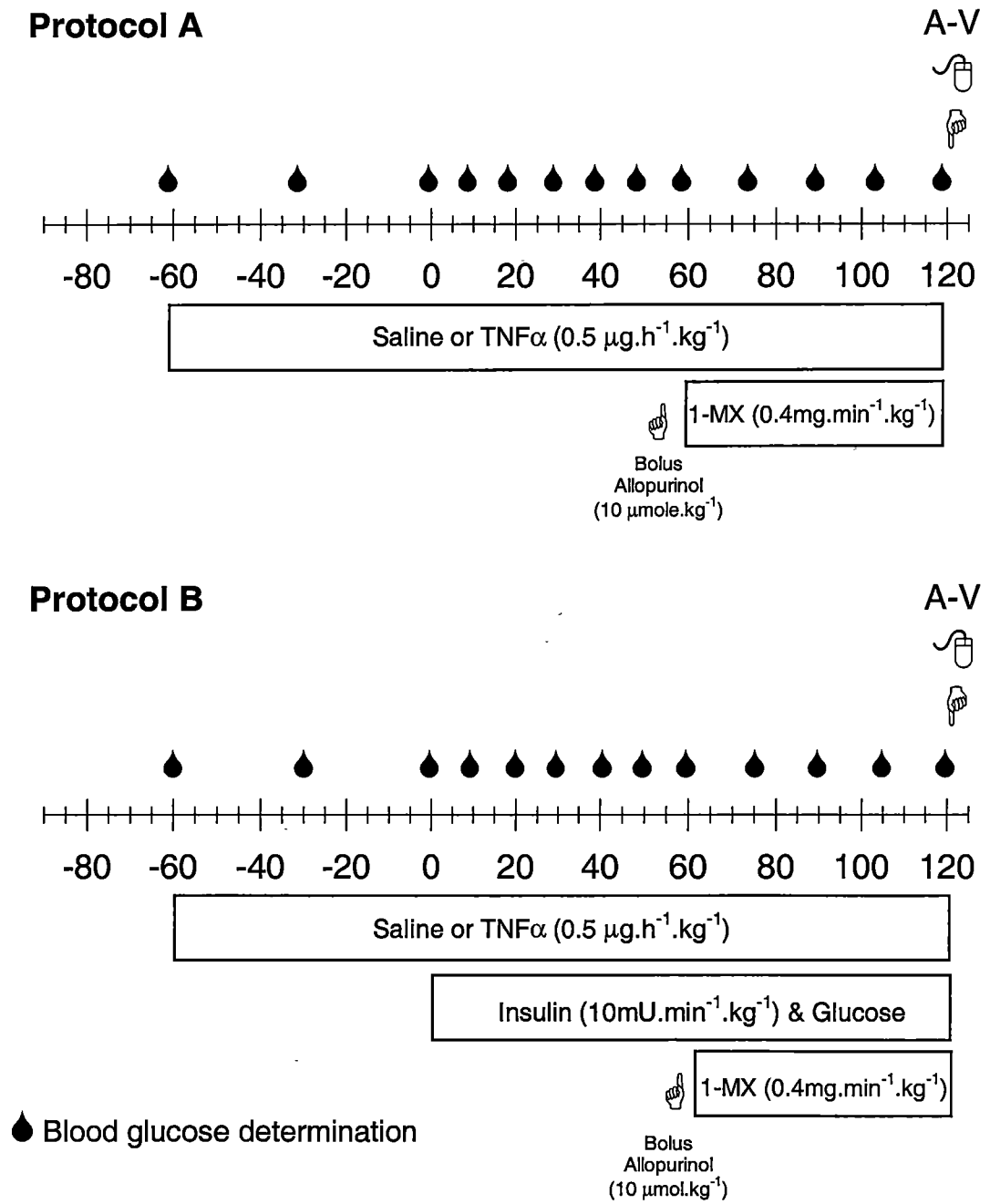


Figure 7-1 Study design.

In both the control (Protocol A) and the euglycaemic clamp groups (Protocol B), either saline or TNF infusion was commenced at time = -60 min. During protocol B, insulin infusion was started at time = 0 min. Duplicate arterial and femoral venous plasma samples were collected at 120 min, as indicated by , for HPLC analysis and plasma glucose determinations. Arterial samples for glucose determinations are indicated by . Venous infusions are indicated by the bars.

7.3 Results

7.3.1 Haemodynamic Effects

Figure 7-2 shows the femoral blood flow (FBF), mean arterial blood pressure and hindleg vascular resistance following saline or TNF infusions and following insulin or TNF plus insulin infusions at the completion of the experiment (120 min). TNF infusion alone had no significant effect on any of these haemodynamic parameters, although it did significantly ($P < 0.05$) decrease heart rate compared with saline infusion (350 ± 8 v's 391 ± 13 bpm). Insulin infusion alone caused a significant increase (70%) in femoral blood flow (0.91 ± 0.11 to 1.51 ± 0.14 ml.min^{-1}) by the end of the experiment. Since blood pressure was unchanged this corresponded to a 35% decrease in hindleg vascular resistance. The insulin-mediated increase in femoral blood flow and decrease in hindleg vascular resistance was completely prevented during infusion with TNF.

7.3.2 Glucose Metabolism

There was no significant difference in arterial blood glucose concentration between any of the treatment groups at either the beginning of the experiment (time = 0 min) or at the end (time = 120 min). During the euglycaemic insulin-clamp experiments, arterial blood glucose was maintained at or above basal values by infusion of glucose. At the conclusion of the experiment, the whole body glucose infusion rate required to maintain euglycaemia during the insulin alone infusions (22.5 ± 0.4 mg.min^{-1}.kg^{-1}) was significantly higher (17%) ($P < 0.001$) compared to the TNF + insulin infusions (18.6 ± 0.4 mg.min^{-1}.kg^{-1}).

