Vascular control of resting metabolism in muscle

by

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Contents

Abstract

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Whilst it is generally accepted that the metabolic rate of muscle will determine its own blood flow by autoregulation the reverse relationship of blood flow controlling metabolism is less understood. Investigations by Clark and colleagues (Clark *et al.* 1995) suggest the existence of two different vascular systems in the perfused rat hindlimb. One of these systems appears to be nutritive for muscle while the other system appears to behave as a functional shunt. When flow is selectively directed through either of these systems by vasoconstrictors or vasodilators profound metabolic changes are observed. One set of vasoconstrictors, Type A, can double the resting metabolic rate of the perfused hindlimb while the other set, Type B, can halve the resting metabolic rate of the perfused hindlimb.

The aim of the present thesis was to obtain further evidence for two discretely different vascular systems in muscle and to investigate the histological nature of these two different vascular systems.. A secondary aim of the thesis was to assess the control of selective vascular recruitment over key metabolic pathways such as lactate metabolism and glycolysis, glycerol metabolism lipolysis and respiration.

The first group of experiments provided further evidence for two different vascular systems in the hindlimb. One of these experiments used fluorescent microspheres to measure blood flow distribution between muscle groups and methyl-xanthine (an indicator of vascular recruitment) to assess the degree of nutritive flow. Another set of experiments provided fluorescent dye-tracing evidence for vessels supplying septa and tendons being non-nutritive conduits for the Type B response.

Investigations into vasoconstrictor and flow-rate control of lactate uptake and release by muscle showed that both Type A norepinephrine (ie., concentrations of norepinephrine below 1 μM) and increased-flow, stimulated lactate release from the perfused hindlimb. However, when 10 mM lactate was added to the perfusion medium to stimulate lactate uptake increased flow was found to increase uptake while Type A norepinephrine decreased lactate uptake. These data indicate a mechanistic difference between flow and vasoconstrictor-stimulated metabolism. Glycerol release from the hindlimb was also regulated by vasoconstrictors suggesting a vascular control of lipid metabolism. However, no concurrent decrease in respiratory quotient or increase in fatty acid release was found during Type A stimulation, thus suggesting a vascular control of the fatty acid reesterification cycle.

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

Declaration

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J.T. Steen

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J.T. Steen

Preface

The experimental work presented in this thesis was conducted between January 1993 and September 1996 in the Division of Biochemistry at the University of Tasmania. All experiments were approved by the Ethics Committee at the University of Tasmania under the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (1990). Work performed by other members of this department has been presented and acknowledged where relevant. In particular I would like to thank John Newman and Prof. Clark for allowing me to include their tendon fluorometry study in this thesis.

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Publications arising from Ph.D. studies

"Vasoconstrictor-mediated release of purines and pyrimidines from perfused rat hindlimb, perfused mesenteric arcade and incubated de-endothelialized aorta" (1994) Richards S.M., Clark M.G., Steen J.T., Dora K.A and Colquhoun E.Q. *Gen. Pharmacol.* **25:** 1679-1690.

"Purine and pyrimidine nucleotide metabolism of vascular smooth muscle cells in culture" James S.G., Appleby G.J., Miller K.A., Steen J.T., Colquhoun E.Q. and Clark M.G. (1996) *General Pharmacology* **27:** 837-844.

"Vascular and metabolic regulation of muscle" Clark M.G., Rattigan S., Dora K.A., Newman J.M.B., Steen J.T., Miller K.A. and Vincent M.A. In:Physiology, Stress and Malnutrition, ed. J. Kinnney and H Tucker, Lippincott-Raven, New York 1996.

"α-Adrenergic stimulation of thermogenesis in a rat kangaroo (Marsuplia, *Bettongia gaimardi*)". Ye-J-M. Edwards S.J., Rose R.R., Steen J.T., Clark M.G. and E.Q. Colquhoun (1996) *Am. J. Physiol.* In Press.

"Resting muscle thermogenesis: novel mechanisms." Steen J.T., Vincent M.A. and Clark M.G. In: Adaptations to the cold: Tenth international hibernation symposium., ed. F. Geiser, A.J. Hulbert and S.C. Nicol, University of New England Press, Armidale 1996.

"Different effects of norepinephrine and flow on lactate balance in the constant-flow perfused rat hindlimb" Ye J-M, Steen J.T., Matthias A., Clark M.G. and Colquhoun E.Q. *Can. J. Physiol. Pharmacol.* (Under Review)

"Serotonin inhibition of 1-methylxanthine metabolism indicative of enhanced non-nutritive flow in muscle" Rattigan S., Appleby G.J., Miller K.A., Steen J.T., Dora K.A., Colquhoun E.Q. and Clark M.G. *Am. J. Physiol.* (Submitted August 1996)

"Evidence for vessels supplying septa and tendons acting as functional shunts in the perfused rat hindlimb." Newman J.M.B., Clark M.G. and Steen J.T. *Microvasc. Res* (In Press).

Chapter 1

General Introduction

Circulatory transport systems have been essential for the evolution of higher level organisms. Whilst the simplest cellular organisms can rely on nutrients diffusing from the extracellular environment this is not possible in larger animals such as vertebrates. As a consequence these larger animals have developed vascular systems to enable nutrient delivery and removal of waste products.

The generally accepted view of the relationship between the vasculature and skeletal muscle is that muscle perfusion occurs as a response to the demand from the muscle tissue. However, the intriguing possibility exists that the blood vessels themselves, under the influence of vasoactive hormones, may control muscle metabolism by regulating the perfusion of muscle capillaries.

The aim of the present thesis was to investigate the mechanisms by which vasoactive hormones increase or decrease perfusion of the muscle capillaries and, in particular, to investigate the effect of these hormones on respiration as well as carbohydrate and fat metabolism.

To achieve this aim Chapter 2 provides a literature review of current theory and controversy surrounding regulation of muscle metabolism by perfusion. This review also considers some possible physiological consequences of muscle metabolic regulation by blood flow. The experimental model used throughout this study is the perfused rat hindlimb. Many other studies have used this technique and a critical review of this literature also appears within chapter 2.

The first two experimental chapters (Chapters 4 & 5) explore the mechanism by which vasoconstrictor hormones positively or negatively regulate muscle perfusion in the surgically isolated rat hindlimb. The experiments described in Chapter 4 were designed to provide evidence for different vascular networks within perfused muscle that could divert flow away from the muscle capillaries. These experiments used fluorescent microspheres to measure blood flow distribution between muscle groups and methyl-xanthine conversion to measure the

extent of capillary perfusion. Chapter 5 used fluorescent dye-tracing techniques to ascertain perfusate distribution, through non-muscle tissues, when flow was directed away from muscle by vasoconstrictors.

Chapters 6 and 7 investigated the metabolic consequences of flow being diverted to, or away from, the muscle by vasoconstrictors. In particular, Chapter 6 focused on the effect of vasoconstrictors on lactate uptake and release from the perfusion medium. Chapter 7 examined the effect of vasoactive agents on key indicators of lipid metabolism such as respiratory quotient, and both glycerol and fatty acid release.

Chapter 2

Literature Review

2.1 Flow regulation of resting muscle metabolism.

In mammals, skeletal muscle represents the bulk of dry tissue weight. It is unusual in its capacity to sustain a wide range of metabolic rates. A less well known but potentially very important feature of muscle physiology is its ability to adjust its metabolic rate according to the delivery of substrate. This relationship between perfusion and metabolism is known as "metabolic conformity" (Hochachka and Guppy 1987).Physiologists have classified body tissues as metabolic conformers or metabolic regulators. Metabolic conformers adjust metabolism according to perfusion whilst metabolic regulators maintain metabolism regardless of perfusion. Brain tissue is a good example of a metabolic regulator whilst liver and skeletal muscle are generally regarded as metabolic conformers. Perhaps the most startling example of muscle metabolic conformity is the recovery, often after hours of ischaemia, of severed fingers after reattachment. In contrast, an ischemic brain can only survive for minutes before being irreversibly damaged.

Although the term "metabolic conformer" may describe the perfusion-metabolism relationship of skeletal muscle, it does not provide a metabolic explanation of how the muscle cells "sense" the degree of perfusion nor of what metabolic cycles are initiated or depressed when flow is increased or decreased. The search for a unifying hypothesis to explain perfusion-metabolism in perfused tissues has recieved considerable attention over the last eighty four years but has thus far remained elusive.

Flow induced metabolism in muscle has been scrutinized by many investigators. As early as 1912, Verzar showed that autoperfused cat muscle consumed more oxygen as blood flow was increased (Verzar 1912). These observations were subsequently repeated by other investigators using different perfusion preparations (Pappenheimer 1941, Wright and Sonnenshein 1965). The

general interpretation of these results was that delivery of oxygen was insufficient for basal metabolism. From these studies a paradox arose regarding oxygen delivery and the extremely high affinity (low K_m) of mitochondrial respiratory enzymes for oxygen. When mathematical models of tissue oxygenation were applied to the perfusion of rat gracilis muscle, assuming an inter-capillary distance of 36 μm and a capillary radius of 3.5 μm, it was predicted that no cells would be at an oxygen tension of less than 25 mmHg at a rate of oxygen delivery where increased flow could further increase the rate of oxygen consumption (Honig *et al.* 1971). These results indicated that the assumptions of uniform capillary spacing and perfusion were probably incorrect and that heterogeneity was an important consideration in perfusion. In a classic study by Whalen and colleagues (Whalen *et al.* 1973) involving flow induced metabolism and measurement of muscle PO_2 it was concluded that tissue oxygen levels were too high to limit mitochondrial respiration. For example, it was found that only 9 out of 138 measurements of gracilis muscle PO_2 were below 8 mmHg whilst the K_m for isolated mitochondria has been measured to be less than 0.5 mm Hg. An interesting aspect of this study by Whalen was the wide variation in the concentrations of tissue oxygen. This heterogeneity has been observed in many other perfused muscle preparations using both microelectrode (Renkin *et al.* 1981, Kunze 1968) and a microtonometer (Van Liew and Rodgers 1975). The reported variability in tissue oxygenation lends further support to the notion of blood flow heterogeneity in perfused muscle. However it should be pointed out that all of the oxygen concentrations were above the K_m for oxygen obtained in isolated mitochondrial studies.

It remains a controversial topic as to whether mitochondrial oxygen concentrations in resting muscle actually falls below the Km of cytochrome oxidase. It is likely that mitochondria *in situ* have oxygen saturation kinetics that are different from those of isolated mitochondria so that isolated mitochondria have a far greater affinity for oxygen than mitochondria in muscle tissue (Wilson *et al.* 1979 a).Wilson and others have demonstrated that in cell suspensions, reduction of cytochrome c is dependent on PO_2 levels of up to 150 mmHg (Wilson *et al.* 1979 b). From this data Wilson and colleagues suggest that the apparent K_m

for oxygen is strongly dependent on the state of reduction of cytochrome c and on the [ATP]/[ADP][Pi] ratio (Wilson *et al.* 1979 b.)

To accept this explaination one must also accept the hypothesis of the cytochrome oxidase catalytic cycle that incorporates a reversible O_2 binding step occurring before a final irreversible reaction (Hochachka and Guppy 1987). If this proposal is correct then the reversible oxygen binding step in the cytochrome oxidase reaction will exert a degree of control over the respiratory chain because all preceding reactions to this point are reversible (Wilson *et al.* 1979 a). However, if the O_2 binding step is irreversible then the mitochondria will behave as an oxygen "sink". The kinetics of cytochrome oxidase *in vivo* are not well understood and are worthy of further investigation.

An alternative hypothesis, to the notion of altered mitochondrial respiration kinetics *in situ* as an explaination for metabolic conformity, postulates that there is a significant oxygen diffusion limitation to mitochondrial energy metabolism. This diffusion limitation prevents the supply of oxygen to mitochondria even though the mitochondria may have a high affinity for the oxygen. Oxygen diffusion gradients exist from the blood cell in the capillary down to the mitochondria within the muscle fibres. This limitation for oxygen diffusion in whole cells could explain oxygen conformity at both the cellular (Kennedy and Jones 1986) and whole organism level (Brown 1992). For example, Himalayan mountaineers certainly feel as if their working muscles are oxygen limited, even though the O_2 concentration in the arterial plasma may be 100 fold greater than the K_m for isolated mitochondria (Hochachka and Guppy 1987). However, experimental evidence for true diffusion limitation of oxygen consumption across the muscle cell from the plasma membrane to the mitochondria is somewhat unconvincing. Some investigators have used fluorescent probes to observe the microdistribution of oxygen within living cells and have noted a "patchy" O_2 distribution suggesting a limited diffusion of O_2 within cells (Benson *et al.* 1980). These data along with previous work reviewed by Chance (1976) are consistent with the notion that diffusional limitation may truly limit cellular metabolic rate.

There is no clear agreement regarding diffusion limitation in perfused skeletal muscle. Freeze clamp studies of myoglobin saturation *in situ* suggest that no metabolism-limiting O_2 gradients exist within living cells (Connett *et al.* 1984, Gayeski *et al.* 1987). Other similar investigations in isolated myocytes contradict these findings with measurements showing that the oxidation state of cytochrome a3 parallels the oxygenation of myoglobin (Kennedy and Jones 1986). This suggests that there are significant diffusion gradients between mitochondria and the extracellular medium so that delivery of oxygen from myoglobin will control the rate of respiration (Kennedy and Jones 1986). Similar results have been obtained using the surgically isolated perfused rat hindlimb model (Seiyama *et al.* 1991). In this study a linear relationship was observed between spectrophotometrically observed cytochrome a +a3 oxidation, and myoglobin saturation. The authors suggested that this relationship could provide evidence for a significant oxygen gradient between cytosol and mitochondrion, thus creating an oxygen diffusion limitation to respiration. If there were no diffusion barrier to prevent the transfer of oxygen from the myoglobin to the mitochondria then the oxidation of the mitochondria would be independent from the oxidation of the myglobin, because of the vastly greater affinity of the mitochondria for oxygen (Seiyama *et al.* 1991). Furthermore this study indicates that the "critical point" of tissue oxygen tension, below which oxygen supply may limit consumption, may be as high as 10mmHg.

Wilson and colleagues have proposed that an oxygen electrode with the same dimension as a mitochondrion may be useful for assessing oxygen gradients across the mitochondrial membrane because these electrodes effectively behave as oxygen sinks with an extremely low K_m for oxygen. At an oxygen tension of 1 mmHg, in a simple aqueous solution, a micro oxygen electrode consumes ten times more oxygen per unit surface area than a mitochondrion at saturating oxygen concentrations. If the electrode model represents true oxygen limitation then it is unlikely that steep gradients to the mitochondrion are sufficient to explain the relationship between oxygen supply and metabolism (Wilson *et al.* 1979). However it has been suggested that it is not valid to extrapolate from the microelectrode to the mitochondrion for at least two reasons (Hochachka and Guppy 1987). Firstly

the solubility of O_2 may vary in different parts of the cell and secondly, local variations in viscosity may occur within cells (Mastro *et al.* 1984).

Thus the answer to the question regarding the limitation of mitochondrial metabolism *in situ* by oxygen remains unanswered. It does however appear that both oxygen gradients and altered *in vivo* cytochrome oxidase kinetics must be considered in any investigation of oxygen delivery and metabolism. In their elegant review of this field of physiology, Connett *et al.* (1990) suggest that the critical tissue PO_2 (where mitochondrial function becomes compromised) varies with metabolic compensations. These incude phosphorylation state, the cytosolic and mitochondrial redox potontial, and the substrate supply to both the mitochondrial and glycolytic subsytems. The difficulty of identifying O_2 limitation in a perfused tissue was also noted by Dubois-Ferriere and Chinet (1981). These authors stressed that O_2 limitation at the cellular level cannot be proved or disproved on the sole basis of flow rate, O_2 extraction coefficients or tissue and venous PO_2 measurements.

It is important to realize that flow-induced oxygen consumption, identified under experimental conditions, is not simply a matter of improving perfusion to an *in vivo* level. It seems likely that muscle metabolism *in vivo* is limited by cardiac output and oxygen supply to the muscle (Chinet and Mesjnar 1989, Brown 1992). The *in vivo* limitation of oxygen supply has been termed as "physiological ischaemia" which regulates resting muscle metabolism (Chinet 1990).

2.2 Sources of heterogeneity

Further evidence for heterogeneity of flow in perfused muscle has come from arterial injection of radioactive microspheres. These uniform microspheres do not distribute evenly through the muscle reflecting the variety of flow rates through different parts of the perfused tissue (Piiper *et al.* 1985, Iversen *et al.* 1989). Clearance of locally injected ^{133}Xe into skeletal muscle has also revealed significant heterogeneity of perfusion within muscle tissue. Other experiments involving washout of inert gasses showed that the washout curves from perfused skeletal muscle were not monoexponential, also indicating the existence of perfusion heterogeneity (Piiper and Meyer 1984). These observed heterogeneities may arise

by various means. A review by Duling and Klitzman (1980) identified three significant elements that were capable of modulating the distribution of total blood flow to tissues. These elements are : (a) the number of open capillaries (capillary density); (b) the presence of non-capillary pathways from artery to vein (shunts); and (c) the range of variation in type, length, and flow pattern of the capillary bed (unequal distribution of flow).

2.2.1 Capillary Density

Krogh put forward the idea that at rest some capillaries carry no flow in resting muscle. In his early work he reported that the density of perfused capillaries could increase up to 35 fold during exercise (Krogh 1919). However, recent results have disputed these findings and the exercise-induced increase in capillary density has been suggested to be closer to 2-4-fold (Duling and Klitzman 1980) There is histological evidence to suggest that capillaries are arranged in units called capillary bundles. It has been reported that after a 5-10 second arterial infusion of indian ink a clustering pattern of perfused capillaries was noted (Renkin 1981). These bundles are fed by one arteriole (Skalek *et al.* 1985) and are collected by one venule. It has been proposed that these arterioles act as "precapillary sphincters" to shut down perfusion to these bundles. These arterioles have been shown to be the most important regulators of capillary perfusion (Sweeney and Sarelius 1989). This is in contrast to the original idea of Krogh who proposed that the capillary was the smallest independently controlled unit.