Arterial plasma insulin concentrations in the insulin treated animals (1226 ± 118 pM) were not significantly different from the insulin + TNF (1388 ± 107 pM) infused rats at the end of the clamp.

Hindleg glucose extraction and uptake were significantly increased during the euglycaemic insulin clamps (Figure 7-3). TNF infusion alone had no effect upon either hindleg glucose extraction or uptake, but significantly decreased both the insulin-mediated hindleg glucose extraction (by 21%) and uptake (by 47%). This

latter effect may be even greater as there was no significant difference between the hindleg glucose uptake during the TNF + insulin infusions and the saline or TNF alone experiments; TNF blocked extraction and flow were each contributory.

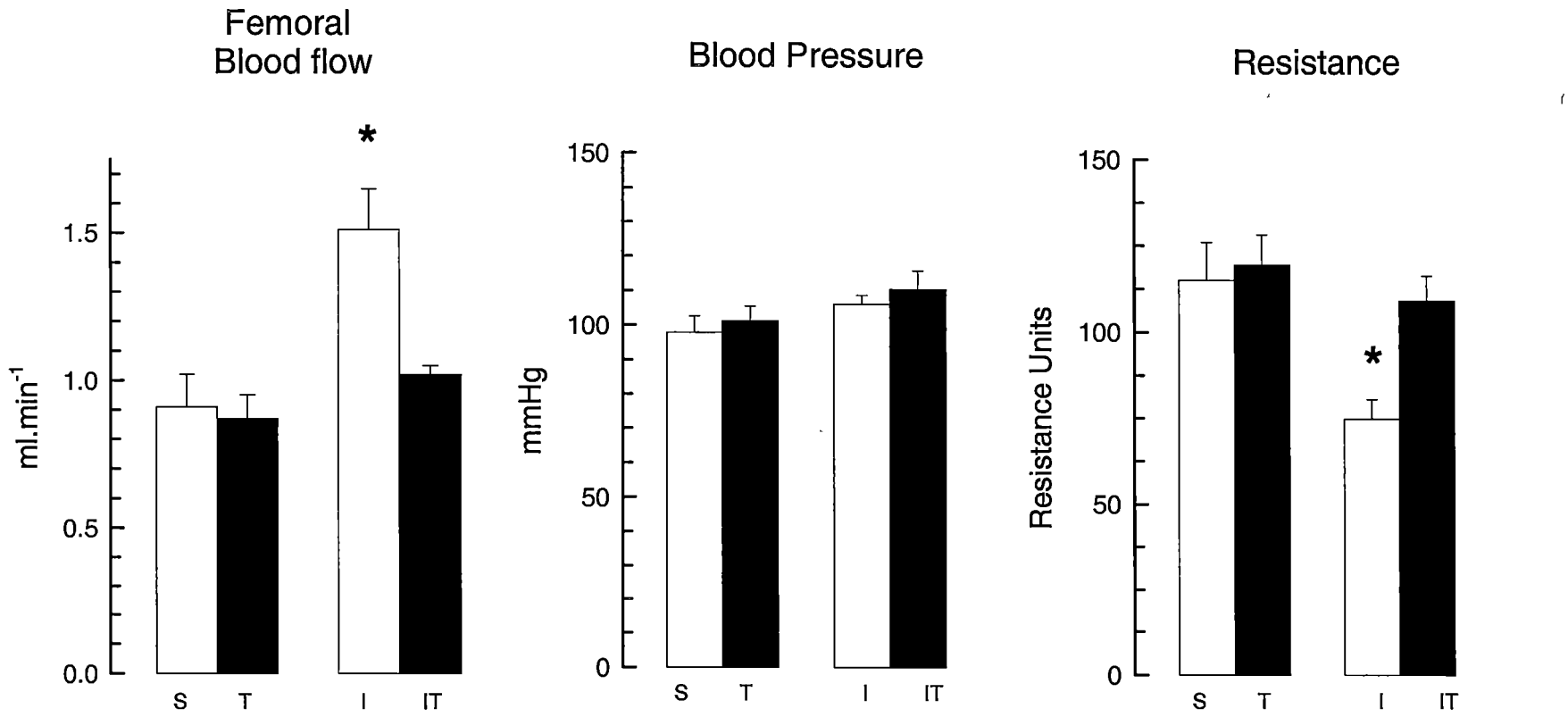


Figure 7-2 Hindlimb femoral blood flow, mean arterial blood pressure and hindleg vascular resistance at end of experiments (120 min). S = Saline and T = TNF alone infusion, as shown in protocol A (Figure 7-1). I = Insulin infusion alone and IT = Insulin + TNF as shown in protocol B. Data were collected from 5 sec sub-samples taken each 15 min interval, as described in Chapter 2. Values are means ± SE for 6 - 10 animals (6 animals in TNF group, 10 in insulin group and 7 in both the saline and TNF + insulin groups). * Significantly different ($P < 0.05$) from saline infusion at 120 min.