2.2.2 Unequal distribution of flow

Experimental evidence for closed capillaries has usually come from studies looking at a "snapshot" of blood flow by perfusion with a vascular marker for a very short period of time. Subsequent experiments have shown that if these markers are infused for longer periods of time, eventually all of the capillaries will become perfused (Renkin 1981). This indicates that rather than having some unperfused capillaries there is a wide variation in the rate at which these capillaries are perfused. Current evidence favors the interpretation of recruitment as an increase in the velocity of capillary flow rather than the opening of unperfused

capillaries (Hargreaves *et al.* 1990, Iversen and Nicolaysen 1991, Snyder *et al.* 1992). Unequal distribution of flow will result in an exchange inefficiency in capillaries with high flow rates (Renkin 1969) whilst some areas of tissue surrounded by slowly perfused capillaries remain underperfused. An interesting study using the primary respiratory organ of the early chick embryo suggests that a third of all A-V channels have a transit time shorter than that required for complete oxygenation (Meur and Bertram 1993). Similar estimates of oxygen tension in individual capillaries have yet to be made in skeletal muscle. The exact cause of this unequal distribution is uncertain. One explanation is that Poiseuilles law predicts that blood will flow preferentially along shorter capillaries with less resistance (Renkin 1969). It has been proposed that although these vessels account for only 13% of capillary density, such capillaries could theoretically carry 70% of blood flow under resting conditions (Harrison *et al.* 1990). An alternative to this, which accentuates the role of the terminal arteriole in the regulation of capillary perfusion, stems from the observation that the least perfused capillaries are fed by the arterioles with the smallest diameter and therefore highest resistance (Sweeney and Sarelius 1989). At the level of the capillary bundle the degree of perfusion of the bundle depends on the perfusion pressure after the terminal arteriole. It follows therefore that if a large pressure drop occurs after the terminal arteriole then fewer vessels in the bundle will be perfused.

2.2.3 Shunts

It is well documented that there are no anatomical shunts in skeletal muscle, whereby large thoroughfare channels allow blood to bypass the capillary bed (Hammersen 1970). It is possible however that muscle possesses what are known as "functional shunts". If these functional shunts exist in muscle they are probably manifested as non-nutritional capillary networks. Such a network would be anatomically distinct from the skeletal muscle circulation. It has been proposed that the non-nutritive circulation is located in the intermuscular septa and tendons (Barlow *et al.* 1959). Many of the terminal vessels of the tendons are wider than capillaries and are therefore capable of carrying a large proportion of blood flow (Grant and Payling Wright 1970). These vessels are well insulated from muscle by

surrounding collagenous tissue. The hypothesis of these vessels acting as functional shunts has been discussed in recent publications (Lindbom and Arfors 1984, Chinet 1990, Schmid-Schonbein 1991).

2.3 Implications of heterogeneity for flow induced metabolism

Lindbom and Arfors (1984) have suggested that the extent of capillary perfusion is dependent on the perfusion presure at the terminal arteriole proximal to the capillary bed. Therefore at low perfusion pressures only the capillaries with least resistance will be perfused. As flow is increased the capillaries will be progressively filled, thus improving perfusion and increasing metabolism. This would account for the flow and oxygen relationships reported by Whalen *et al.* (1973) and Ye *et al.* (1990). However, perfusion heterogeneity may distort the relationship between net flow and metabolism. For example, increasing flow to muscle will not increase the muscle metabolism if the flow is being directed through functional shunts such as the short capillaries envisaged by Harrison (Harrison *et al.* 1990). The way in which perfusion heterogeneity distorts the relationship between total flow and metabolism has been discussed by Piiper (1992). Based on hypothetical models, Piiper argues that if heterogeneous flow exists then the relationship between oxygen delivery and the O_2 uptake/O_2 requirement[1] ratio will be a gradual departure from linearity. This is different from the single critical point of oxygen delivery predicted from the homogenous flow model (Fig 2.1). Thus heterogeneity and shunting may create an "apparent" affinity for O_2 in perfused muscle which is substantially lower than that observed for isolated cells.

[1] In this hypothetical model, O_2 requirement is the maximum resting respiration rate under saturating conditions of oxygen delivery. Thus when the rate of oxygen uptake satisfies the muscle's requirement for oxygen this theoretical ratio will equal 1.

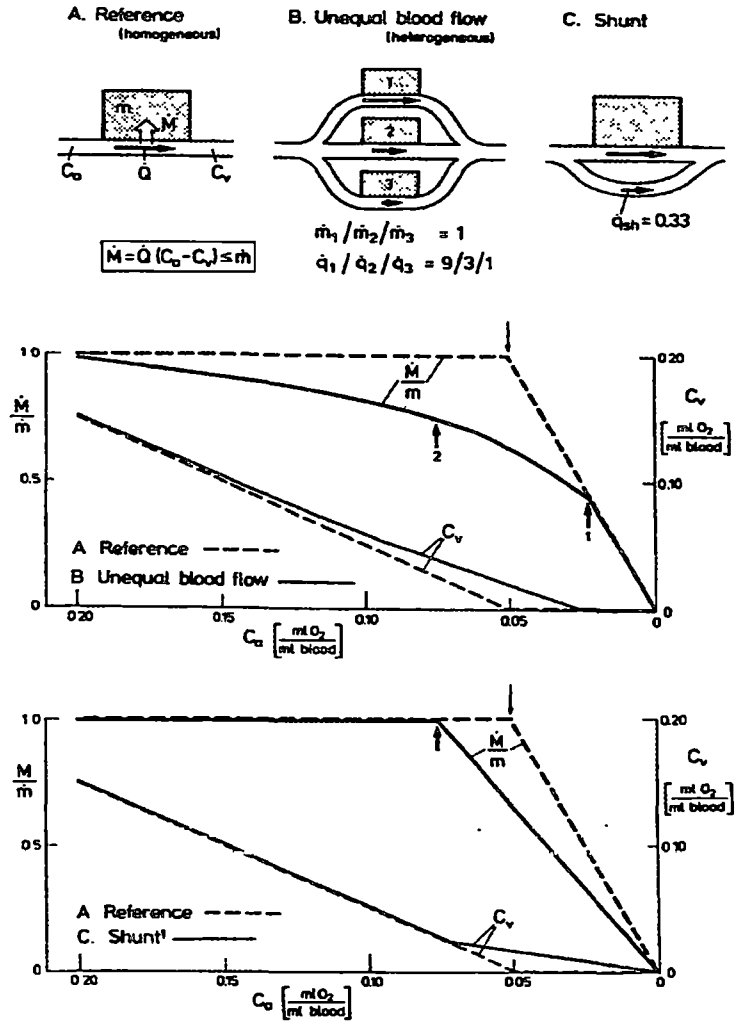

Figure 2.1. Models for perfusion limited O_2 supply and their dependence on arterial O_2 content (Ca) at constant blood flow (Q) and O_2 requirement (m). Venous O_2 content (Cv) and the O_2 uptake/O_2 requirement ratio (M/m) are shown. The behavior of the reference (homogenous) model is represented by dashed lines. Arrows mark onset of critical O_2 supply conditions (Piiper 1992).

The presence or absence of heterogeneity or shunts in different perfused muscle preparations may explain why there is such a large variation in the reported effects of increased flow on muscle metabolism. Some studies using the perfused rat hindlimb were not able to reach a flow induced maximum oxygen consumption (Mc Dermott *et al.* 1989, Ye *et al.* 1990). The hindlimb preparation in these studies consumed oxygen in proportion to the rate of oxygen delivery demonstrating a classic metabolic conformer. Another study using slightly different

perfusion conditions demonstrated that flow induced oxygen consumption reached an apparent "critical" rate of oxygen delivery. Further increases in the flow rate at this point did not further stimulate oxygen consumption (Gorski *et al.* 1986). In that study the muscle behaved as a metabolic regulator. It has been suggested that this observation of a critical rate of oxygen delivery is the result of muscle cells being "protected" by haemodynamic mechanisms from receiving non-limiting amounts of oxygen and substrates (Chinet 1990). This view is supported by the demonstration that the relative phosphocreatine content of muscle tissue tends to be larger in oxygen conformers than in oxygen regulators at arterial PO_2 levels above 40 mmHg (Gutterierez *et al.* 1989).

2.4 Vasoconstrictive control of heterogeneity and metabolism

The potential for vasoconstriction to regulate heterogeneity was noted as early as 1941 by Pappenheimer. His experiments demonstrated that stimulation of vasoconstrictor nerves in the autoperfused dog gastrocnemius muscle resulted in a decrease in oxygen consumption. In the same study it was shown that epinephrine infusion caused an increase in oxygen consumption. Pappenheimer interpreted these results to mean that epinephrine increased nutritive flow to muscle whilst vasoconstrictor nerve stimulation decreased nutritive flow. A later study by Sonnenschein and Hirvonen (1961) investigating the effect of epinephrine, norepinephrine, sympathetic nerve stimulation and arterial clamping on force development in working cat muscle provided further evidence for vasoconstrictor regulated muscle metabolism. This study showed that the infusion of epinephrine or norepinephrine or sympathetic stimulation diminished maximal muscle force in a similar manner to arterial clamping. Some disparity in the effects of sympathetic stimulation or vasoconstrictor infusion among these studies appears throughout the literature. For example Duran and Renkin found that high frequency sympathetic stimulation in the autoperfused dog hindlimb preparation caused an inhibition of oxygen consumption with a rise in resistance. However, low frequency stimulation while increasing vascular resistance increased oxygen consumption (Duran and Renkin 1976). This incongruity of results in similar studies is most likely due to small variations in experimental procedure such as frequency of nerve stimulation

or vasoconstrictor concentration (reviewed by Clark *et al.* 1996). This is particularly well exemplified in a study by Dubois-Ferriere and Chinet who noted that the vasoconstrictor-mediated metabolic effect of norepinephrine was dependent on the concentration of norepinephrine. Infusion of 0.1 μM norepinephrine caused an increase in the constant flow perfused mouse hindlimb. Addition of 10 μM norepinephrine further increased the perfusion pressure but caused a decrease in oxygen consumption (Dubois-Ferriere and Chinet 1981). Importantly these authors concluded that the norepinephrine effect was a result of haemodynamic control by the vasculature rather than a direct effect on muscle because the increase in metabolism could only be obtained in the perfused rather than incubated (perifused) mouse muscle preparation. The notion of vasoconstrictors being able to increase or decrease metabolism in perfused muscle preparations but not incubated muscle was more fully appreciated by Clark and colleagues who categorized vasoconstrictors according to their effect on muscle metabolism (Clark *et al.* 1995). Thus vasoconstrictors were classified into two groups, designated as "type A" which stimulated metabolism and "type B" which inhibited metabolism (Table 2.1). It is significant that many of the vasoconstrictors in each group will obviously have different modes of action. For example vasopressin and norepinephrine do not share common receptors. This further supports the hypothesis that these effectors work by vasoconstriction rather than by a direct effect on the muscle itself. The vasoconstrictor control of metabolism is not restricted to oxygen consumption. Type A vasoconstrictors increase lactate, glycerol, urate and uracil efflux as well as increasing pressure and oxygen consumption. Type B vasoconstrictors, on the other hand, decrease the efflux of the metabolites. The exact reason for vasoconstrictors being able to regulate metabolism in a positive or negative manner probably lies with the ability of vasoconstrictors to regulate perfusion of capillaries (Newman *et al.* 1996).

Table 2.1 TypeA and Type B vasoconstrictors in the perfused rat hindimb (Clark *et al.* 1996).

Type A	Type B
Norepinephrine	Noreinephrine (> 1 μM)
Phenylephrine	Serotonin
Angiotensin II	Capsaicin (> 1 μM)
Capsaicin	High-frequency sympathetic nerve stimulation (> 4 Hz)
Low-frequency sympathetic nerve stimulation (0.5-4 Hz)	

Vasodilators have also been noted to affect the metabolism of perfused muscle by haemodynamic mechanisms. Ye and colleagues noted that the vasodilator, nitroprusside attenuated the flow induced pressure development and oxygen consumption (Ye *et al.* 1990). This must mean that significant control of heterogeneity occurs in the vessels before the capillaries and that the perfused hindlimb in this case is not maximally dilated. Many investigations involve the arterial infusion of dilators to ensure maximal perfusion of the hindlimb. The assumption that vasodilators will improve perfusion may be erroneous because dilation may increase pre-capillary heterogeneity in the same way that low pressure increases capillary heterogeneity. This notion is supported by several in vivo studies which show that vasodilators increase rather than decrease heterogeneity (reviewed by Clark *et al.* 1996).

2.5 Flow Sensors.

There is evidence to suggest that the delivery of oxygen, beyond the critical rate may continue to stimulate its own consumption. For example, rat hindlimb perfusions using virtually identical surgical techniques and perfusion media have

shown that increasing the flow rate will stimulate oxygen consumption without an apparent plateau (Ye *et al.* 1990), but increasing arterial oxygen tension whilst keeping the flow rate constant causes an increase in oxygen uptake to an apparent critical level followed by a plateau (Seiyama *et al.* 1990). Flow through muscle itself has been identified as a possible regulator of muscle metabolism and a probable regulator of fatigue during exercise (Stainsby *et al.* 1991). In a review on the mechanism of fatigue of exercising muscle *in situ*, Stainsby and colleagues point out that the onset of fatigue does not correlate well with a depletion of energy stores or intracellular pH due to lactate acidosis. On the other hand reduced blood flow through the muscle correlated well with fatigue to an extent where reduced flow and fatigue developed in parallel so that the arterio-venous oxygen extraction from the perfusate remained constant. This matching response did not seem to be related to changes in the availability of oxygen to the mitochondria (Stainsby *et al.* 1991). These authors concluded that blood flow affected excitation-contraction coupling by an unknown mechanism, probably related to a flow sensor within the vascular bed.

Further evidence for flow being a prime regulator of metabolism can be found in the studies of Rubio and coworkers who demonstrate a positive dromotropic response caused by flow in the perfused guinea pig heart (Rubio *et al.* 1990). These authors provide evidence that endothelial shear-stress, causing the release of paracrine substances, is the basis of the flow effect and not simply the delivery of substrate or oxygen. Interestingly these results also imply a role for extracellular calcium. A possible sequence of events may be increased flow causing shear stress and release of a paracrine substance which causes an influx of calcium into the cardiac tissue. If this is indeed the mechanism for the positive inotropic effect of flow then it is possible that the same mechanism may be responsible for vasoconstrictor- and flow-induced metabolism in skeletal muscle.

2.6 Flow regulation of metabolic pathways.

Flow and vasoconstrictor regulation of muscle metabolism is not restricted to oxygen consumption and exercise performance. Increased flow has been shown to cause an increase in glucose uptake in the perfused rat hindlimb (Grubb and

Snarr 1977) which has been interpreted as a saturation of the plasma-membrane glucose transporters. In the same study it was noted that at supraphysiological flow rates the rate of glucose uptake reached a plateau despite evidence that this plateau was not a consequence of glucose transporter saturation. The authors concluded that at high flow rates the perfusate would be directed through functional shunts thus effectively reducing the flow rate within the muscle (Grubb and Snarr 1977).

Glucose uptake has also been shown to be positively or negatively influenced by vasoconstrictors, presumably by regulating the proportion of nutritive flow to non-nutritive flow. For example Richter *et al.* (1982) have reported an alpha adrenergic stimulation of glucose uptake in the perfused rat hindlimb. This result highlights the importance of the perfused vasculature for metabolic regulation because previous studies involving isolated incubated muscle were unable to find an alpha mediated glucose uptake (Young *et al.* 1985). Higher doses of norepinephrine (10 μM) approaching doses thought to occur at sympathetic synaptic clefts (Esler *et al.* 1990) and serotonin depress the rate of insulin-mediated glucose uptake in the perfused hindlimb suggesting the diversion of flow to functional shunts by these vasoconstrictors (Rattigan *et al.* 1993, 1995). Glycolysis and lipolyis may also be regulated by perfusate flow distribution (Clark *et al.* 1994).

2.7 Biological examples of flow regulating metabolism

During exercise *in vivo* it is widely accepted that blood flow to the working muscles will be set to meet the needs of metabolism. There are however lesser known *in vivo* examples of a converse relationship whereby blood flow regulates metabolism to fulfill an important physiological role. An excellent example of this is diving animals, in particular diving seals, which have been studied extensively for over sixty years. It was first noted by Irving (1938) that, if the rate of oxygen consumption were unregulated, the oxygen reserve was insufficient for all tissues during a long dive. This implied that the metabolic rate of non-critical organs, in particular skeletal muscle, was depressed during long dives. Furthermore, in later studies, it became apparent that anaerobic metabolism did not fully compensate for this decline in oxygen consumption (Hochachka and Guppy 1987). An important

clue to this metabolic regulation lies in the observation that decreased cardiac output and vasoconstriction occured during diving, thus suggesting that both the alteration of blood flow rate and the diversion of flow away from inactive muscles was regulating the metabolism of these animals (Cherepanova *et al.* 1993).The effect of diving on heart rate, muscle blood flow and vasoconstriction has been shown to be predominantly mediated by catecholamines (Matyukhin *et al.* 1988). Radiolabelled microspheres have been used to demonstrate the circulatory distribution to the organs and tissues during an episode of diving. The restriction of blood flow to a particular muscle began immediately after the animal was forcibly submerged and maintained for the duration of the dive (Blix *et al.* 1983). These experimental conditions are far from ideal as the forced diving used in this study is known to be adversely stressful for the seals resulting in an exaggerated vasoconstriction response (Cherepanova *et al.* 1993).

A more popular technique for estimating muscle blood flow is the clearance of xenon which is injected into the muscle. The disappearance of xenon from the muscle is monitored radiometrically. Using this technique it has been demonstrated that xenon clearance in the back, lower limb, upper limb and pelvic muscles was dramatically reduced during a brief dive (Cherepenova *et al.* 1993). Whilst these authors concluded that the reduced xenon clearance was a consequence of reduction of total muscle flow it would also seem likely that vasoconstriction during diving may be selectively diverting flow through functional shunts.

These shunts may be similar to those vessels that have been identified by Grant and Payling-Wright (1970) as supplying septa and tendons in terrestrial mammalian muscle. This suggestion becomes more attractive when evidence for the critical site of vasoconstriction during diving is considered. It has been argued that the site for vasoconstriction during diving must lie on large blood vessels outside the muscle. If these sites were in the muscle itself then locally released vasodilators would override the vasoconstriction (Cherenpenova *et al.* 1993). These supply arteries to muscle have been shown to remain constricted in diving ducks (Folkow *et al.* 1966) and seals (White *et al.* 1973). It is worth noting that there is indirect evidence for a similar vasoconstrictor response to diving in humans (Heistad *et al.* 1968, Elsner *et al.* 1971). Such a control of muscle blood flow by

vasoconstriction proximal to the muscle has been referred to as an "extramuscular throttle"

Another practical example of flow regulating metabolism may be the association between insulin resistance and hypertension in humans (Lind and Lithell 1993). This has been termed as Syndrome X which is also associated with reduced lipid clearance and obesity (Lind and Lithell 1993). Whilst alternative explanations do exist it is possible that that hypertension, along with the overactive sympathetic drive that is associated with the condition may cause insulin resistance (Clark *et al.* 1996). Julius and colleagues argue that hypertension may precede insulin resistance and that changes in the microcirculation alter delivery of insulin and glucose to the skeletal muscle cell causing impaired muscle glucose uptake (Julius and Jamerson 1994). It is not known at this stage if hypertension induces functional vascular shunting but this idea provides a plausible hypothesis for the development of insulin resistant diabetes. That is to say, that if functional shunting predominates during hypertension then insulin would be denied access to the receptor on the muscle cell.

2.8 Muscle as a potential thermogenic tissue controlled by blood flow

The muscle shivering response to cold is well documented. However there is considerable evidence to suggest that nonshivering thermogenesis (NST) may exist in skeletal muscle, particularly in animals that have been previously adapted to cold. For example, rats that have been treated with curarè (an agent that prevents muscle movement and therefore shivering) double their metabolic rate during cold exposure, as does the oxygen consumption in the leg muscle during the same cold exposure (Jansky 1995). A 30% increase in blood flow to muscle, estimated using microspheres, occurs in rats upon exposure to cold (Foster and Frydman 1978). Estimates of the contribution of skeletal muscle to non shivering thermogenesis based on the observed blood flow increase and total cytochrome oxidase activity of muscle suggest that about 50% of NST could be accounted for by muscle tissue in cold-acclimated rats (Jansky 1995). The precise figure for the contribution of skeletal muscle to regulatory nonshivering thermogenesis (thermogenesis in

response to cold challenge) probably lies between 25 and 50% (Jansky 1995, Chinet and Mesjnar 1989).

The main tissue involved in the cold response in most rodents is universally accepted to be brown fat which possesses a proton conductance pathway across the inner mitochondrial membrane that is regulated by a protein called thermogenin (Nicholls 1979). However brown fat can not account for all cold induced thermogenesis (reviewed in Jansky 1995). The recent observation that exercise training in rats before cold exposure reduces brown fat mitochondria proton conductance by 40% compared with sedentary rats suggest that brown fat and muscle thermogenesis are oppositely regulated and thus compensate for each other (Larue-Achagiotis *et al.* 1995). If this is indeed correct then estimates of the brown adipose tissue contribution made on sedentary laboratory animals may be significantly overestimated. Maximum estimates for the contribution of brown fat to nonshivering thermogenesis in small cold adapted mammals range from 45 to 61% (Rafael *et al.* 1985, Puchalski *et al.* 1987) leaving the possibility of a significant contribution by skeletal muscle. Furthermore, larger animals such as adult pigs (Trayhurn *et al.* 1989) and adult humans (Astrup 1985) do not possess significant quantities of brown fat and in some animals such as birds (Saarela *et al.* 1991) and marsupials (Hayward and Lisson 1992) brown fat may be absent entirely. Significantly, nonshivering thermogenesis has been demonstrated in birds, despite the absence of brown adipose tissue in these animals (Duchamp *et al.* 1993). Similar studies of nonshivering thermogenesis in marsupials have yet to be undertaken.

Further evidence for brown fat not being the only thermogenic tissue comes from pharmacological studies on whole body thermogenesis. Norepinephrine, which is known to be a key hormone in thermogenesis is both an alpha and beta adrenergic agonist. The thermogenic response to norepinephrine in brown fat cells is mediated by α and β-adrenergic receptors (reviewed by Nedergaard *et al.* 1996). However the α-adrenergic agonist phenylephrine has been shown to stimulate thermogenesis in rats (Borst *et al.* 1994, Rothwell 1994), dogs (Liard 1989) and in marsupial rat kangaroos (Ye *et al.* 1996). This suggests that the α-adrenergic

effect of norepinephrine may be an important thermogenic signal that does not act upon brown fat (Ye *et al.* 1996).

This alpha adrenergic effect could be a result of selective vasoconstriction to increase muscle blood flow (Clark *et al.* 1995) combined with a positive inotropic effect on total cardiac output (Ye *et al.* 1996) causing a flow-induced thermogenesis which has been previously noted in perfused preparations (reviewed in Bonen *et al.* 1994). The role of blood flow to an active thermogenic tissue has been highlighted by Trayhurn (1994) who suggests that for a tissue to be regarded as functionally thermogenic a number of criteria should be met. In this review the blood flow to the tissue is regarded as critical since high flow rates enable heat to be rapidly transferred to the rest of the body. Moreover, a substantial flow rate also ensures that the substrates required to fuel thermogenesis are provided at rates appropriate to the demands of the tissue

The precise cellular mechanism that is responsible for muscle nonshivering thermogenesis is unclear. Obviously muscle is not a dedicated thermogenic tissue so any proposed mechanism would need to be compatible with the motor function of muscle. This implies that the thermogenic mechanism must be able to be turned off during work. This tenet is of primary importance when a muscle thermogenic mechanism is considered. One such process that may contribute to muscle thermogenesis is Na/K cycling whereby ions are actively pumped against a gradient by an ATPase and then move back passively across a membrane via a channel, thus consuming ATP with no net work done. Shiota and Masumi (1988) concluded that norepinephrine-induced oxygen consumption in the constant flow perfused rat hindlimb was attributable to increased Na/K ATPase activity. It should be noted that the observations of Shiota and Masumi have been questioned because of the absence of a perfusion colloid in their studies to prevent edema (Richards *et al.* 1991). Generally there is scarce support for Na/K cycling as a major contributor to nonshivering thermogenesis (Dubois-Feriere and Chinet 1981) with most observations of significant Na/K cycling associated respiration being restricted to cut or lesioned muscle preparations (reviewed in Clausen *et al.* 1991). Under basal conditions or during muscle contraction in intact muscle fibres only 4-10% of total

energy expenditure can be attributed to active Na/K transport (Clausen *et al.* 1991).

A more probable target for nonshivering thermogenesis in muscle involving ion cycling is the Ca^{2+} ATPase situated on the sarcoplasmic reticulum of skeletal muscle (Chinet *et al.* 1992). Under normal physiological conditions more than 90% of Ca^{2+} cycles between SR and contractile filaments. Only a few percent of Ca^{2+} ions entering the cytoplasm are cycled across mitochondrial and plasma membranes It has been proposed that release and active uptake of Ca^{2+} by the sarcoplasmic reticulum may account for up to 25-40% of total metabolic energy in the intact mouse soleus under basal and near-basal conditions (Chinet *et al.* 1992). Most estimates of the contribution of SR Ca^{2+} cycling in tissues have been obtained by observing the oxygen consumption (or heat production) decrease after the addition of Ca cycling inhibitors such as dantrolene or butane dionemonoxime (BDM) to prevent Ca release from the SR[2]. However, the use of inhibitors creates a problem in the interpretation of these studies. It is well known that Ca^{2+} is a potent cell activator at levels insufficient to induce muscle contraction (McCormack and Denton 1989) and may therefore stimulate other metabolic processes which will also appear to be dantrolene or BDM sensitive. Other investigations that do not involve the use of such inhibitors suggest that the maintenance of SR Ca^{2+} gradients would cost about 20% of basal O_2 consumption rate (Clausen *et al.* 1991). The precise role of SR Ca^{2+} cycling in skeletal muscle thermogenesis is unclear.

It has been known for some time that some species of fish are endothermic. Billfish dive to depths of up to 400m in cold water regions during the day and are able to maintain an elevated cranial temperature at these depths. These fish possess a novel thermogenic organ which has originated from the superior rectus muscles of the eye. These unique muscle cells lack contractile filaments but remain highly oxidative (Block 1991). Isolated mitochondria from this tissue suggest that respiration is tightly coupled to ATP production (Ballantyne *et al.* 1992). Evidence

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[2] Dantrolene and BDM prevent SR calcium cycling by different mechanisms. The effect of BDM appears to be related to either a direct action on calcium channels or to the reversible inhibition of passive calcium transfer through the sarcoplasmic reticulum Ca^{2+} channel (Van der Bent *et al.* 1990). The precise mechanism by which dantrolene prevents the discharge from the SR is uncertain but probably relates to blockade of the SR Ca channel. (Van der Bent *et al.* 1990).

for calcium cycling being the key thermogenic event in these cells stems from the observations that the Ca^{2+} ATPase is the most abundant protein in the cell. Furthermore this enzyme has a low coupling ratio (calcium ions pumped per ATP consumed) suggesting a less efficient isoform in this muscle (Block 1991). Along with brown fat in mammals the fish heater organ is the only recognized dedicated thermogenic tissue in animals (Block 1991).

A recent study comparing the content of the Ca^{2+} ATPase and Ca^{2+} release channel (ryanodine receptor) in cold acclimated or thermoneutral ducklings noted a 30-50% increase in the expression of these proteins during prolonged cold exposure (Dumonteil *et al.* 1993). Based on this observation these authors concluded that Ca^{2+} cycling could be a major contributor to muscle NST. The condition known as malignant hyperthermia which affects genetically susceptible pigs and humans, is caused by a loss of Ca^{2+} regulation in muscle resulting in an increase in the level of myoplasmic Ca^{2+} (Cheah *et al.* 1989). This event has been proposed to stimulate oxygen consumption by the Ca^{2+} ATPase or by Ca^{2+} causing fatty acid production which may stimulate state 4 respiration in isolated mitochondria (Cheah *et al.* 1989). This condition, although pathological, indicates a possible role for Ca^{2+} in muscle nonshivering thermogenesis.

Mitochondrial uncoupling whereby substrates are oxidized by the respiratory chain but the resulting proton gradient is dissipated via proton conductance is the mechanism by which brown fat mitochondria produce heat. The same mechanism existing in muscle is difficult to envisage because of the absence of the uncoupling protein and the requirement for muscle to perform a major contractile function apart from heat production. This is reflected in the finding that cold adapted muscle mitochondria have the same coupling efficiencies (RCR) as muscle mitochondria from thermoneutral animals. However the mitochondria in muscles of cold adapted animals are more numerous and smaller (Himms-Hagen 1976). Other studies involving cold acclimated ducklings have found histochemical *in situ* evidence for loose coupling of muscle mitochondria (Duchamp *et al.* 1992).The mechanism and regulation of such uncoupling is unknown at this stage. It has been noted by many investigators that free fatty acids (FFA) are able to depolarize the membrane potential of isolated mitochondria and stimulate

respiration (e.g. Barre' *et al.* 1986, Andreyev *et al.* 1989, Matthias 1995). The uncoupling mechanism of FFA on isolated mitochondria is likely to be the FFA functioning as a proton carrier across the inner mitochondrial membrane. The crossing of the FFA anion across the membrane has been suggested to be facilitated by the ATP/ADP antiporter (Andreyev *et al.* 1989). A functional role for fatty acids as uncouplers in intact cells (other than brown adipose tissue) is less clear as fatty acids within the cell appear to be mostly associated with fatty acid binding protein rather than dissociated molecules (Oscai *et al.* 1990). Nonetheless there have been demonstrations of fatty acids increasing respiration in perfused liver in association with a lowered mitochondrial ΔpH (representative of lowered proton motive force) and lowered cytosolic ATP/ADP ratio suggesting an uncoupling role for fatty acids in this system (Soboll *et al.* 1984). However, it has also been demonstrated that oligomycin, an inhibitor of ATP synthesis, will significantly inhibit the fatty acid induced respiration suggesting that fatty acids act partly through a mechanism coupled to ATP synthesis (De beer *et al.* 1974, Nobes *et al.* 1990a). An elegant study by Nobes *et al.* (1990) examining the mechanism of stimulation of respiration in isolated hepatocytes provides further evidence that the uncoupling observed in isolated mitochondria upon fatty acid addition does not occur in intact cells. Rather the increase in oxygen consumption is due to increased substrate supply which raises Δp (proton motive force) and increases the rate of cycling of protons through the non-ohmic proton leak across the mitochondrial inner membrane, and part is due to cytoplasmic ATP turnover by unidentified reactions (Nobes *et al.* 1990 a). Simply stated, non-ohmic proton leak describes the non-linear relationship between mitochondrial membrane potential and proton conductance. Thus at high mitochondrial membrane potentials, in the absence of ATP synthesis, a large proportion of respiration is attributable to non-ohmic proton leak (Nobes *et al.* 1990 b). In a recent study investigating the contribution to proton leak to Standard Metabolic Rate in the perfused rat hindlimb it was found that about half of basal respiration is attributable to proton leak (Rolfe *et al.* 1996a). Furthermore it was found that proton leak exerted a significant degree of control over respiration (Rolfe *et al.* 1996b). Proton leak has been shown to occur in isolated mitochondria, and the mitochondria of isolated cells and whole organs.

It has been correlated with three important regulators of metabolic rate, namely body mass, thyroid hormone status and phylogeny (Brand *et al.* 1994). The role of proton leak in thermogenesis is at this stage only partially understood and deserves further consideration.

2.9 The perfused rat hindlimb

The rat hindlimb perfusion technique was first described by Ruderman *et al.* (1971). The present thesis is based on this technique so a thorough review of this popular method is necessary. The perfused rat hindlimb is a relatively recent perfusion technique. Perfusion of liver was first described as early as 1915 (Mautner and Pick 1915). Perfusion was via the portal vein by gravity feed using either Ringer's solution or diluted blood as perfusate. The methods for perfusion of other organs such as stomach (Lim *et al.* 1927) and pancreas (Babkin and Starling 1926) also have experimental origins in the earlier part of this century. Techniques for the perfusion of hindlimbs from larger animals, such as dogs and cats, were also developed around this time.

Probably the first experiment conducted on the perfused rat hindlimb was reported by Robinson and Harris in 1959. However, no systematic attempt to assess the viability of the preparation and compare the tissue with the *in situ* counterpart was made until 1971 (Ruderman *et al.* 1971). That investigation measured oxygen consumption, utilization of glucose and ketone bodies, lactate production, efflux of K^+, tissue concentrations of creatine phosphate and the adenine nucleotides, appearance under the electron microscope and the response to insulin and sciatic nerve stimulation. The results implied that the behavior of the hindlimb, when perfused with a Krebs-Henseleit bicarbonate buffer containing 4g/100 ml bovine serum and 7-8g/100 ml human erythrocytes, closely resembled that of the hindquarter in the living rat. It is important to note that these studies were conducted under contant flow (9-12 ml/min) conditions to produce a perfusion pressure of between 80 and 100 mm Hg. While constant-flow perfusion models are simpler to operate than constant pressure one must keep in mind the fact that the hindlimb *in situ* is perfused under constant pressure conditions. However, this does not detract from the

usefulness of the constant flow model, as long as this particular limitation is considered when discussing any experimental results.

Further modification of the perfusion method arose from the difficulties and health risks associated with the use of red blood cells, particularly from blood bank sources (Bonen *et al.* 1994). Hindlimb perfusion in the absence of erythrocytes has been demonstrated to be a viable experimental technique (Shiota and Sugano 1986, McDermott *et al.* 1989) with several important advantages. Firstly, venous PO_2 can be measured directly with an in-line oxygen electrode and secondly, there is no metabolic interference due to erythrocytes (Bonen *et al.* 1994). Characterization of erythrocyte-free hindlimb perfusions has shown that oxygen consumption is similar to the rate obtained when the preparation is perfused with a buffer containing erythrocytes (reviewed in Bonen *et al.* 1994, Rolfe and Brand 1996). Furthermore, estimates of high energy phosphates (PCr and ATP) in muscle samples from erythrocyte and erythrocyte free perfusions are virtually identical to *in vivo* levels (Reimer *et al.* 1975, Colquhoun *et al.* 1988, Bonen *et al.* 1994). This observation further validates the perfused hindlimb as a viable and relevant experimental model. Recently, rat hindlimbs have been perfused at 25°C rather than 37°C (e.g. Colquhoun *et al.* 1988). The advantages of perfusing at this temperature are that the flow rate can be lowered thus reducing the cost of albumin and the onset of edema (Colquhoun *et al.* 1988, Bonen *et al.* 1994).