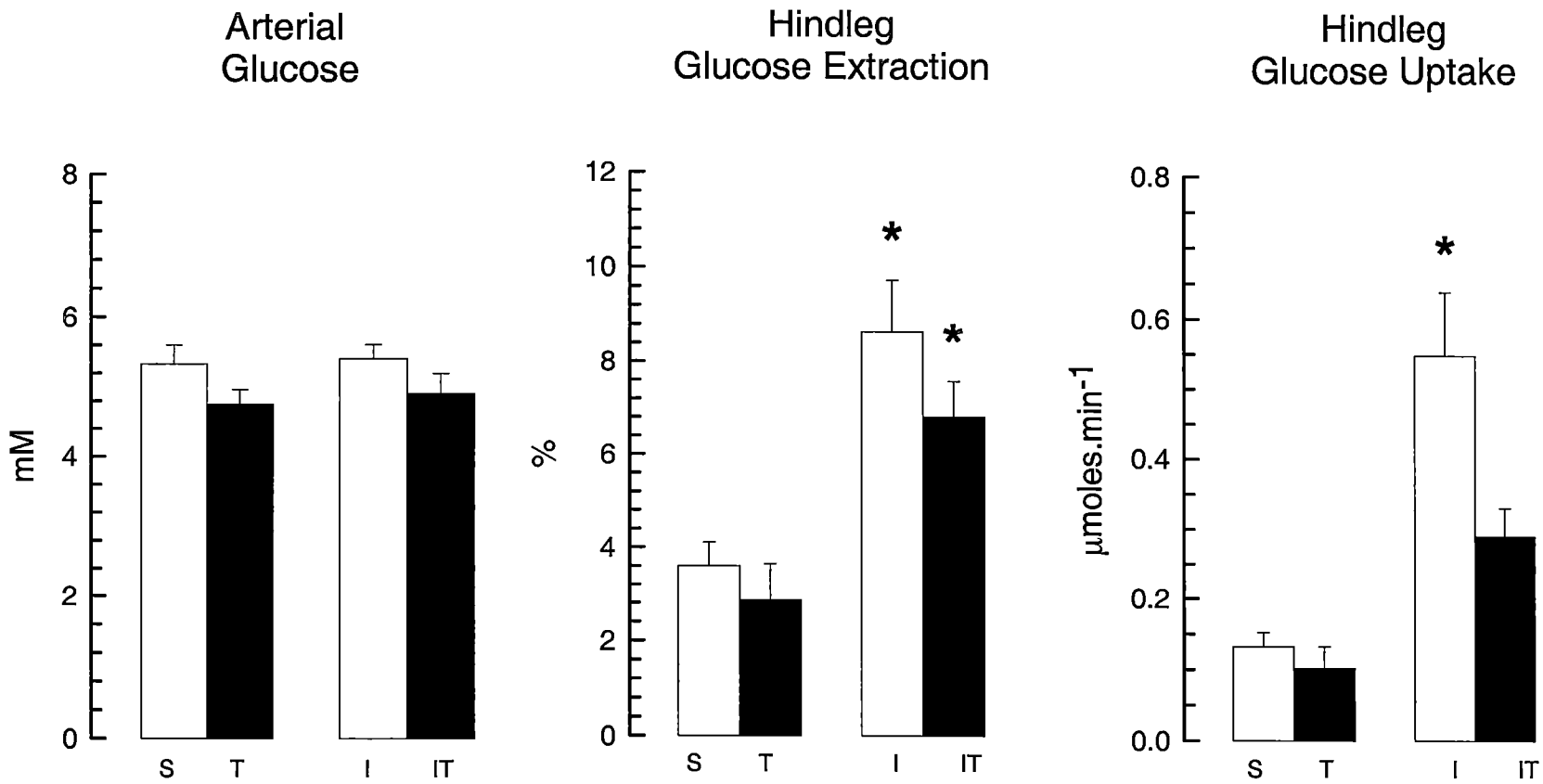


Figure 7-3 Systemic and hindleg glucose values of control groups (saline or TNF alone) and insulin clamp groups (insulin or TNF + insulin) at 120 min.

Values are means ± SE for 6 - 10 animals in each group. * Significantly different ($P < 0.05$) from saline values at 120 min.

7.3.3 Arterial Plasma TNF levels

Arterial plasma TNF concentrations in the TNF infusion only group (1404.3 ± 210.6 $pg.ml^{-1}$) were not significantly different from the TNF + insulin animals (1270.4 ± 216.9 $pg.ml^{-1}$) ($P = 0.67$). Animals infused with insulin only (and not infused with TNF) had arterial plasma TNF values of 14.2 ± 7.9 $pg.ml^{-1}$, and these were significantly lower than the TNF-infused animals ($P < 0.001$). The level of TNF in saline infused rats was generally undetectable.

7.3.4 [^{3}H] 2-Deoxyglucose Uptake

[^{3}H] 2-deoxyglucose (2DG) was administered for the final 45 min of each experiment. Figure 7-4 shows uptake values for soleus and plantaris muscles removed at the completion. TNF alone tended to cause a small decrease in 2DG uptake in both the soleus and plantaris muscles, but this was not significant. Insulin infusion alone resulted in a marked increase in 2DG uptake in both the soleus (5.7-fold; from 1.9 ± 0.4 to 11.0 ± 0.4; $P < 0.001$) and plantaris (6.3-fold; from 1.2 ± 0.1 to 7.6 ± 0.6; $P < 0.01$). However, when combined with TNF infusion, the insulin-mediated increase in 2DG uptake by both soleus and plantaris muscles was significantly blocked (43% and 57%, respectively).