A discussion of the perfused rat hindlimb would not be complete without a thorough examination of the 1986 study by Gorski and colleagues (Gorski *et al.* 1986). In this study microspheres were used to trace the regional distribution of perfusate flow through three different surgical preparations of the perfused hindlimb. These studies revealed that flow did not distribute equally between muscle groups. Instead red muscle groups received more flow than white muscle groups, presumably due to the greater capillary density in red muscle. Furthermore the distribution of microspheres indicated that it is exceptionally difficult to restrict perfusion to a single leg of one animal. The hindlimb model used in this thesis involves single hindlimb perfusion by the tying-off of the contralateral femoral artery. According to Gorski, approximately half of the flow goes to the leg whilst slightly less than half of the flow is sent to the trunk. Interestingly, 2% of the flow goes to the "non-perfused" leg.

These results suggest that "macro-changes" in flow between muscle in the hindlimb model also need to be considered when interpreting results.

Chapter 3

General methods and materials

3.1 Animal Care

Male hooded Wistar rats from the University's colony were used in all experiments. Five to eight rats were housed per cage under temperature-controlled conditions (12h:12h light/dark cycle). Rats were provided with a commercial diet (21.4% protein, 4.6% lipid, 68% carbohydrate and 6% crude fiber with added vitamins and minerals from Gibson's Hobart) and water *ad libitum.*

All experiments were carried out under the auspices of the University of Tasmania animal ethics committee and in accordance with federal animal care recommendations (publication).

3.2 Perfused rat hindlimb

Surgery was performed as described by Colquhoun *et al.* (1988), which was a modified version of that used by Ruderman *et al.* (1971). The detailed operative procedure was as follows (see Fig 3.1):

Rats were anaesthetized with an intraperitoneal injection of aqueous pentobarbital sodium (6 mg/100 g body weight). After a midline abdominal incision, the skin was folded back and the epigastric vessels were ligated (for anatomical nomenclature see Greene, 1968). The abdominal wall was then incised from the pubic symphesis to the xiphoid process. Ligatures were placed around the left superficial epigastric vessel (1.), the internal spermatic vessels (2.) and the other vessels supplying the testes, the neck of the bladder (3.) and the seminal vesicles (4.). The testes and seminal vesicles were removed. Two ligatures were placed around the descending colon {proximal to the inferior mesenteric artery (5.)} and excision occured in between these ligatures. The colon and large intestine were separated from the connective tissue the level of the renal vessels (6.), and a ligature was placed around the duodenum taking care to tie off the superior mesenteric vessels (7.). The intestine was excised below the duodenal ligature and the entire gastro-intestinal tract was removed. Ligatures were placed around the illiolumbar vessels (8.), ureter (9.), tail near the anus (10.) and tarsus

of the foot (11). In the case of the two hindlimb perfusion ligatures were placed around the tarsi of both feet.

Next, two pairs of loose ligature were placed around the aorta and the vena cava. The first pair (13.) were located immediately below the renal vessels, and the second pair (14.) above the illiolumbar vessels. Heparin (1000 units per ml) (0.1ml/100 g body wt) was injected into the vena cava at the junction between the right renal vein and the vena cava and allowed to circulate. The vena cava (15.) was tied off and the second pair (14.) above the illiolumbar vessels. Heparin (1000 U/ml, 0.1 ml/100 g body wt) was injected into the vena cava and allowed to circulate for at least 3 min. The vena cava (15.) was tied off (13.), and cannulated by passing a Teromo 18G needle with 16G catheter through the vessel wall (X). The catheter tip was positioned 3 mm above the aortic bifurcation and tied in place. The aorta (16.) was tied off (13.) and a small incision (X) allowed insertion of a Terumo 18G catheter filled with 0.9% NaCl. The catheter was pushed gently until the tip was at the same level as the venous catheter, and then secured.

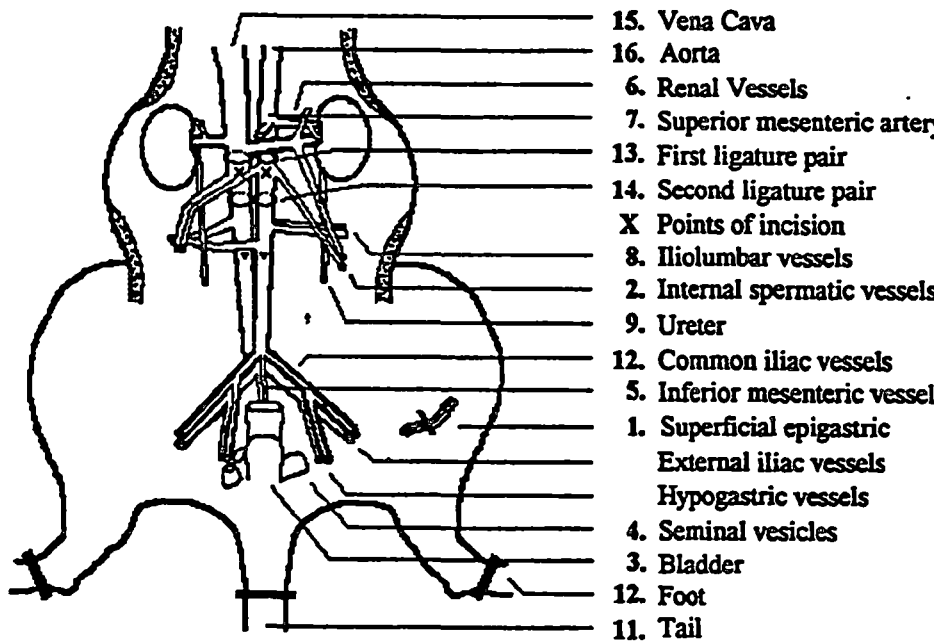

Figure 3.1 Vessels ligated during surgical isolation of the rat hindlimb.

The preparation was then transferred to the perfusion apparatus, and the arterial cannula was connected to the oxygenated perfusion inflow line. The outflow line was connected to the oxygen electrode (Figure 3.2). Approximately 2 min elapsed from the time that the vena cava was ligated and the circulation was reestablished. A further ligature was placed

around the abdomen of the rat at the level of the L3,L4 vertebrae to restrict flow to the upper torso. The animal was then sacrificed with an over-dose of pentobarbital sodium injected into the heart. The entire operative procedure required 10-25 min.

It is worth noting that the sympathetic nerve, running below the aorta, is crushed during the cannulation procedure, effectively halting further transmission (Cowen *et al.* 1982). Recent experiments using Guanethidine (5 μM), a blocker of sympathetic transmission, have demonstrated that no sympathetic output to the hindlimb occurs after surgery since this agent has no effect on resting perfusion pressure and oxygen consumption (Hall J.L., unpublished observations, University of Tasmania 1996). Furthermore, the perfused hindlimb is fully vasodilated which further supports the notion of absent sympathetic drive in the perfused hindlimb system (Clark *et al.* 1995)

Similar experiments, using agents which block motor nerve transmission, have demonstrated that there is no motor nerve output to the hindlimb after cannulation. Tubocurarine, at doses which prevent sciatic-nerve induced muscle contraction, has no effect on basal pressure or oxygen consumption (Ye J-M, Ph.D. thesis, University of Tasmania, 1995).

3.3 Perfusion medium

The standard perfusion medium was prepared according to Côté *et al.* (1985). Bovine serum albumin (BSA) (2% w/v) was added to Krebs-Henseleit bicarbonate buffer (118 mM NaCl, 4.74 mM KCl, 1.19 mM KH_2PO_4, 1.18 mM $MgSO_4$, 25 mM $NaHCO_3$) including 1.27 mM $CaCl_2$ and 8.3 mM glucose. BSA was dialyzed 5 times against distilled water before spectrophotometric determination of protein content by the Biuret assay (Clark 1964). The final solution was filtered (0.45 μM) before use. The pH of this buffer at 25°C (the perfusion temperature used in all cases in this thesis), after gassing with 95% O_2: 5% CO_2 was between 7.25 and 7.30.

2.4 Perfusion apparatus

Perfusions were conducted in a thermostatically-controlled cabinet (Fig. 3.2). Unless specified otherwise, the perfusion temperature was 25°C. This reduced basal oxygen uptake by the hindlimb and improved O_2 solubility. The perfusion medium reservoir was gassed with 95% O_2:5% CO_2 to facilitate full oxygenation. Gassed perfusate was pumped at a constant flow rate by a peristaltic pump (Masterflex, Cole

Parmer USA) from a reservoir through the apparatus. Before reaching the hindlimb the medium passed through a water-jacketed glass heat exchange coil, and an oxygenator. The oxygenator consisted of a glass jar containing 3 meters of silastic tubing which was continuously gassed with 95% O_2: 5% CO_2 to ensure constant arterial PO2 levels.

Perfusion pressure was continuously monitored in a sidearm proximal to the aorta. Changes in perfusion pressure were continuously monitored in a sidearm proximal to the aortic cannula. Since arterial flow was held constant, changes in perfusion pressure reflected changes in vascular resistance.

Continuous measurement of venous effluent oxygen content was achieved by an in-line 0.5 ml Clark-type oxygen electrode contained in a temperature-controlled water jacket.

Arterial PO_2 at the perfusion flow rate was measured before and after the completion of experiments. Calibration of the oxygen electrode was made routinely by using temperature-equilibrated 100% O_2 and air before and after each experiment. The electrode response was assumed to be linear in the range of PO_2 200-700 mmHg.

Freshly prepared solutions of agonists and antagonists were infused continuously (LKB Brommer , 2232 Microprepex S) into the perfusion line proximal to a small, stirred bubble trap and the arterial cannula. An infusion rate of <2% of the flow rate ensured the vehicle had no measurable effect on venous PO_2 or perfusion pressure.

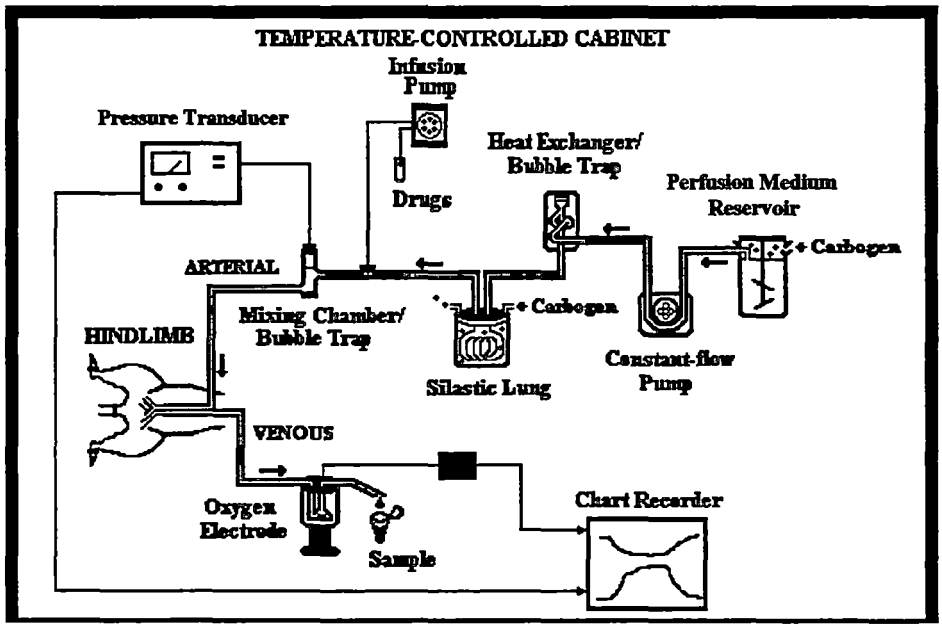

Figure 3.2 Perfusion apparatus for rat hindlimb. Perfusions were conducted at 25°C in a non-recirculating mode.

3.5 Calculation of oxygen consumption.

Oxygen consumption rates (VO_2) for the perfused hindlimb was calculated by multiplying the arteriovenous difference (A-V) in oxygen content by the flow rate of the perfusate per gram of hindlimb skeletal muscle (SKM). VO_2 in μmols/g/h was calculated as follows:

$$VO_2 = \frac{(A\text{-}V) \times 0.03388^{*} \times 60 \times \text{flow rate (ml/min)}}{\text{SKM mass} \times 22.4^{\#}}$$

* μl of oxygen dissolved per 100 μl of perfusate medium per mmHg PO_2 at 25°C (Christoforides *et al.* 1969).

\# At standard temperature and pressure the volume of 1μmol is 22.4 μl.

Perfused skeletal muscle mass was determined by perfusion with Evans' blue dye at the end of the experiment. Stained muscle was dissected from the carcass and weighed (Eldershaw *et al.* 1994). The perfused muscle mass of the hindlimb preparation was constantly found to be 8.3% of the total mass of the rat.

3.6 Chemicals and pharmacological agents.

(-) Norepinephrine bitartrate, 5-hrdroxytryptamine creatine sulfate, prazosin HCl, (±)-propanolol HCl, (-)-isoproterenol HCl, arginine vasopressin and human angiotensin II were from Sigma, USA. BSA (fraction V), pentobarbital sodium, lactate dehdrogenase and NADH were from Boehringer Mannheim, Australia. Heparin was from David Bull, Australia. All other chemicals were of analytical grade from Ajax, Australia. 95% O_2: 5% CO_2 (carbogen) and O_2 were from CIG, Australia.

Chapter 4

Serotonin inhibition of 1-methylxanthine metabolism indicative of enhanced non-nutritive flow in muscle

4.1 Introduction

The constant-flow perfused rat hindlimb responds to vasoconstrictors by increasing or decreasing metabolism (Table 2.1). Vasoconstriction is essential for both types of responses (Clark *et al.* 1995). Furthermore, the effects appear to involve discrete changes in flow patterns within the skeletal muscle which are in turn controlled by vasoconstrictor sites. These sites can be distinguished on the basis of their oxygen and Ca^{2+} requirements (Dora *et al.* 1992). Recently, further evidence has been obtained suggesting that NE and 5-HT, in association with vasoconstriction at different sites, may control different capillary flow routes in the hindlimb. Those findings were based on post-equilibration red blood cell efflux, vascular entrapment of fluorescein-labeled dextran and vascular corrosion casting using methyl methacrylate (Newman *et al.* 1996).

Increased exchange of nutrients either during Type A vasoconstrictor action or during exercise could imply increased exposure to capillary endothelial surfaces. This could result from the greater total surface area of the nutritive capillaries of muscle which result from the Type A vasoconstrictor mediated change in flow pattern. Conversely, Type B vasoconstrictors, that appear to decrease nutrient exchange by the perfused rat hindlimb may act to decrease total surface area of capillary exposure. A potential marker for capillary exposure could thus involve the metabolism of an infused substrate by a specific capillary endothelial enzyme. Xanthine oxidase (XO) is a potential candidate enzyme. It is widely spread amongst the mammalian tissues. Liver and intestine have the highest activity but there are detectable activities in heart, spleen, kidney and skeletal muscle (Parks and Granger 1986). In skeletal muscle, XO occurs primarily in the capillary endothelial cells and is restricted to the smaller vessels (Jarasch *et al.* 1986). The present study examines the effects of a Type B (5-HT)

vasoconstrictor on the metabolism of exogenous 1-MX, a substrate for endothelial xanthine oxidase (Bergman and Dikstein 1956). Relative flow between individual muscles as well as non-muscle tissue was monitored by tissue recovery of infused fluorescent microspheres (15 μm).

4.2 Materials and Methods

4..2.1 Hindlimb perfusions.

Hindlimb surgery was essentially as described in Chapter 3. The effluent was periodically sampled (at times indicated) for measurement of flow and purines by high-performance liquid chromatography (HPLC). The hindlimb was allowed to equilibrate for 40 min before infusions were commenced.

5-HT (35 μM) in 0.9% NaCl was freshly prepared before use and infused at a rate of 1 in 100 of the flow rate. Solutions of 1-MX or XAN were made by dissolving in 0.1 N NaOH and dilution in 0.9% NaCl. Infusions were made into the perfusion line prior to a small bubble trap that was continuously mixed by a magnetic stirrer. Infusion of vehicle alone had no effect.

4.2.2 Microsphere infusions.

Constant-flow perfusions were conducted as described above. Equilibration time was 40 min, followed by a 10 min infusion of either 5-HT or vehicle. At steady state for either 5-HT or vehicle, a bolus of 250,000 yellow-green 15 μm FluoSpheres® (Fluorescent Microsphere Resource Center, University of Washington, Seattle) was injected over 10 s, inducing a pressure spike of less than 5 mmHg. Perfusion continued for a 10 min washout period with agonist infusion unchanged.

4.2.3 Extraction of microspheres.

Muscles including soleus, plantaris, gastrocnemius red, gastrocnemius white, tibialis, extensor digitorum longus, remaining calf muscles, vastus, remaining thigh and trunk muscle, as well as bone, skin and subcutaneous white adipose tissue of the perfused leg were dissected free. In addition tissues were collected from unperfused regions to check for leakage. These included the tied off foot of the perfused leg, the contralateral (unperfused) leg, associated skin, spinal and abdominal regions and the tail. All tissue samples were briefly blotted and weighed, then digested using the method of Van Oosterhout *et al.*. (1995). Tissues were placed in 50 ml Greiner

centrifuge tubes and digested in 5-10 volumes of 2 M ethanolic KOH (2 M KOH in 95% ethanol) containing 0.5% Tween-80 (Sigma), with heating to 58-60°C for 2-6 hr (depending on tissue size and effectiveness of digestion).

Tubes were centrifuged for 25 min at 2000 g. Supernatants were aspirated, retaining 1-2 ml at the base of the tube. Pellets were washed using 0.25% Tween-80 in distilled water, followed by distilled water alone. Following the final wash, supernatants were carefully aspirated to avoid disturbing the pellet but to retain minimal aqueous phase. A volume (5 ml) of 2-ethoxyethyl acetate was used to dissolve the microspheres, releasing the lipophilic fluorescent dye. Samples were allowed to extract overnight (4°C) before being vortexed and centrifuged (2000 g, 20 min).

Fluorescence intensity of the organic phase was measured against a solvent blank using an Aminco-Bowman Spectrophotofluorometer with excitation and emission settings at 495 nm and 510 nm respectively Calculation of microsphere numbers in each sample was achieved by reference to a standard curve produced from dilution of a known number of microspheres dissolved in the solvent. The data were expressed in terms of percentage of total infused.

4.2.4 Purine analyses.

Perfusate samples (0.5 ml) were taken from the venous outflow, centrifuged briefly to remove any remaining blood cells, added to 0.1 ml of 2 M $HClO_4$ on ice and centrifuged for 5 min at 8000 g. The supernatant (0.4 ml) was neutralized with 2.5 M K_2CO_3. $KClO_4$ was allowed to precipitate at 0°C and removed by centrifugation at 8000 g for 5 min. This supernatant was used directly for HPLC analysis or stored at -20°C until use.

Reverse-phase HPLC was conducted essentially as described by Wynants *et al.*. (1987) with an LKB instrument fitted with a Varian Polychrom Model 9065 Diode Array Detector equipped with LC star Workstation. Nucleosides and catabolites were separated on a Hibar Li Chrosorb Select B column (25 cm; 5 μm particles Merck) under isocratic conditions at 0.7 ml/min and identified by retention time and absorbance spectra. For a more rapid analysis of 1-MX and 1-MU, a buffer containing 10 mM sodium acetate pH 4.0 with 6% acetonitrile was used.

Rat hindlimbs were intentionally perfused with cell-free perfusate to avoid complications due to the release or uptake of purine and purine catabolites by red blood cells. Under the conditions chosen of constant flow at 5.0±0.1 ml/min per hindlimb (0.33 ml/min per g wet wt of muscle at 25°C) with albumin-containing buffer responses to vasoconstrictors and indices of high energy phosphate status were similar to blood perfused hindlimbs at 37°C (Clark *et al.* 1995). However, there was a small constant release of purines and purine catabolites as previously reported (Clark *et al.* 1990). Effluent concentrations after equilibration (40 min) were (in μM) uric acid, 5.34±0.28; hypoxanthine, 0.09±0.05 and xanthine, 0.22±0.05 (n=5). These concentrations remained constant for up to 2 h providing the conditions of perfusion were unaltered. However, peak resolution on HPLC was good and determination of 1-MX and 1-MU was unhindered.

4.2.5 Cytochrome oxidase.

Rats were anaesthetized, tissues were removed and homogenised in 2-5 ml 50 mM potassium phosphate buffer, pH 7.35. Cytochrome C (Boehringer-Manheim) was dissolved in water to make a 2% (w/v) solution, then reduced with excess ascorbate and passed through a small column of G.25 Sephadex. The cytochrome oxidase reaction was assayed in a recording spectrophotometer set at 550 nm and 37°C with a continuously stirred cuvette (Wharton and Tzagoloff 1967). Reactants were 50 mM potassium phosphate buffer, pH 7.35 (2.0 ml), water (250 μl), reduced cytochrome C (100 μl) and homogenate (10 μl).

4.2.6 Xanthine oxidase and dehydrogenase.

The total rate of xanthine oxidation was monitored at 293 nm and 37°C in 50 mM phosphate buffer pH 7.4 containing 0.1 mM EDTA essentially described by Wajner and Harkness (1989). Xanthine oxidase and the combined activities of oxidase plus dehydrogenase were determined using oxygen and NAD^+ as electron acceptors, respectively.

4.3 Results

4.3.1 Effects and metabolism of 1-MX

Figure 4.1, a representative set of traces, shows the effect on venous PO_2 and perfusion pressure of constant infusion of 0.35 μM 5-HT, or vehicle alone and the effect of step-wise increasing doses of 1-MX (5-100 μM) in combination with each of the above. 5-HT, alone increased the venous PO_2 from 370 to 490 mmHg resulting in a net decrease in $\dot{V}O_2$ from 7.2 to 3.8 $\mu mol.h^{-1}.g^{-1}$. Infused 1-MX (5-100 μM) showed no vasoactivity whether added alone, or in combination with 5-HT.

Since 1-MX showed neither vasoconstrictor nor vasodilator activity (≤100 μM) it was infused into the perfusion medium to achieve 23 μM, final concentration, throughout the experiment with the purpose of assessing its conversion to 1-MU by endogenous xanthine oxidase of the hindlimb. Expectations based on findings by others (Day *et al.* 1988) were that metabolism of 1-MX would result only in the formation of 1-MU and neither 1-MX nor 1-MU would be taken up and assimilated by tissue. The data of Fig. 4.2 shows that these expectations were confirmed and that once steady state was reached (approx. 20 min after commencement of infusion) the sum of 1-MX + 1-MU was constant (approximately 23 μM) and thus recovery was quantitative.

Furthermore, Table 4.1 shows that infusion of 20 μM allopurinol resulted in a marked decrease in the ratio of 1-MU:1-MX from 1.14±0.02 to 0.16±0.02 (P=0.0001). This change did not reverse when the allopurinol was removed (Fig. 4.2) and the ratio of 1-MU:1-MX continued unchanged at approximately 0.10 for at least another 20 min. The recovery (1-MU + 1-MX) was close to 100% before, during and after the period of allopurinol infusion (Fig. 4.2, Table 4.1). Unlike 1-MX and 1-MU, the recovery of allopurinol + oxypurinol was not quantitative, possibly being due to uptake and further metabolism of oxypurinol.

Infusion of xanthine (16 μM) also inhibited the conversion of 1-MX to 1-MU (Table 4.1, Fig. 4.3). However, since basal values for XAN were only 0.22±0.05 μM it appears that interference with 1-MX conversion to 1-MU would be minimal and may even be less following 5-HT when levels of XAN decrease below basal (see

below).

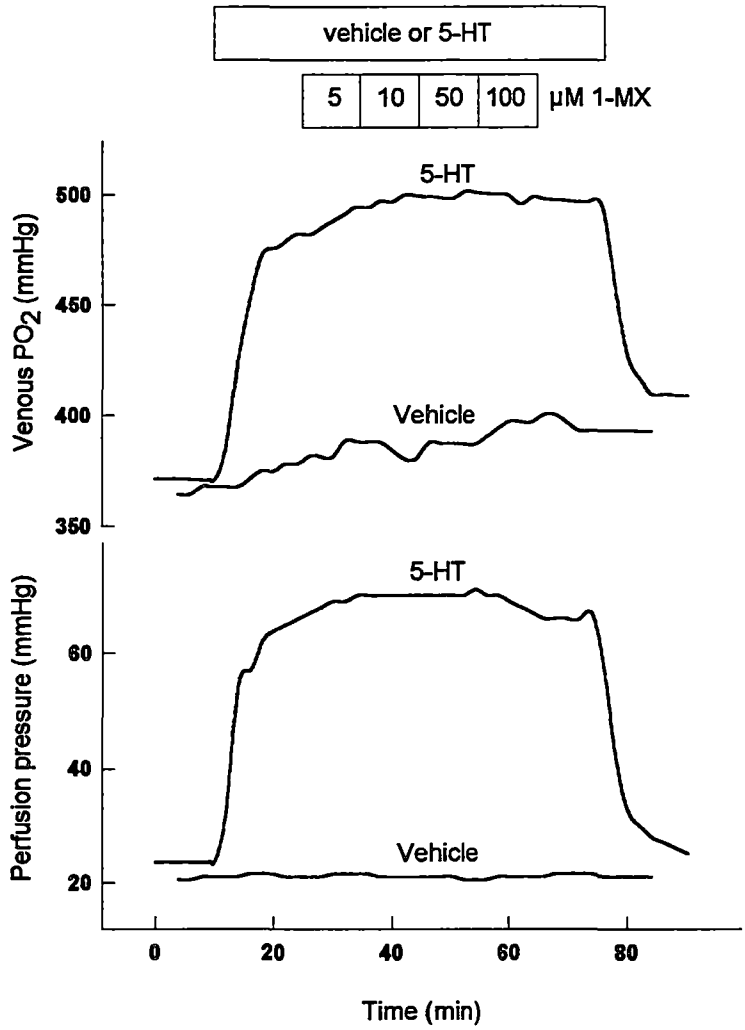

Fig. 4.1. Time course for the effects of 1-MX on venous PO_2 and perfusion pressure of the constant-flow perfused rat hindlimb. Infusions of vehicle alone or 0.35 μM 5-HT were commenced at 10 min and continued for 65 min. 1-MX was infused at incremental doses of 5-100 μM starting at 25 min. Venous PO_2 and perfusion pressure were continuously recorded. A representative trace for each group, is shown.

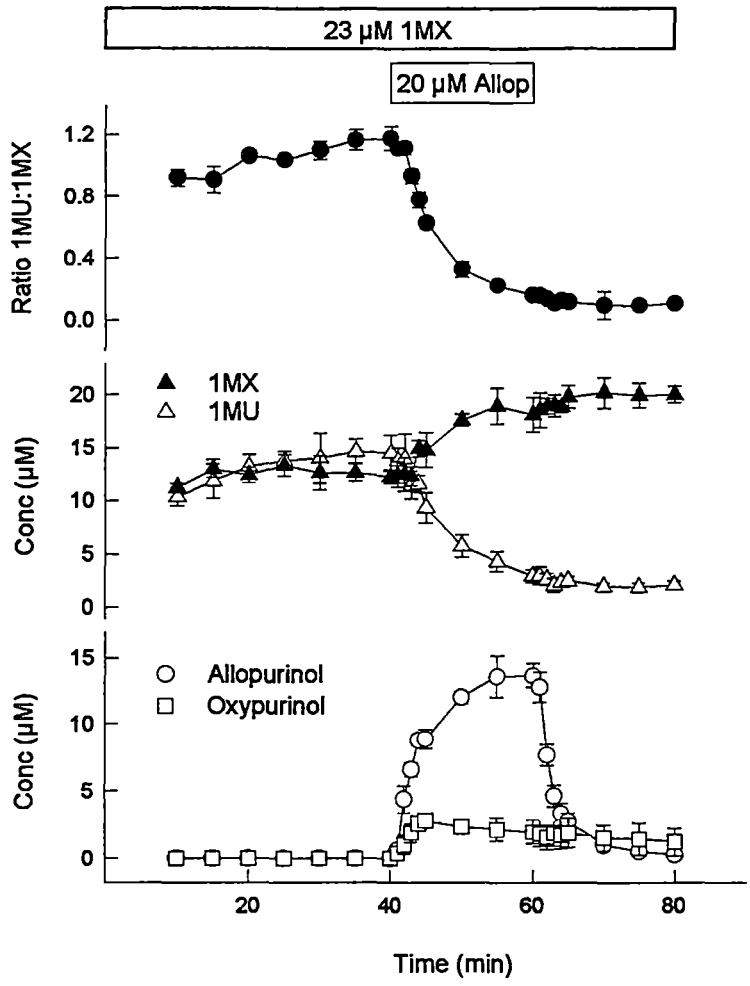

Fig. 4.2. Effect of infusion of 20 µM allopurinol on the conversion of 1-MX to 1-MU by the constant-flow perfused rat hindlimb. 1-MX (23 µM) was infused for the duration of the perfusion (approx. 80 min) and 20 µM allopurinol was introduced at 40 min and maintained until removal at 60 min. Samples of perfusate were collected for analysis of 1-MX, 1-MU, allopurinol and oxypurinol. Values shown are means ± SEM (n=3). When not shown error bars are within the symbol.

The results of Fig. 4.4 show the effect of 0.35 µM 5-HT on the conversion of 1-MX to 1-MU when 23 µM 1-MX was continuously infused into the constant flow perfused hindlimb. 5-HT (0.35 µM) resulted in a marked decrease in the ratio of 1-MU:1-MX from 1.14±0.02 to 0.71±0.05 ($P<0.05$) (Table 4.1). This was reversed upon 5-HT removal (Fig. 4.4). Examination of individual results for perfusate

Table 4.1. Effects of allopurinol, xanthine and 5-HT infusions on the metabolism of 1-MX by the constant flow perfused rat hindlimb.

	Recovery,%	[1-MX]	[1-MU]	[1-MX]/[1MU]
Control	102±4	11.0±0.5	12.5±0.5	1.14±0.02
Allopurinol (20 μM)	92±10	18.1±1.6*	3.0±0.6*	0.16±0.02
Xanthine (16 μM)	107±9	13.8±1.0	10.8±2.0	0.79±0.17*
5-HT (0.35 μM)	102±6	13.8±0.7	9.5±0.1*	0.71±0.05*

*, Significantly different ($P<0.05$) from control values based on 1-way analysis of variance and pairwise comparison procedures by Dunnett's method using SigmaStat (Jandel Scientific Software).

concentration of 1-MU and 1-MX indicated that the decrease in the ratio of 1-MU:1-MX due to 5-HT infusion resulted from an equal change in the concentrations of each of approx. 2.8μM (Fig. 4.4). In addition, changes in the ratio of 1-MU:1-MX coincided closely with the changes in perfusion pressure due to the vasoconstrictor activity of 5-HT (data not shown). Infusion of 5-HT also reversibly decreased the release of total endogenous purines (XAN + HX + UA) from 6.78±0.14 to 4.14±0.13 μM ($P<0.05$). Guanosine and adenosine were undetectable (i.e. less than 0.05 μM) and thus changes due to 5-HT were insignificant. Uric acid accounted for approx. 93% of the total endogenous purines released by the hindlimb, whether or not 5-HT was present.

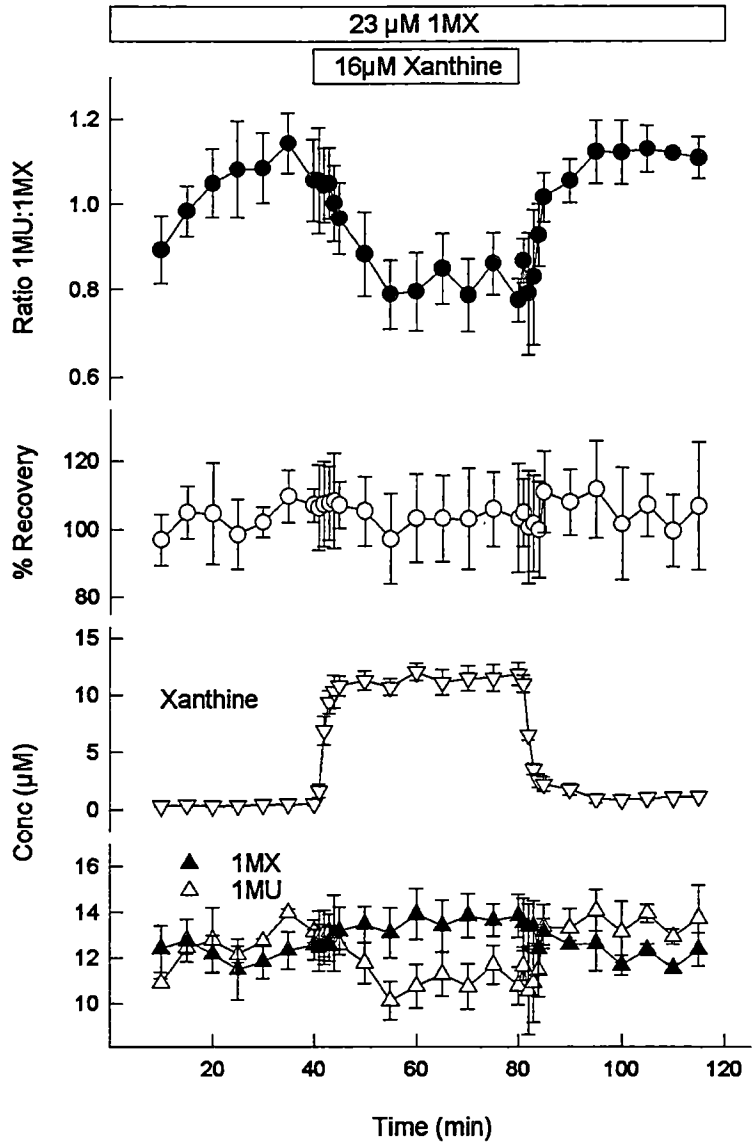

Fig. 4.3. Effect of infusion of 16 µM XAN on the conversion of 1-MX to 1-MU by the constant-flow perfused rat hindlimb. Values shown are means ± SEM (n=3).

4.3.2 Microsphere recovery

The data of Fig. 4.5 show that when compared to control (vehicle), 5-HT infusion did not alter microsphere recovery in muscle, spine, skin, white adipose tissue, or tissues of the lower abdomen. Only bone showed a significant decrease. Of the muscles only two, soleus and gastrocnemius red, showed a significant increase.

Most of the microspheres that were infused were recovered in the tissues shown (Fig. 4.5A). Thus recoveries in muscle, spine, bone, skin, white adipose tissue and the

abdominal region of the perfused leg constituted 72.9±4.1 and 75.7±2.6% of the total infused for vehicle and 5-HT, respectively. Perfusate contained an additional 1.1±0.4 (vehicle) and 0.8±0.4% (5-HT) and the non-perfused regions including the foot of the perfused leg, accounted for 2.3±0.4 (vehicle) and 1.6±0.2% (5-HT). Thus total recovery of microspheres was 76.4±4.8 (n=5, vehicle) and 77.8±2.9% (n=5, 5-HT). The difference was not significant. The remaining 22-23% of microspheres could not be found in other parts of the carcass and may represent the accumulated loss from processing of the eighteen individual tissue samples listed in Section 4.2.6.