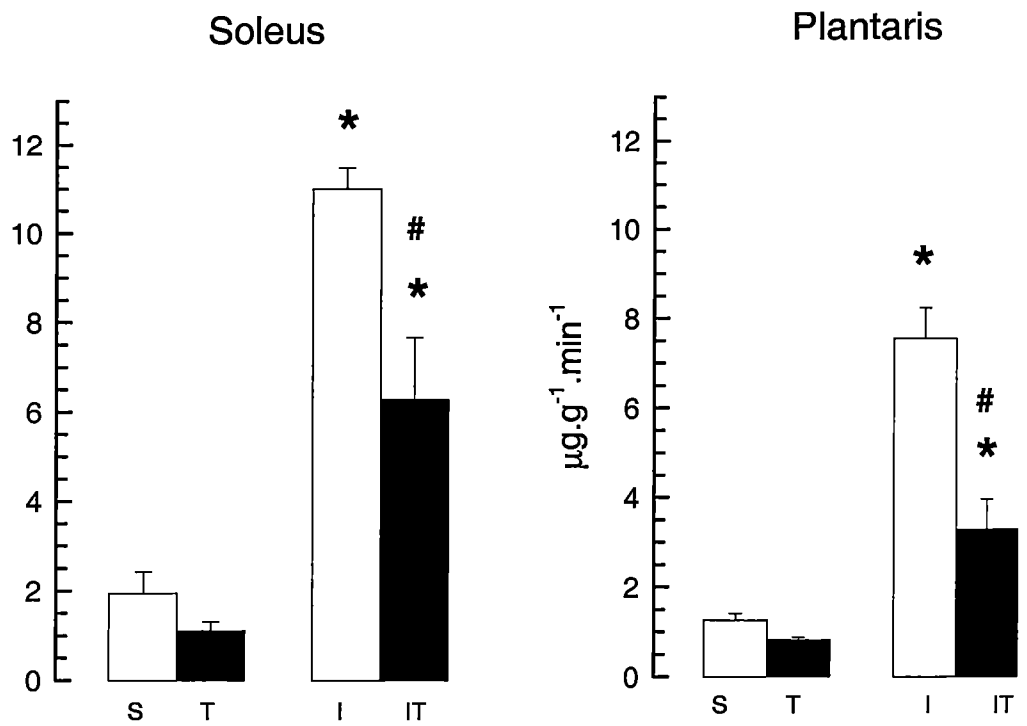


Figure 7-4 [^{3}H] 2-deoxyglucose uptake values for soleus and plantaris muscle. Values are means ± SE for 5-6 animals in each group. * Significantly different ($P < 0.05$) from saline values, # Significantly different from insulin alone.

7.3.5 1-MX Metabolism

No significant difference was found between the experimental groups in arterial plasma concentrations of 1-MX (Figure 7-5) or oxypurinol ($P = 0.51$ and $P = 0.41$ respectively), the metabolite of allopurinol and inhibitor of xanthine oxidase. Insulin infusions alone significantly increased hindleg 1-MX metabolism (Figure 7-5). This resulted from the combined trend of insulin to increase 1-MX extraction and marked effect to increase femoral blood flow (Figure 7-2). When TNF was combined with the insulin, the increase in 1-MX metabolism was completely abolished. TNF infusion alone did not affect 1-MX metabolism.

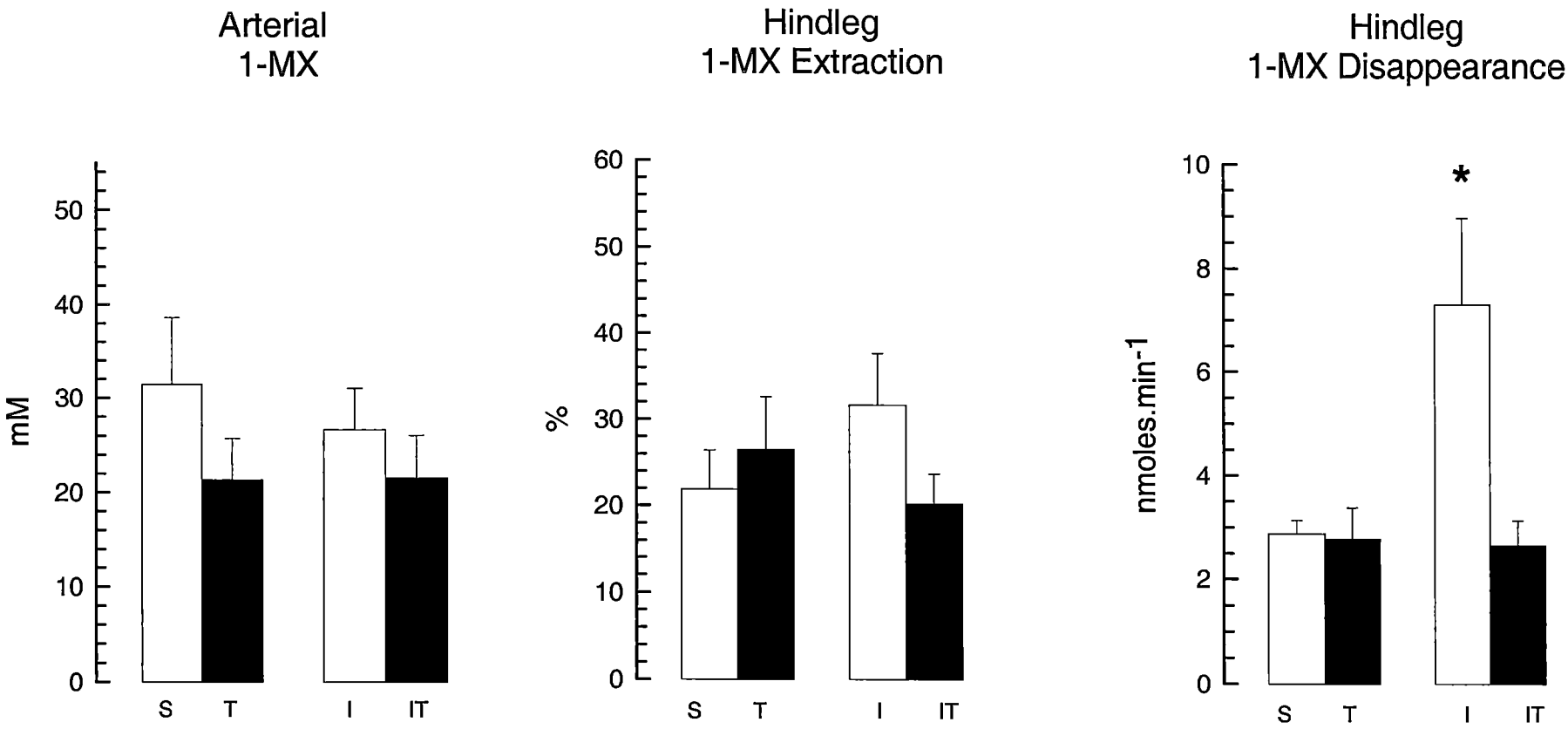


Figure 7-5 Systemic and hindleg 1-MX values of control groups (saline or TNF alone) and euglycaemic insulin clamp groups (insulin alone or insulin + TNF) at 120 min.