Of the microspheres recovered and shown in Fig. 4.5A, 40±4.6 and 46.7±2.2% were found in muscles of the perfused leg from vehicle and 5-HT infusions, respectively. The non-muscle tissues of bone, skin and white fat of the perfused leg accounted for 14.0±1.8% (vehicle) and 8.6±1.4% (5-HT). Again, there was no significant effect of 5-HT when compared to vehicle (P>0.05). Finally, and as expected, some microspheres were recovered from perfused muscles surrounding the lower spine (below the L3 tie) and perfused tissues of the lower abdomen (below the L3 tie). For vehicle and 5-HT infusions, these represented 13.8±1.4 and 11.8±2.0% for spinal region and 5.2±0.9 and 8.5±1.3% for the lower abdomen, respectively.

A breakdown of figures for microsphere recovery in muscle (Fig. 4.5A) is shown in Fig. 4.5B. When compared to vehicle alone, 5-HT significantly increased microsphere recovery in soleus and gastrocnemius red muscles; all other muscles were unaffected. Although not shown, values for microspheres recovered/g fresh weight of muscle were not uniform and showed a ranked order: sol=g.red>tib=calf=plant>g.white=vast>thigh. This order was not affected by 5-HT.

4.3.3 Cytochrome oxidase

Total activity of cytochrome oxidase was determined as a potential indicator of relative oxidative capacity of the tissues of the hindlimb. Values were (μmol.min^{-1}.g tissue^{-1}) soleus (25.2±1.6, n=9); thigh (7.2±0.7, n=3); white adipose tissue (3.2±0.3, n=9); bone (4.2±0.6, n=9); and skin (3.9±0.4, n=9).

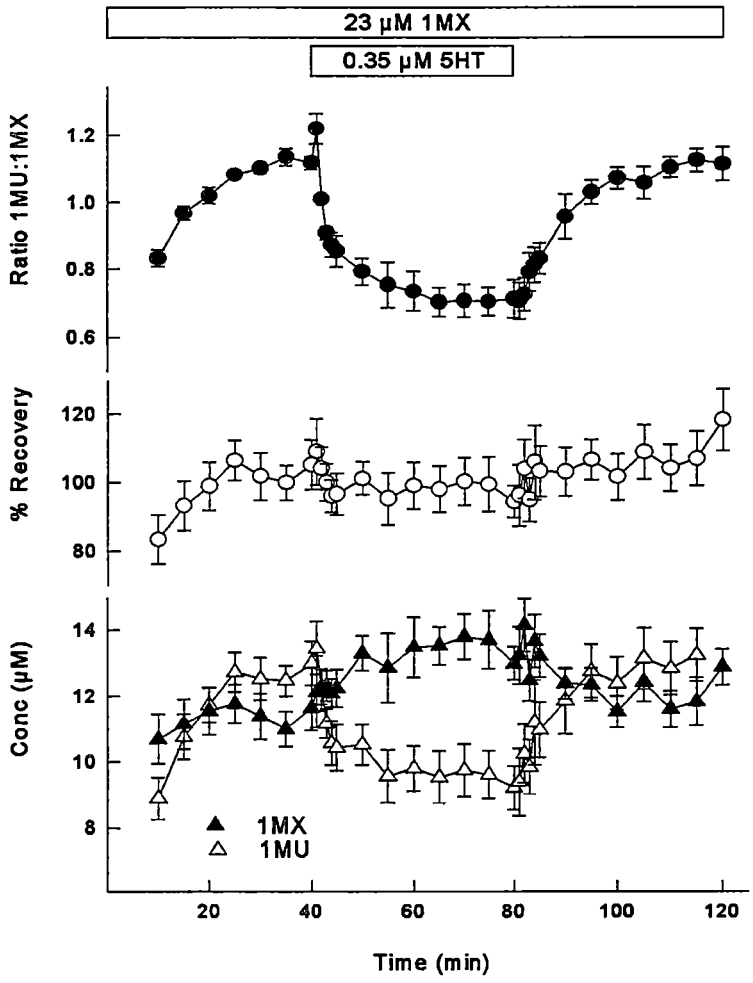

Fig. 4.4. Effect of 5-HT on the conversion of 1-MX to 1-MU. Following equilibration, 1-MX (23 µM) was infused for the duration of the perfusion (approx. 120 min) and 0.35 µM 5-HT was introduced at 40 min and maintained until removal at 80 min. Samples of perfusate were collected for analysis of 1-MX and 1-MU using HPLC. Values shown are means ± SEM (n=5). When not visible error bars are within the symbol.

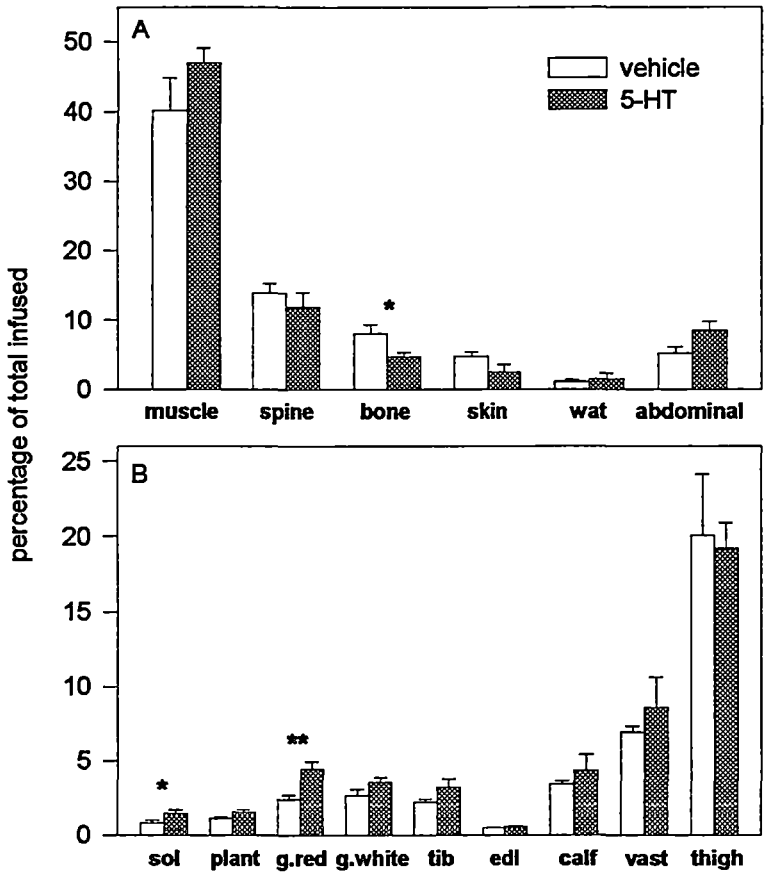

Fig. 4.5. Recovery of fluorescent microspheres from tissues (A) and individual muscles (B) of the perfused rat hindlimb following 5-HT or vehicle treatment. Microspheres recovered from muscle included soleus (sol), plantaris (plant), gastrocnemius red (g.red); gastrocnemius white (g.white); tibialis (tib); extensor digitorum longus (edl); remaining muscles on the lower leg after removal of the sol, plant, g.red, g,white, tib and edl (calf); vastus group (vast); remaining muscles on the upper leg and trunk after removal of the vastus group (thigh). *Spine* included muscles of the lower back between the L3 tie and tail. *Wat* represented subcutaneous white adipose tissue. *Abdominal* included tissue of the urogenital region between the L3 tie and tail. Values are means ± SEM for n=5. *, $P<0.05$; **, $P<0.01$ for 5-HT *versus* vehicle alone.

4.3.4 Xanthine oxidase

Values for the gastrocnemius-plantaris-soleus muscle group (n=3), with and without 5-HT showed xanthine oxidase to be 0.31±0.05 and 0.27±0.07 units/g protein and xanthine dehydrogenase 0.63±0.07 and 0.65±0.16 units/g protein, respectively.

4.4 Discussion

The present study was undertaken to explore the possibility that 1-MX metabolism could be used as an indicator of changes in flow distribution while total (global flow) held constant in the isolated perfused rat hindlimb. It was reasoned that the site-specific vasoconstrictor 5-HT (0.35 μM) that has previously been found to decrease nutrient uptake and metabolite release in a dose-dependent manner over the range 50 nM to 10 μM (Dora *et al.* 1991) by altering the pattern of perfusate flow within the hindlimb (Newman *et al.* 1996) would correspondingly decrease the exposure of exogenously added substrate to enzyme(s) located in the vasculature. Decreased exposure, for example, to endothelial xanthine oxidase might therefore be expected to lead to decreased metabolism of 1-MX, a substrate of xanthine oxidase, during a single pass through the vascular system. Experiments were based on three assumptions. Firstly, that 1-MX at the concentrations used would be non-vasoactive. Secondly, 1-MX would be exclusively metabolized to 1-MU and that recovery of 1-MX + 1-MU would be quantitative. Thirdly, 1-MX was metabolized to 1-MU exclusively by xanthine oxidase in capillary endothelium.

Methylxanthines as inhibitors of cyclic AMP phosphodiesterases, are capable of altering Ca^{2+} ion transients and as a consequence can have potent relaxing activities on preconstricted smooth muscles (Leijten and Van Breeman 1984). In addition, some such as caffeine (1,3,7 trimethylxanthine), cause transient contractions in smooth muscles by releasing Ca^{2+} from intracellular and extracellular sources (Van der Bent and Bèny 1991). However the concentration generally required for these effects is 100 μM or greater. In the present study 1-methylxanthine was found to have neither vasoconstrictor nor vasodilator effect in the perfused rat hindlimb over the concentration range at which its metabolism to 1-MU could be readily studied (5-100

μM). These data suggested that the primary assumption was valid and metabolism of 23 μM 1-MX could be studied in the constant-flow perfused rat hindlimb under basal and 5-HT Type B (Clark *et al.* 1995) constricted states without itself altering the balance of perfusate flow distribution.

The second assumption that 1-MX was exclusively metabolized to 1-MU would also seem to have been met. Thus regardless of the perfusion conditions (basal, allopurinol or xanthine infused or 5-HT vasoconstricted) the sum of perfusate levels of 1-MX + 1-MU was always quantitative (100±5%) during the experimental period (Table 1).

Allopurinol and xanthine were used to assess the third assumption that 1-MX was metabolized to 1-MU exclusively by xanthine oxidase. Allopurinol and/or its metabolite, oxypurinol are well recognized inhibitors of xanthine oxidase and allopurinol is used clinically to prevent uric acid formation in the treatment of gout (Emmerson *et al.* 1987). Figure 4.2 shows the effect of infusing 20 μM allopurinol on the conversion of 1-MX to 1-MU by the perfused hindlimb. Almost complete blockade of the conversion occurred and coincided with the formation of oxypurinol, a product of, and more potent inhibitor of, xanthine oxidase (Hille and Massey 1981). Thus although the infusion of allopurinol was withdrawn at 60 min the inhibition of xanthine oxidase was sustained well beyond the point when allopurinol levels had decreased and the levels of oxypurinol were still high. Xanthine also significantly inhibited the metabolism of 1-MX to 1-MU (Table 4.1) but the inhibitory effect was immediately reversible following xanthine removal (Fig. 4.3) as expected for a competitive inhibitor. Overall, the data suggest that the third assumption was valid and 1-MX was metabolized to 1-MU predominantly by XO in capillary endothelial cells.

Xanthine oxidase normally exists in two forms, either as XO or as xanthine dehydrogenase (XD) (Parks and Granger 1986). XD may be reversibly converted to XO through thiol oxidations or may be irreversibly converted to XO through proteolysis (Parks and Granger 1986). Although there have been reports that XO activity may be lost from cells during hypoxic exposure or reperfusion the time required for these changes is much longer (e.g. 24 h) than any possible hypoxia-induced effect that may occur during surgical procedures from hindlimb perfusion

(Hassoun *et al.* 1994). Thus reversible alterations in the interconversion of XD and XO were unlikely to have contributed to the change in metabolism of 1-MX in the time course of 5-HT-induced vasoconstriction. Indeed, there was no indication that the effects were due to direct 5-HT inhibition of the enzyme as xanthine oxidase and xanthine dehydrogenase activity in the gastrocnemius-plantaris-soleus muscle group were not statistically different with and without 5-HT.

It is relevant to note that assessment of changes to the distribution of flow may not be restricted to substrates for XO. Another candidate enzyme that is endothelial and could theoretically be used is alkaline phosphatase. However it must be borne in mind that substrates should fulfill the three objectives discussed above for 1-MX in that they should not be vasoactive, recovery should be quantitative and metabolism within the hindlimb restricted to the endothelial enzyme in question. The present choice of 1-MX was prompted by the observations by Day *et al.*. (1988) that 1-MX derived from theophylline could be used as an *in vivo* biochemical probe of allopurinol efficacy in humans.

Since the inhibitory effect of 5-HT on both oxygen uptake and 1-MX metabolism could result from the redistribution of flow between tissues of the constant-flow perfused hindlimb it was imperative that flow assessment to each tissue was made. Accordingly fluorescent microspheres were used. Major redistribution of flow did not occur as a result of 5-HT-mediated vasoconstriction. Thus, when compared to vehicle alone, 5-HT had either no effect (plantaris, gastrocnemius white, tibialis, extensor digitorum longus, remaining calf vastus and thigh), or increased the percentage of microspheres recovered (soleus and gastrocnemius red) from muscles with only bone showing a significant decrease. Since bone can only contribute in a minor way to hindlimb metabolism (e.g. it has a relatively low cytochrome oxidase activity), it is unlikely that the decrease in flow to this tissue induced by 5-HT could solely account for the decrease in oxygen uptake and 1-MX metabolism. Based on previous metabolic and contractile performance data from 5-HT-mediated effects on the constant-flow perfused rat hindlimb (Clark *et al.* 1995), as well as more recent data on distribution of flow (Newman *et al.* 1996), it seems most likely that 5-HT acts on muscle tissue predominantly to decrease the nutritive:non-nutritive flow ratio within each muscle.

In summary, the findings from the present chapter suggest that 1-MX metabolism to 1-MU by the constant-flow perfused rat hindlimb is a potential marker for the decrease in nutritive flow that accompanies 5-HT mediated vasoconstriction. At the concentration used (23 μM) 5-HT mediated a decrease of 36% in the steady state ratio of 1-MU/1-MX indicative of a considerable change in flow pattern within the hindlimb (Newman *et al.* 1996) leading to a markedly lower uptake of nutrients and release of products (Clark *et al.* 1995). Since there was no evidence of a redistribution of flow between tissues of the hindlimb, a redistribution of flow (nutritive to non-nutritive functional shunting) within each muscle was likely. Future studies will focus on the effects of Type A vasoconstrictors on the metabolism of 1-MX by the constant-flow perfused rat hindlimb.

Chapter 5

Evidence for vessels supplying septa and tendons acting as functional shunts in the perfused hindlimb.

5.1 Introduction

Data from the previous chapter indicate the presence of shunts in muscle. However, it is generally accepted that there are no non-capillary pathways from artery to vein (arterio-venous anastomoses) in muscle (Hammersen 1970). The currently favoured view of blood flow shunting in muscle is that the muscle possesses "functional shunts" which manifest themselves as non-nutritional capillary networks. One possible form that a non-nutritional capillary network may take is that of a high flow rate capillary. Unequal distribution of flow will result in an exchange inefficiency in capillaries with high flow rates (Renkin 1969) whilst some areas of tissue surrounded by slowly perfused capillaries will be under-perfused. Poiseuille's law predicts that blood will flow preferentially through shorter capillaries with less resistance (Renkin 1969). It has been proposed that although these vessels account for only 13% of capillary density, such capillaries could theoretically carry 70% of blood flow under resting conditions (Harrison et al.1990).

Another possibility for functional shunts is that they are anatomically distinct from the skeletal muscle circulation. As long ago as 1961 Barlow, Haigh and Walder proposed a dual circulatory system in muscle based on observations of ^{24}Na clearance from semi-isolated muscle (Barlow *et al.*1961). These authors proposed that the lack of a relationship between blood flow and ^{24}Na clearance could easily be explained if part of the circulation could bypass the capillary bed of the muscle. Furthermore, high doses of adrenaline could selectively divert flow to this functional shunt. The suggestion by Barlow *et al.*that this non-nutritive bypass was supplying septa and tendon was further developed in an anatomical study by Grant and Payling-Wright (1970).

Most of these early clearance studies employed β or γ emitting radioisotopes such as ^{24}Na or ^{131}I to detect regional blood flow. However, the use of these isotopes

in perfusion is not entirely desirable from a safety viewpoint. More recently, tendon blood flow has been measured using surface laser Doppler flowometry in clinical applications (Astrom and Westlin 1994, Shino *et al.*1991). Using this technique, tendon blood flow can be measured in a non-toxic and non-invasive manner.

In this chapter a similar technique, using surface fluorometry, was used to estimate perfusate flow through the tibial tendon of the biceps-femoris muscle in the perfused rat hindlimb during Type A and Type B stimulation. The results from these surface fluorometry studies suggested that Type B vasoconstriction caused an increased flow rate through the tendon which was accompanied by decreased metabolic rate in the hindlimb. Furthermore, Type A vasoconstriction caused a decreased flow rate through the tendon accompained by an increase in hindlimb metabolic rate. These data suggest that the funtional shunts involved in vasocontrictor mediated inhibition of metabolism may indeed be the vessels that supply septa and tendons.

5.2 Materials and Methods

Single hindlimb perfusions of 180-200 g rats were conducted at 25°C as described in Chapter 3. A minor modification was made to the surgery to expose the tibial tendon of the biceps-femoris muscle by removing the skin from the area around this tissue below the knee (Fig. 5.1). The perfusion cabinet was also modified to exclude ambient light from the hindlimb and avoid interference with the flourometry measurement. The hindlimb was clamped in place so that a uv/visible, excitation/emission probe could be placed on the tendon. This probe was connected via fibre optic cables to an Aminco-Bowman flourometer (2 cables for λex = 495 nm, 4 cables for λ.em = 528 nm). FITC-dextran (Sigma, Mr 150 000) (0.003 % wt./vol.) was infused into the arterial perfusion line of the hindlimb in pulses of six minutes duration. The relative flourescence of the biceps femoris tendon vessels during vasoconstriction or at rest was recorded on one channel of a two channel chart recorder. The venous effluent was pumped through an Hitachi (F1000) HPLC fluorescence detector. The signal was recorded on the second channel of the chart recorder. Emission wavelength in this case was set at 450 nm so that the signal would not overload the light detectors.

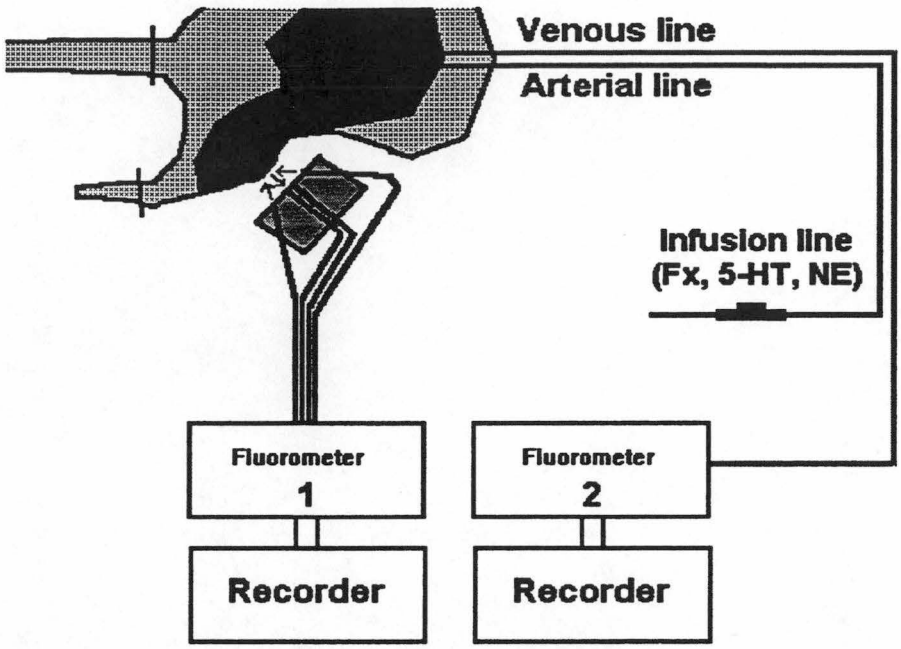

Figure 5.1 Diagram of the perfused rat hindlimb set up for biceps-femoris flow fluorometry.

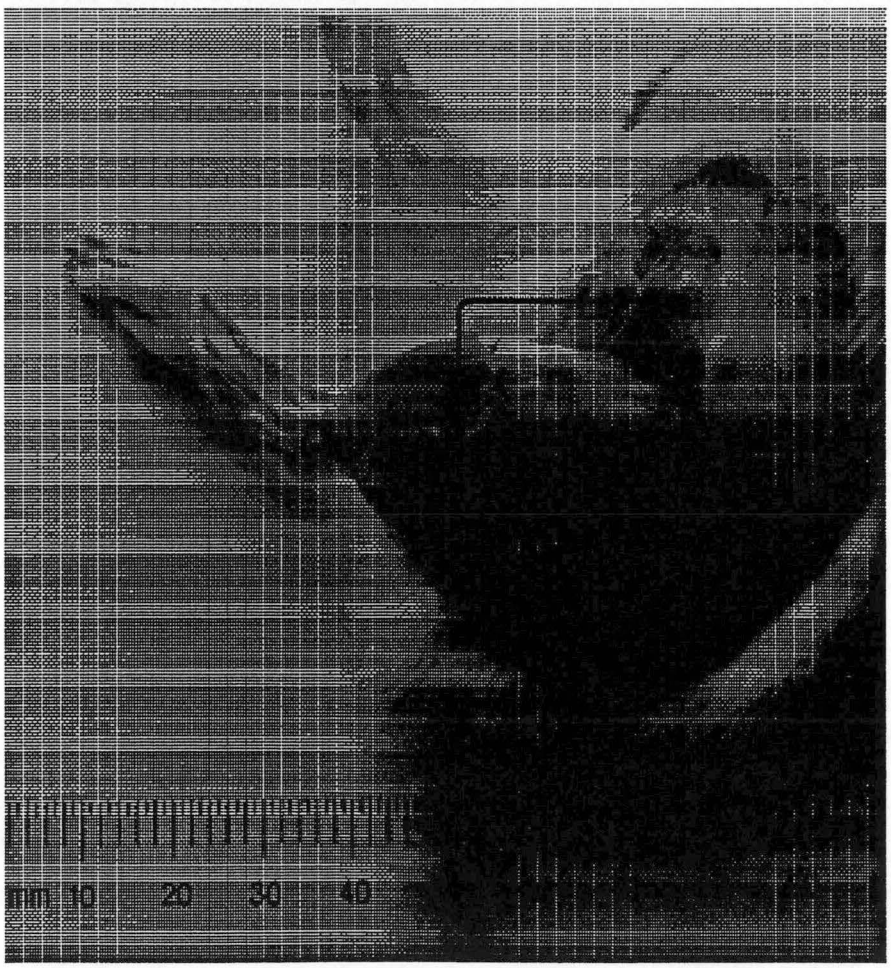

Figure 5.2 Photograph of the exposed biceps-femoris tendon.

5.3 Results

Figure 5.3 shows the results from a typical experiment measuring the fluorescent signal from the biceps femoris tendon during successive doses of 5-HT. The first two pulses of FITC-dextran (indicated by solid bars), in the absence of any 5-HT-induced vasoconstriction, produced peaks of fluorescence that were approximately the same height. However, if a FITC-dextran pulse was infused into the arterial line of the hindlimb in the presence of 5-HT the fluorescence signal from the tendon increased. This increase in fluorescence was particularly notable at 5-HT concentrations of 0.1, 0.3 and 1 μM. The increase in tendon fluorescence corresponded to a decrease in hindlimb oxygen consumption. After the removal of 5-HT the oxygen consumption recovered to the pre-5HT rate of 6 μmol/g/h. The fluorescence signal from the tendon also returned to approximately the pre-5HT level. The maximum fluorescence shown in the top panel of Figure 5.3 is the signal from the fluorometer measuring the fluorescence from the venous output of the rat. Since the venous return signal was always 100 % (arbitrary units) it can be assumed that each FITC-dextran pulse was the same and the arterio-venous recovery was 100%.

Figure 5.4 shows a typical experiment measuring the effects of norepinephrine (Type A) on the fluorescent signal from the same biceps femoris tendon. The first two control pulses of FITC-dextran produced fluorescence signals that were the same height. Pulses of FITC-dextran infused during norepinephrine-induced vasoconstriction produced diminished fluorescence signals from the biceps femoris tendon. The magnitude of the fluorescence signal from the biceps-femoris was inversely related to increase in oxygen consumption. For example, 100 nM norepinephrine increased oxygen consumption by 60 % and decreased the fluorescence signal from the tendon by 70%. The recovery of fluorescence shown in the top panel of figure 8.4 was not altered by norepinephrine throughout the duration of the perfusion.

The results of the experiments shown in figures 5.3 and 5.4 are summarized in figure 5.5 (n=3). On the left hand side of figure 5.5 it can be seen that as the dose of norepinephrine increases, oxygen consumption also increases. However, the inverse relationship occurs with the fluorescence signal from the biceps-femoris tendon. It is noteworthy that the NE dose which begins to increase oxygen consumption is also

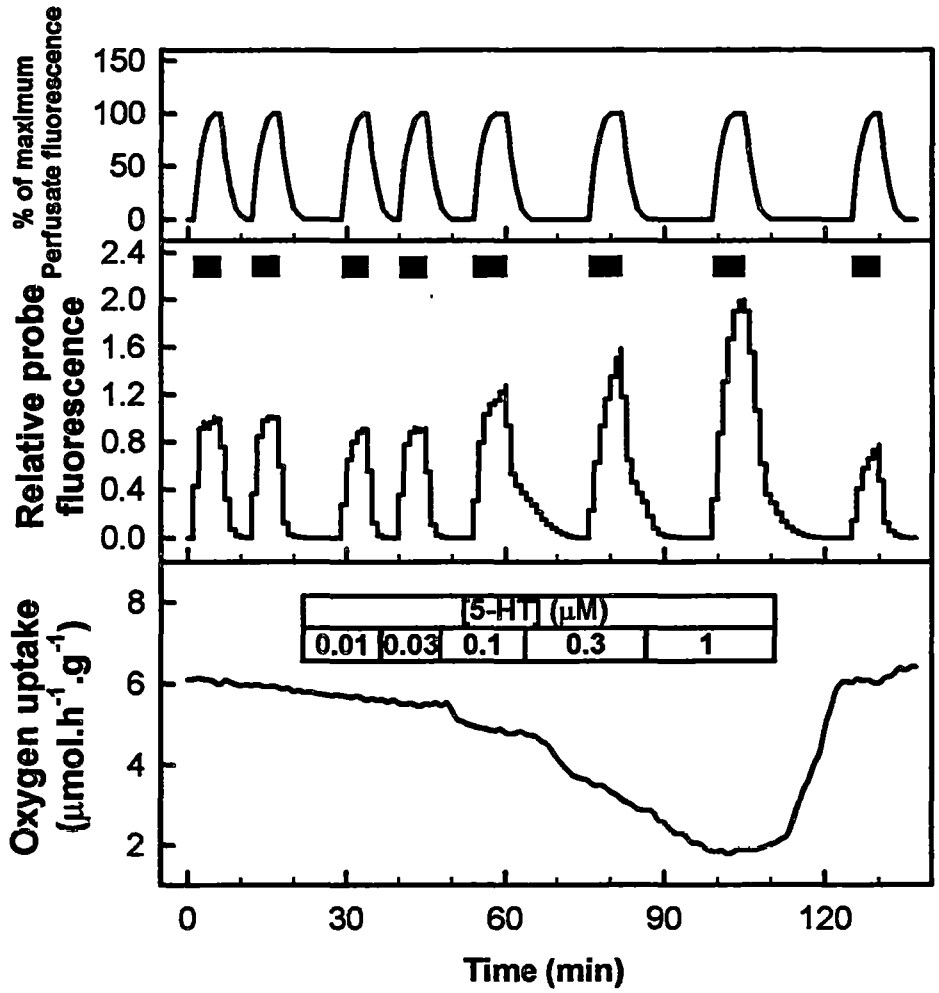

Figure 5.3 Typical trace of oxygen consumption and biceps-femoris fluorescence (Relative probe fluorescence) and total fluorescence (% of maximum perfusate fluorescence) during a 5-HT dose curve. FITC dextran pulses are indicated by solid bars.

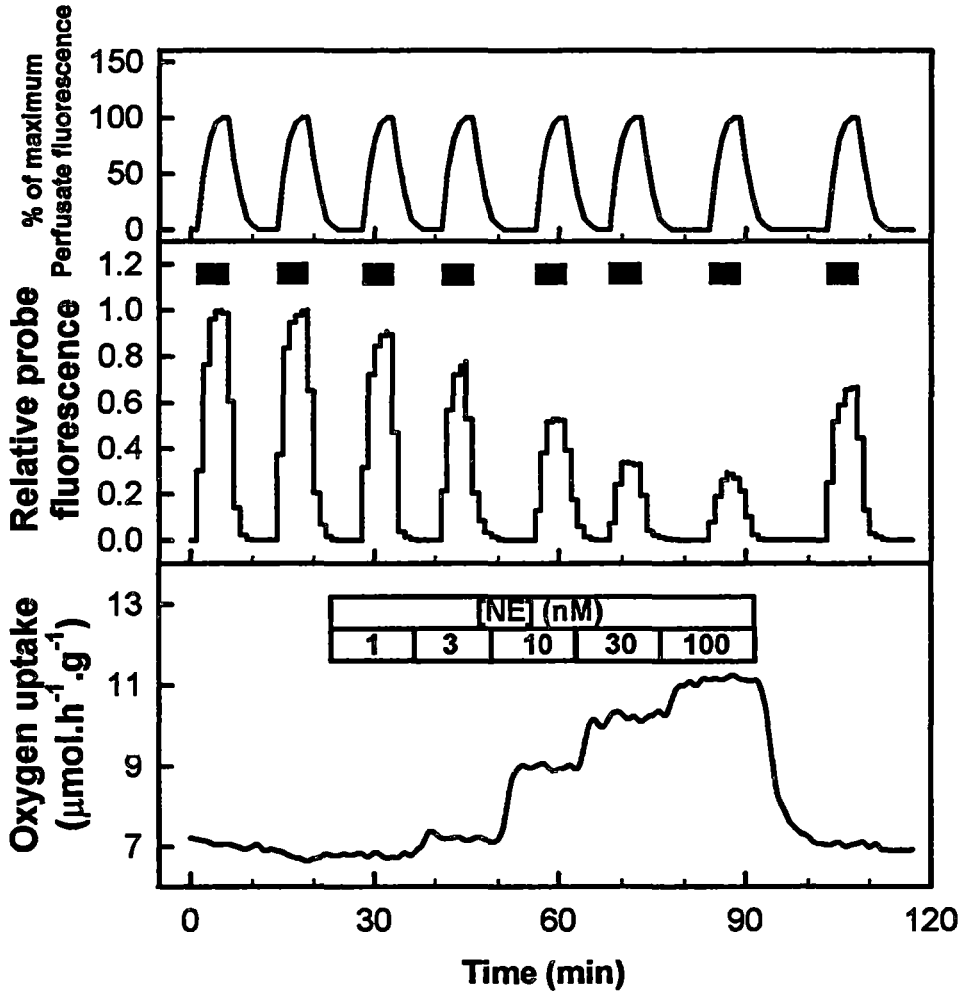

Figure 5.4 Typical trace of oxygen consumption and biceps-femoris fluorescence (Relative probe fluorescence) and total fluorescence (% of maximum perfusate fluorescence) during a norepinephrine dose curve. FITC dextran pulses are indicated by solid bars.

the dose that starts to cause a decrease in the fluorescence signal. The recovery position at the end of the experiment after the removal of norepinephrine is indicated by an open square. This post-norepinephrine fluorescence and oxygen consumption was the same as the control at the beginning of the experiment. The right hand side of the graph in figure 5.5 shows the dose-response of oxygen consumption and biceps-femoris fluorescence to 5-HT. These curves are opposite to the curves observed by increasing the dose of norepinephrine. As the concentration of 5-HT is increased the

oxygen consumption of the hindlimb decreases. This decrease in oxygen consumption is accompanied by an increase in tendon fluorescence

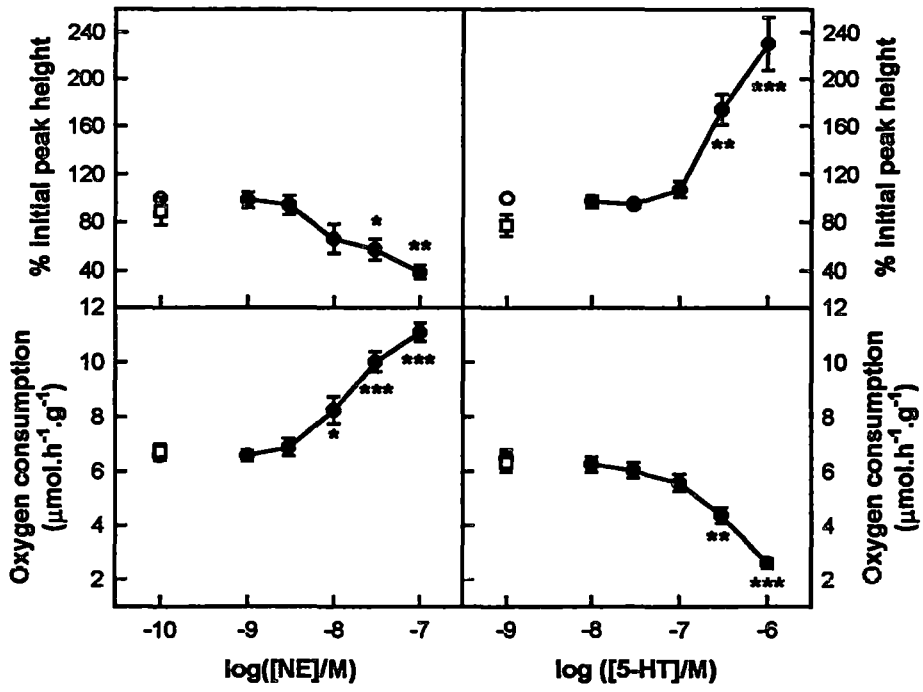

Figure 5.5 Hindlimb oxygen consumption and biceps-femoris tendon fluorescence during norepinephrine (NE) or serotonin (5-HT) dose curves. Recovery rates of oxygen consumption and biceps-femoris tendon fluorescence after the dose curve regimen are indicated by open squares, rates of oxygen consumption and fluorescence prior to the dose curve are indicated by open circles (n=5, * P<0.05, **P<0.001, *** P<0.001).

5.4 Discussion

The main finding from this chapter was that vasoconstrictors which regulate hindlimb oxygen consumption also regulate flow through the connective tissue of the biceps-femoris muscle. The inverse relationship between tendon blood flow and oxygen consumption suggests that the degree of perfusion of the tendon may be an important regulator of total hindlimb metabolism by reducing muscle nutritive vessel

perfusion. The suggestion of vessels supplying septa and tendon acting as functional shunts is not new. The concept of these vessels acting as non-nutritive flow routes in muscle perfusion has been discussed in several recent reviews (Clark *et al.*1996, Chinet 1990, Duling and Damon 1987) based on evidence from several earlier investigations (Barlow *et al.*1961, Grant and Payling-Wright 1970, Sparks and Mohrman 1977, Ley *et al.*1988). However, this is the first report of vasoconstrictors selectively directing flow to, or away from tendon vessels.