Values are means ± SE for 6 - 10 animals in each group. * Significantly different ($P < 0.05$) from saline values.

7.4 Discussion

Two findings emerge from this study. Firstly, acute administration of TNF led to marked insulin resistance with decreased insulin-mediated 2DG uptake by individual muscles, decreased hindlimb glucose uptake and decreased whole body glucose infusion. Secondly, the inhibitory effect of TNF appears to be wholly haemodynamic in that insulin-mediated increases in femoral blood flow and capillary recruitment were totally blocked. Most striking was the effect of TNF on insulin-mediated increases in capillary recruitment as measured by 1-MX metabolism. This substrate has been used by this research group in two previous studies *in vivo* (Rattigan *et al.* 1997b, Rattigan *et al.* 1999) as a marker for capillary (nutritive flow) in muscle. In addition, it has been shown that in the constant flow perfused rat hindlimb 1-MX metabolism is decreased when the proportion of nutritive:non-nutritive flow is decreased pharmacologically (Rattigan *et al.* 1997a) and increased when the ratio is increased, for example, with exercise (see Chapter 3). In addition, we have shown *in vivo* that insulin increases the metabolism of 1-MX independently of changes in total flow, leading to the conclusion that insulin mediates an increase in capillary recruitment as part of its action to increase glucose uptake by muscle. Moreover, in a recent study we have shown that the vasoconstrictor, α-methyl serotonin, which decreased the proportion of nutritive flow in perfused muscle, caused an acute state of insulin resistance *in vivo*. Thus insulin-mediated increases in femoral blood flow, hindleg glucose uptake and hindleg 1-MX disappearances were all markedly inhibited (Rattigan *et al.* 1999). Indeed there are striking similarities between the effects of α-methyl serotonin in that study and the effects of TNF in the present study, suggesting that the mechanisms may be similar. From isolated perfused hindlimb studies (Newman and Clark 1999), and the increase in blood pressure *in vivo* (Rattigan *et al.* 1999), it would appear likely that α-methyl serotonin acts *in vivo* to constrict vessels preventing access to the nutritive capillaries and thereby preventing insulin's action to recruit capillaries. TNF, however, does not increase blood pressure in the way which α-methyl serotonin does and so its effects are unlikely to involve a redistribution of blood flow to the detriment of insulin's action to recruit capillaries. Rather, the effect of TNF, directly or indirectly, is more likely to involve an inhibitory effect at the level of signal transduction. Candidate targets include tyrosine phosphorylation of the insulin receptor or insulin receptor substrate-1, or the association of

phosphatidylinositol 3-phosphate kinase with phosphorylated insulin receptor substrate-1 (Nolte *et al.* 1998). Most favored among these is the activation of p55 and/or p75 TNF receptor leading to IRS-1 serine phosphorylation that then blocks insulin signalling (Hotamisligil 1999). What is not clear is whether the effects of TNF occur at the skeletal muscle cell where a putative vasodilator capable of increasing capillary recruitment might be released or at the vascular tissue where insulin may act directly to enhance flow. These issues are beyond the scope of the present study and may only be resolved when tissue specific receptor deleted animals are compared. It is unlikely that TNF directly inhibits xanthine oxidase, as there was no effect of TNF on hindleg 1-MX disappearance when added alone (Figure 7-5).

The finding that TNF administration prior to and during the hyperinsulinaemic euglycaemic clamp causes insulin resistance is not new and has been shown by others (Ling *et al.* 1994). In that study TNF was administered initially as a 10 $\mu g.kg^{-1}$ bolus followed by a continuous infusion of 10 $\mu g.kg^{-1}$ over 3 hours. Thus a total of 20 $\mu g.kg^{-1}$ was administered. In the present study, we infused 0.5 $\mu g.kg^{-1}$ per hour over 3 hours, giving rise to a total of 1.5 $\mu g.kg^{-1}$. This is perhaps more physiological, given that mini osmotic pump delivery of TNF at 0.5 $\mu g.kg^{-1}$ per hour for 4-5 days has been shown to give a serum concentration of 309 ± 47 $pg.ml^{-1}$ (Miles *et al.* 1997), which compares favourably with serum levels of around 200 $pg.ml^{-1}$ for genetically obese insulin resistant animals (Kimura *et al.* 1998). In humans the levels of TNF in serum are somewhat lower. For example, TNF serum levels in Type II diabetics (90 ± 10 $pg.ml^{-1}$), and obese patients (78 ± 12 $pg.ml^{-1}$) and control subjects (20 ± 8 $pg.ml^{-1}$) (Winkler *et al.* 1998), although, levels in patients with peritoneal adhesions after abdominal surgery are as high as 261 ± 88 $pg.ml^{-1}$ (Saba *et al.* 1998).

At first glance the present findings might seem at odds with the recent report by Nolte *et al.* ((Nolte *et al.* 1998) that exposure of isolated incubated muscles to TNF (up to 6 $nmol.L^{-1}$ or 102 $ng.ml^{-1}$ for 4 hours) had no effect on insulin signaling or insulin-mediated glucose uptake. This may mean that the inhibitory effects of TNF on insulin action are targeted exclusively at the vascular tissue, which is a minor component of incubated muscles and if modified could not affect insulin action which reaches the muscle by diffusion in this preparation.

In conclusion, acute administration of TNF causes insulin resistance in muscle and may involve effects exerted directly or indirectly at the vascular level to prevent insulin action to increase total limb blood flow and capillary recruitment. These two effects of insulin are likely to be separate as increasing limb blood flow (Rattigan *et al.* 1997b) does not necessarily increase capillary (nutritive) flow. Finally, the findings suggest that at least 50% of the increase in muscle glucose uptake due to insulin is mediated by a haemodynamic contribution involving capillary recruitment.

CHAPTER 8

Final Discussion

8.1 Summary of Thesis

The most recognisable action of insulin is to increase glucose uptake into skeletal muscle, by increasing glucose transporter translocation to the plasma membrane and activation of downstream glucose metabolic pathways (Kahn 1994). In recent times, a number of studies have reported that in addition to its metabolic action to increase muscle glucose uptake, insulin has a haemodynamic effect upon the vasculature to increase total blood flow to the muscle (Baron 1994). However, some dispute these findings, claiming no blood flow increase occurs within skeletal muscle in the presence of insulin (DeFronzo *et al.* 1981, Jackson *et al.* 1986, Natali *et al.* 1990, Richter *et al.* 1989). Other researchers support the notion that insulin possesses this capability, but there is debate as to whether or not this flow increase is simply a phenomenon that occurs at higher than physiological insulin doses, or when the subject is exposed to insulin for a long period of time (Yki-Jarvinen and Utriainen 1998). Furthermore, it is contentious whether or not the insulin-mediated increase in total flow is actually involved in the determining overall rates of insulin-stimulated glucose disposal (Laine *et al.* 1998). If it were, then an increase in total blood flow to a limb should result in a corresponding increase in glucose uptake. To this end, Baron *et al.* (1991, 1994) demonstrated that insulin induced a dose-dependent increase in leg blood flow that closely paralleled its effect on leg muscle glucose uptake (Baron *et al.* 1991, Baron *et al.* 1994). Furthermore, this relationship held in patients who were obese or suffered from NIDDM, but the magnitude of each effect of insulin was decreased (Baron 1994, Laakso *et al.* 1992). In addition to this, impaired insulin-mediated increases in total blood flow have been noted in patients with hypertension (Baron *et al.* 1993a), obesity (Baron *et al.* 1990), NIDDM (Laakso *et al.* 1992), IDDM (Baron *et al.* 1991), and aging (Meneilly *et al.* 1995).

All of the studies mentioned so far in this chapter measured only total blood flow into skeletal muscle and they did not investigate the possibility that changes in blood flow

distribution could occur within the muscle itself. It was not realised that insulin could have other haemodynamic actions until Rattigan *et al.* (1997) published a paper showing that insulin not only increased total blood flow within skeletal muscle, but also stimulated metabolism of 1-MX, a putative indicator of capillary recruitment within the muscle (Rattigan *et al.* 1997b). However the increase in capillary recruitment may not be the result of increased total flow as similar increases in total flow induced using epinephrine were not accompanied by increases in 1-MX metabolism or glucose uptake (Rattigan *et al.* 1997b). In addition, the increase in capillary recruitment appeared to account for a significant part of the insulin-mediated increase in glucose uptake into skeletal muscle. Thus, when a state of acute insulin resistance was induced in rats *in vivo* using the vasoconstrictor, α-methyl serotonin, insulin-mediated glucose uptake was significantly decreased. Furthermore, the insulin-stimulated increase in total blood flow was abolished and insulin's ability to increase 1-MX metabolism was almost entirely removed (Rattigan *et al.* 1999).

As mentioned previously in this thesis, the method used to measure capillary recruitment in these studies by Rattigan *et al.* (1997, 1999) was based on specific metabolism of 1-methylxanthine by capillary endothelial xanthine oxidase. The work presented in this thesis makes several advances to this knowledge by (a) consolidating the applicability of the 1-MX method for capillary recruitment and by (b) introducing a second technique (laser Doppler flowmetry) for the measurement of capillary recruitment (nutritive flow) within skeletal muscle, suitable for use in both in hindlimb perfusion and *in vivo*. Chapter 3 shows that exercise in skeletal muscle (as induced via sciatic nerve stimulation) gave rise to an increase in 1-MX metabolism. Since muscle exercise is known to lead to substantial increases in capillary recruitment, this strengthened the proposition that 1-MX metabolism was indeed an index of capillary recruitment.

Armed with the information that 1-MX metabolism is now likely to be a valid indicator of capillary recruitment, a further advance was made concerning TNF-induced insulin resistance (see Chapter 7). TNF infusion produced an insulin resistant state in rats *in vivo* within 3 hours. Not only was the insulin-mediated increase in total flow prevented from occurring, but also the insulin-mediated

capillary recruitment was completely prevented. Thus, it is likely that TNF exerts its effect by acting directly or indirectly upon the vasculature to prevent insulin's action to increase capillary recruitment and total hindleg blood flow.

Not all of the models of insulin resistance investigated so far can be explained by impaired haemodynamics. Sciatic nerve severance (see Chapter 6) leads to an insulin resistant state in perfusion as early as 3 hours post-surgery. However, no change was seen in either total flow to muscle (as measured by fluorescent microsphere entrapment) or nutritive flow (as measured by 1-MX metabolism). Thus haemodynamic changes would not appear to account for the insulin resistance caused by severing the sciatic nerve.

The need for other methods to support the findings obtained using the 1-MX method prompted the investigation of laser Doppler flowmetry (LDF) as a possible means of measuring nutritive and non-nutritive flow in perfusion (see Chapter 4) and *in vivo* (see Chapter 5). Also, work by collaborators in the United States to adapt the 1-MX method for use in human subjects has commenced. However, at this time, it is premature to discuss the findings in this thesis.

Early laser Doppler flowmetry equipment was developed mainly to study microvascular flow in superficial tissue layers, and the majority of the literature reports the use of LDF for measurement of cutaneous perfusion (Forst *et al.* 1998, Gonzalez-Darder and Segura-Pastor 1994, Holloway and Watkins 1977). Those studies aimed to measure the changes in perfusion of the skin capillaries under a variety of conditions. However, there are a number of studies where LDF is purported to measure microvascular perfusion in muscle (Leahy *et al.* 1999). One study published in 1987 reports a method for monitoring blood flow within muscle using insertable single-fibre LDF probes (Salerud and Oberg 1987). Further to this, Cai et al. (1996) modified the LDF probes to minimise the trauma to muscle tissue upon insertion of the LDF probe (Cai *et al.* 1996). Another study reported that changes in local tissue perfusion, of the exposed rabbit tenuissimus muscle, induced by various stimuli could be quantitated using high-resolution LDF (Linden *et al.* 1995). Thus, those studies and others (Skjeldal *et al.* 1993, Tyml and Ellis 1985) concluded that LDF can be used to measure muscle perfusion. However, work from

Kuznetsova *et al.* (Kuznetsova *et al.* 1998) and from this laboratory using stationary LDF probes, insertable LDF probes and scanning LDF systems (see Chapters 4 and 5) all suggest that LDF is specifically measuring the distribution of capillary blood flow within muscle. As discussed in Chapter 4, the larger surface probes were unable to distinguish between regions of nutritive and non-nutritive flow due to their size and the average signal reflected predominantly nutritive flow. In contrast, the smaller, impaled microprobes showed a distinct heterogeneity in their response depending on whether they were inserted into a 'nutritive' or 'non-nutritive' site, or indeed a mixture of the two. An important advance as a result of this work is the demonstration of differing patterns of flow, albeit nutritive and non-nutritive flow, within skeletal muscle. These discrete sites are seen upon insertion of the impaled microprobes and the findings support the results seen using the 1-MX method. Chapter 5 demonstrates that when insulin is present *in vivo*, there is a significant increase in nutritive flow as measured using LDF. Figure 9-1 shows a correlation between data obtained by Rattigan et al. (Rattigan *et al.* 1997b) using the 1-MX metabolism to measure changes in capillary recruitment due to insulin, epinephrine and saline, and data presented in this thesis (Chapter 5) showing changes in LDF under the same conditions *in vivo*. Thus, application of both the 1-MX method and LDF appear to be indicating similar outcomes. Furthermore, LDF is capable of measuring these changes in a more direct manner than 1-MX metabolism.

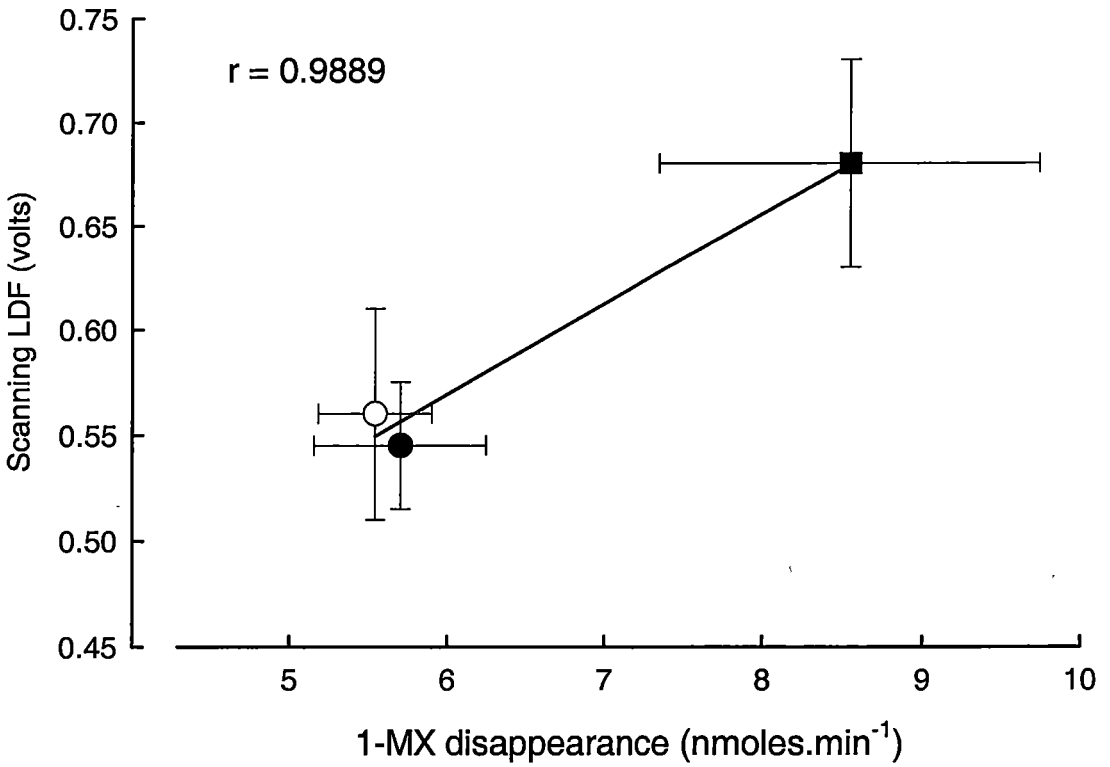


Figure 8-1 Correlation between 1-MX disappearance and LDF *in vivo* in the presence of insulin (■), epinephrine (○) or saline (●).

8.2 Other Studies

Early work in rats *in vivo* shows that changes in capillary perfusion within muscle can be measured using contrast-enhanced ultrasound. Relative blood volume during a steady state of insulin infusion was significantly higher than that observed during infusion of saline alone (see Table 8-1) (personal communication, Jonathan Lindner, University of Virginia Medical Centre, Charlottesville, USA). The relative microvascular blood volume is significantly increased during the course of the experiment of in both saline ($P < 0.05$) and insulin-treated ($P < 0.001$) animals. However, the size of the increase is much smaller in animals infused with saline than those infused with insulin. Post-infusion, there is a significant increase in relative capillary blood volume in insulin-treated rats compared with the saline group ($P <$

0.05). This increase in relative muscle blood volume corresponded with significant insulin-mediated increases in 1-MX metabolism seen in the same animals.

Table 8-1 Change in relative microvascular blood volume due to infusion of either insulin or saline, as measured using contrast-enhanced ultrasound of the rat hindleg.

	Relative Blood Volume *(Basal)*	Relative Blood Volume *(Post infusion)*
Saline infusion	4.7 ± 0.7	7.9 ± 2.0*
Insulin infusion	4.1 ± 1.7	12.5 ± 3.3** #

Values are means ± SD. *, $P < 0.05$ relative to basal. **, $P < 0.001$ relative to basal. #, $P < 0.05$ relative to saline post infusion (Student's t test). Personal communication from Jonathan Lindner, University of Virginia Medical Centre, Charlottesville, USA.

Similar data to that presented above can be seen from work by Coggins *et al.* using the human forearm clamp model (Coggins *et al.* 1999). In an attempt to measure insulin-mediated changes in capillary flow distribution in human skeletal muscle, Coggins *et al.* (1999) used contrast-enhanced ultrasound to measure microvascular blood volume (Coggins *et al.* 1999). Microvascular flow velocity was also determined from the relationship between the ultrasound pulsing interval and video intensity during constant intravenous infusion of albumin microbubbles. Results showed that upon infusion of a physiological dose of insulin or saline for 3 hours, there was a significant increase in glucose uptake as compared with saline controls. Furthermore, insulin also led to a significant increase in microvascular volume (MV VOL) and decreased microvascular velocity (MV VEL), without significantly increasing total forearm blood flow as shown in Table 8-2 (Coggins *et al.* 1999). Their data further supports the rat data communicated by Lindner (see above) and

work using LDF and 1-MX showing that insulin administration leads to increased capillary recruitment within skeletal muscle *in vivo* in rats.

Table 8-2 Changes in microvascular volume (MV VOL) and velocity (MV VEL), forearm blood flow, and glucose uptake as a result of forearm insulin infusion in healthy human subjects.

	Glucose uptake µmol/min/100ml		[Insulin] µU/ml		MV VOL		MV VEL		Forearm blood flow ml/min	
Time	**0hr**	**3hr**	**0hr**	**3hr**	**0hr**	**3hr**	**0hr**	**3hr**	**0hr**	**3hr**
Insulin	1.2	3.6*	5	58*	14	22*	0.32	0.16*	66	86
Saline	1.4	1.4	4	4	13	14	0.25	0.2	63	71

Values shown are the means from 10 subjects (6 infused with insulin and 4 with saline). *, $P < 0.05$ versus 0 hr. Reproduced from Coggins *et al.* (Coggins *et al.* 1999).

Another area which warrants further study is the possibility that other methods targeted at measuring capillary blood flow distribution within skeletal muscle may be developed using the same principles as the 1-MX method is based on. Infusion of substances that, like 1-MX, are metabolised by capillary enzymes could be used to measure nutritive flow in muscle. Examples of possible candidates include angiotensin-converting enzyme (ACE) which is located in the endothelial cells of skeletal muscle capillaries (Schaufelberger *et al.* 1998), and alkaline phosphatase. Similarly, methods aimed at measuring non-nutritive flow could involve targeting connective tissue enzymes, such as UDP glucosyl transferase, or enzymes involved in collagen synthesis. Another enzyme to measure non-nutritive flow has already been investigated recently by Clerk *et al.* (2000). That study demonstrated that during a state of high non-nutritive flow (produced by serotonin infusion) where oxygen

consumption was decreased, triglyceride hydrolysis was significantly enhanced as shown in Table 8-3 (Clerk *et al.* 2000). This indicates that lipoprotein lipase activity is greater when non-nutritive flow predominates and appears to be attributable to resident adipocytes located on the non-nutritive connective tissue vessels (Clerk *et al.* 2000).

Table 8-3 Effect of serotonin on triglyceride (TO) hydrolysis, VO_2 and perfusion pressure in the perfused rat hindlimb at constant-flow

	Control	Serotonin
TG hydrolysis (nmol FFA/h/g)	184 ± 28	602 ± 132*
VO_2 (μmol/g/h)	16.7 ± 0.6	10.2 ± 1.0#
Perfusion pressure (mmHg)	70.6 ± 5	170 ± 27*

Perfusions were performed at constant-flow. Values are means ± SE. *, $P < 0.05$; #, $P < 0.001$ for serotonin v's control. Table modified from Clerk *et al.* (Clerk *et al.* 2000).

9.3 Towards the Future

The most important point emerging from this thesis is the congruence between two independent methods to measure capillary recruitment within skeletal muscle. Agents known to increase nutritive flow in perfusion also show an increase in LDF signal. Conversely, agents known to decrease nutritive flow in perfusion, result in a decrease in LDF signal and a decline in 1-MX metabolism. Furthermore, *in vivo* under hyperinsulinaemic euglycaemic clamp conditions, insulin increases both 1-MX metabolism and LDF signal.

Further development of both the 1-methylxanthine metabolism method and laser Doppler flowmetry for use in human subjects will pave the way for application of these techniques in the clinical setting. In order to be applicable for humans studies, the LDF probe would have to be redesigned to incorporate the properties of the currently used surface probe for measuring signals from no less than 1 mm^3, and be suitable for implantation. With this attended to, it will be possible to assess insulin-mediated muscle haemodynamic responses using LDF. These methods, together with additional techniques including contrast-enhanced ultrasound, could be used clinically to monitor the state of microvascular perfusion or nutritive flow in obese, hypertensive or Type II diabetic patients.

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