In their detailed anatomical study of these tendon vessels, Grant and Payling-Wright (1970) noted that the functional shunt vessels in the biceps-femoris muscle were about twice as wide as the muscle capillaries. According to Poiseuilles law ($dV/dt \propto r^4$), at the same vessel length and perfusion pressure, these functional shunts would be capable of carrying sixteen times the flow of a typical capillary. This simple calculation combined with the fact that these vessels are located in a metabolically inactive tissue makes the hypothesis of a significant non-nutritive flow pathway through tendon highly plausible.

In the same study, Grant and Payling-Wright (1970) provided evidence that the blood vessels in tendon were pharmacologically different from the blood vessels of muscle. For their preparation they noted that adrenaline caused dilation or dilation followed by constriction of skeletal muscle blood vessels whereas in tendon vessels adrenaline caused constriction only. Furthermore, histamine caused constriction of tendon vessels whereas in muscle it caused vasodilation. Further studies on the pharmacology of the tendon vessels compared to muscle are required but it is possible that there are more differences in the response to vasoconstrictors that could account for the selective vasoconstriction caused by serotonin and angiotensin. For example, it is plausible that serotonin receptors causing vasoconstriction of large vessels (Newman *et al.*1996) are predominantly located on the larger arterioles branching from the transverse arterioles (Figure 5.6). When these vessels constrict, flow is diverted to the connective tissue supplied by the distal end of the transverse arteriole. A similar explanation for the action of Type A norepinephrine is also possible. During norepinephrine stimulation the dominant vasoconstriction would occur in the vascular bed of the connective tissue (Figure 5.6). This vasoconstriction would divert flow to the muscle bed. It is quite likely that norepinephrine also causes vasoconstriction of

smaller vessels within the muscle bed because exercise will cause dilation of Type A vasoconstriction (Rattigan *et al.*1996). However, the predominant vasoconstriction would occur in the connective tissue if flow is to be diverted to muscle.

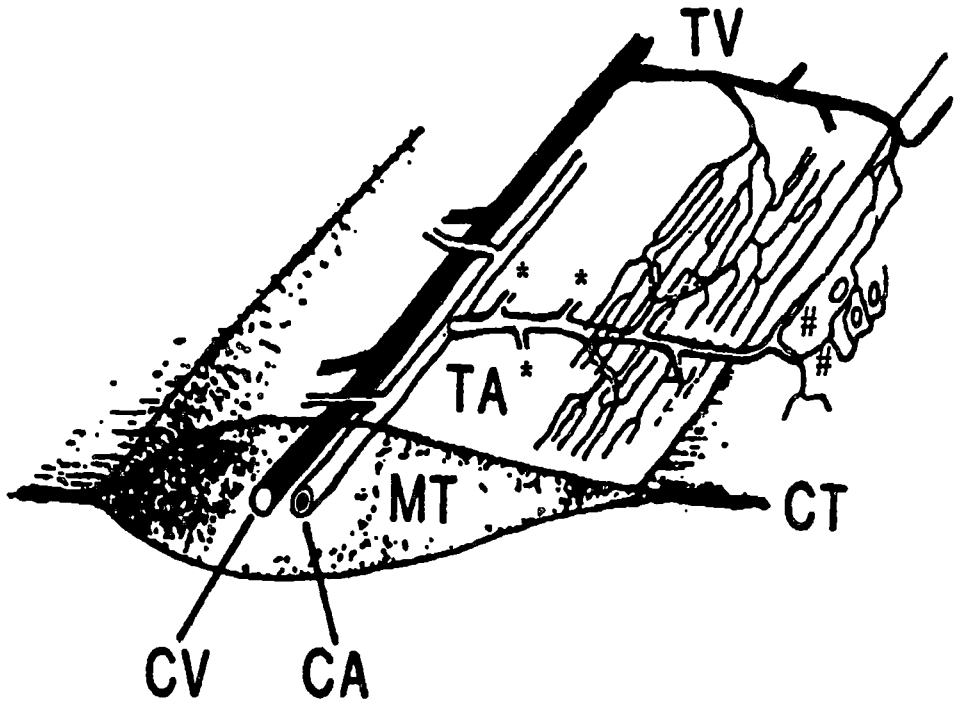

Figure 5.6 Diagram of rabbit tenuissimus muscle in cross-section. MT, muscle tissue; CT, connective tissue; CA,CV, central arteriole and venule; TA, TV, transverse arteriole and venule. *, 5-HT constriction site, # NE constriction site. (Adapted from Ley *et al.*1988).

Chapter 6

Vasoconstrictor-mediated control of lactate metabolism.

6.1 Introduction

The importance of skeletal muscle in whole body lactate metabolism is well recognized with the production and release of lactate by this tissue, particularly during exercise. Lactate released from muscle is subsequently utilized by liver as a precursor of gluconeogenesis and glyconeogenesis[1], forming the basis of the Cori cycle (Consoli *et al.* 1990, McDermott and Bonen 1992). The hepatic glucose production from lactate can be enhanced by the sympathetic nervous system (Saccà *et al.* 1983, Brochman 1991) involving α_1-adrenoceptors (Taylor *et al.* 1986, Ciprés *et al.* 1995). In addition, skeletal muscle can take up and remove lactate from the circulating plasma (Pagliassotti and Donovan 1990, McDermott and Bonen 1992, Poole and Halestrap 1993). Although the pathways involved in removal of lactate by muscle have not been comprehensively identified (McDermott and Bonen 1992, Guttierrez *et al.* 1994), oxidation via pyruvate and glyconeogenesis are the probable metabolic fates (McLane and Holloszy 1979, McDermott and Bonen 1992, Wickler and Gleeson 1993).

Catecholamines play an important role in regulating muscle lactate metabolism (McDermott and Bonen 1992), with the stimulation of lactate release (Richter *et al.* 1982) or lactate oxidation (McDermott and Bonen 1992) frequently linked to β-adrenoceptors. Despite this, studies involving perfused muscle have noted an α-adrenergic stimulation of lactate release when lactate levels in the perfusion medium were zero (Hettiarachchi *et al.* 1992, Clark *et al.* 1995) or low (i.e. 1.5-2 mM, Richter *et al.* 1982). These increased rates of skeletal muscle lactate release were accompanied by increases in oxygen consumption (Richter *et al.* 1982, Hettiarachchi *et al.* 1992, Ye *et al.* 1995) and glucose uptake (Richter *et al.* 1982). The α-adrenergic effects appear difficult to explain given the paucity of evidence for functional α-adrenoceptors on skeletal muscle (Clark *et al.* 1995), but the finding that vasodilators oppose the lactate release mediated by α-adrenoceptor as well as other vasoconstrictors such as angiotensin II and vasopressin in the perfused hindlimb

[1] Glyconeogenesis is the synthesis of glycogen from gluconeogenically derived glucose (Pagliosotti and Donovan 1990)

(Hettiarachchi *et al.* 1992, Ye *et al.* 1995) implies a key role for the vasculature in controlling lactate metabolism.

The perfused hindlimb preparation has been proven to be a useful model for the study of the relationship between vascular function and skeletal muscle metabolism because it maintains the structural integrity of these two tissues (Bonen *et al.* 1994). Apart from that, hindlimb includes all three muscle fiber types with different glycolytic capacities, namely slow-twitch oxidative (SO), fast-twitch oxidative-glycolytic fibers (FOG) and fast-twitch glycolytic fibers (FG) (Ariano *et al.* 1973 and references therein). SO muscle fiber is known to have high mitochondrial oxidative enzyme (such as succinate dehydrogenase, SDH) activity and lower glycolytic enzyme (phosphofructokinase, PFK) activity. In contrast, FG muscle type has higher PFK activity but lower SDH activity. Pagliassotti and Donovan (1990) have demonstrated that oxidative (SO and FOG) muscle fibers are more capable of removing circulating lactate. The higher lactate removal rate in oxidative muscle fibers may be due to a monocarboxylate transporter (MCT1) in these fibers which favors lactate uptake (McCullagh et al. 1996). Hence, changes in the recruitment of the muscle fibers may also alter the hindlimb lactate balance. In addition to the heterogeneity of muscle fibers in the hindlimb, blood flow among the muscle fibers is also heterogeneous. For example, the blood flow rate under resting conditions is much higher in SO fibers than others (Folkow and Halicka 1968). Hence, changes in the recruitment of the muscle fibers may also alter the hindlimb lactate balance.

In the perfused hindlimb using lactate-free media, increasing perfusion flow rate stimulates both lactate release and oxygen consumption (Ye *et al.* 1990). In comparison, the effects of vasoconstrictors can be stimulatory or inhibitory even when the perfusion flow is maintained constant (Clark *et al.* 1995). According to their metabolic effects, vasoconstrictors can be categorized into two types. Type A vasoconstrictors such as norepinephrine, vasopressin, angiotensin II stimulate metabolism while the metabolic effects of type B vasoconstrictors such as serotonin are opposite (Clark *et al.* 1995). Type A vasoconstrictors all stimulate net lactate release (Hettiarachchi *et al.* 1992, Ye at al. 1995b). Studies using dye washout kinetics and vascular casting in the same preparation indicated that more vascular space was recruited when type A vasoconstrictors increase hindlimb metabolism.

Perfused vascular space decreased during type B vasoconstriction (Newman *et al.* 1996). Based on these data, it has been hypothesized that the effects of type A vasoconstrictors on metabolism in the constant-flow perfused hindlimb are caused by site-selective vasoconstriction which increases capillary recruitment (Clark *et al.* 1995).

In vivo, the circulating lactate ranges between 1-2 mM, and this level can rise to more than 10 mM during exercise (Poole and Halestrap 1993). When the lactate concentration is higher than 4-6 mM, resting skeletal muscle takes up lactate instead of releasing it (McLane and Holloszy 1979, Pagliassotti and Donovan 1990, Poole and Halestrap 1993). Therefore, the studies described in this chapter attempted to test whether the presence of high lactate concentration (10 mM) in the perfusate can negatively affect the stimulatory effects of NE and increased flow on lactate release.

6.2 Materials and Methods

6.2.1 Metabolite assays

Samples (2.0 ml) of venous effluent were collected at each new steady state, or as indicated during the time courses. The collected perfusate was centrifuged for 5 min at 3,000 rpm to remove erythrocytes and kept frozen (-20°C) until assays could be conducted. Lactate was assayed by an enzymatic method (Bergmeyer 1974). Lactate release or uptake was calculated from the arteriovenous difference in perfusate lactate concentrations multiplied by flow rate and divided by the weight of perfused muscle. Arterial and venous perfusate glucose concentrations were determined and glucose uptake was calculated according to the same principle.

The gastrocnemius-plantaris-soleus muscle group was sampled for the measurement of muscle lactate, phosphocreatine and creatine. The skin covering the calf muscle was surgically removed and gently separated from other muscles to give complete exposure without disturbing perfusate flow to the tissue. Then, the muscle group was lifted above the others at the distal end and freeze-clamped *in situ* with liquid N_2 precooled tongs before being cut free from the leg. A sample of the gastrocnemius-plantaris-soleus muscle group was similarly taken from an anesthetized rat in vivo. Phosphocreatine and creatine were determined by HPLC with an ion-paired reversed phase column as previously described (Ye *et al.* 1996 b).

6.2 Results

6.2.1 Basal lactate flux, oxygen uptake and perfusion pressure.

Table 6.1 shows the steady state basal values of perfusion pressure, oxygen uptake and lactate release in the constant flow perfused hindlimbs in the presence, or absence, of lactate. There was a net release of lactate at a rate of 6.1 ± 0.01 $\mu mol \cdot g^{-1} \cdot h^{-1}$ (n=7) from the hindlimb perfused with the lactate-free medium. Addition of 10 mM lactate to the arterial perfusate totally reversed the lactate balance from efflux to uptake (Table 6.1). The change in lactate balance was associated with a 35% increase in oxygen consumption ($P<0.01$) with no apparent effect on the basal perfusion pressure.

Table 6.1. Basal lactate flux, oxygen uptake, and perfusion pressure.

	n	Lactate release ($\mu mol \cdot g^{-1} \cdot h^{-1}$)	Oxygen uptake ($\mu mol \cdot g^{-1} \cdot h^{-1}$)	Perfusion pressure (mm Hg)
Lactate-free	7	6.1 ± 0.01	7.8 ± 0.22	37.1 ± 1.10
10 mM Lactate	13	-16.0 ± 2.00**	10.5 ± 0.16**	36.2 ± 0.45

Negative lactate release = lactate uptake. **P<0.01 vs lactate-free perfusions.

6.2.2 Effects of NE.

In the absence of added lactate, NE induced lactate release at all doses (Fig. 6.1). When lactate was present, creating, as noted above, a net uptake, NE exerted a dose-dependent inhibition of that uptake. At concentrations above 0.1 μM, the presence of NE resulted in net release.

The dose-dependent responses for perfusion pressure and oxygen uptake induced by NE in the perfused hindlimb were similar regardless of the presence or absence of lactate in the perfusate (Fig. 6.1). Significant responses to NE were evident at a dose of 3.3 nM. Oxygen uptake was maximal at 0.33 μM and began to decline beyond this dose even though the perfusion pressure continued to rise. Perfusion pressures were slightly lower at each dose of NE in the presence of lactate ($P<0.01$) while corresponding values for oxygen uptake were slightly higher in the

hindlimbs perfused with 10 mM lactate. At higher concentrations of NE (≥ 0.1 μM), the differences in oxygen uptake between these two groups (+/- lactate) decreased.

6.3.3 Effect of increasing perfusion flow rate

Figure 6.2 shows the effect of increasing perfusion flow rate from 5 to 10 ml/min (0.33 to 0.66 $ml \cdot min^{-1} \cdot g\ muscle^{-1}$) on perfusion pressure, oxygen uptake and lactate uptake. This increase in flow significantly increased both perfusion pressure and oxygen uptake ($P<0.01$) whether or not lactate was added. Oxygen uptake at the steady state was approximately 18% higher at 10 ml/min in the presence of lactate (12.50 ± 0.75 vs 14.7 ± 0.47 $\mu mol \cdot g^{-1} \cdot h^{-1}$, $P<0.05$). In the absence of lactate, increasing flow stimulated lactate release (from 6.16 ± 0.18 to 10.1 ± 0.94 $\mu mol \cdot g^{-1} \cdot h^{-1}$, $P<0.01$) and this change paralleled the change in oxygen uptake. In the presence of 10 mM lactate, the increase in flow rate from 5 to 10 ml/min stimulated lactate uptake by two fold (16.25 ± 1.87 vs 37.20 ± 8.54 $\mu mol \cdot g^{-1} \cdot h^{-1}$, at 20 min, see Fig.6.2).

6.3.4 Time course of the changes induced by NE and flow.

The experiments shown in Fig.6.3 were conducted in the presence of 10 mM lactate. The changes in perfusion pressure, oxygen uptake and lactate balance induced by NE (0.4 μM) or increased flow (10 ml/min) were sustained and reversible. The stimulatory effects on perfusion pressure and oxygen uptake were observed when NE was infused or when the flow rate was increased. Despite these similarities, different changes in lactate metabolism occurred during the two conditions. Increased flow accelerated lactate uptake but NE still caused a net lactate efflux.

Analysis of glucose concentrations from the perfusate samples taken at steady state level showed that both NE and increased flow elevated glucose uptake by 144% and 253%, respectively (Fig. 6.4).

Table 6.2 Muscle creatine compounds and lactate

	n	PCr	Cr	Lactate	Ratio of PCr/Cr
			$\mu mol \cdot g^{-1}$		
In vivo	5	26.5 ± 2.04	24.8 ± 1.69	2.10 ± 0.20	1.07 ± 0.02
Perfused (lactate-free)	5-6	23.7 ± 0.95	23.9 ± 2.40	1.22 ± 0.14	1.00 ± 0.04

The gastrocnemius-plantaris-soleus muscle group was freeze-clamped under liquid N_2 for the assay. Phosphocreatine (PCr) and creatine (Cr) were determined by HPLC. The muscle sample *in vivo* was freeze-clamped under anesthesia whilst the perfused muscle was taken from the hindlimb perfused at 5 ml/min for 120 min for the lactate-free perfusions.

6.3.5 *Involvement of α- and β- adrenoceptors on NE-induced changes.* In order to clarify whether β-adrenoceptors were involved in the observed changes induced by 0.4 μM NE, the β-adrenergic blocker, 10 μM (+/-)-propranolol, was infused 10 min before and during NE infusion. The results in Fig. 6.5 indicate that NE-induced oxygen uptake and net lactate efflux were not significantly affected by propranolol although the perfusion pressure was higher in the presence of propranolol. Vasopressin, a vasoconstrictor, also caused lactate efflux. Isoproterenol, a β-agonist caused a small increase in lactate uptake. The α-adrenoceptor-mediated responses were similar to those induced by vasopressin but different from those mediated by the β-adrenergic agonist isoproterenol which elicited an increase in lactate uptake as well as oxygen consumption (Fig 6.5).

6.3.6 Muscle metabolites.

The metabolic validity of the perfusion system under the conditions used in the present experiments has been well established (Bonen *et al.* 1994. Clark *et al.* 1995). The results in Table 6.2 further confirm that the hindlimb when perfused at 5 ml/min has an adequate oxygen supply as indicated by the ratio of phosphocreatine to creatine being similar to that of the muscle sampled in vivo.

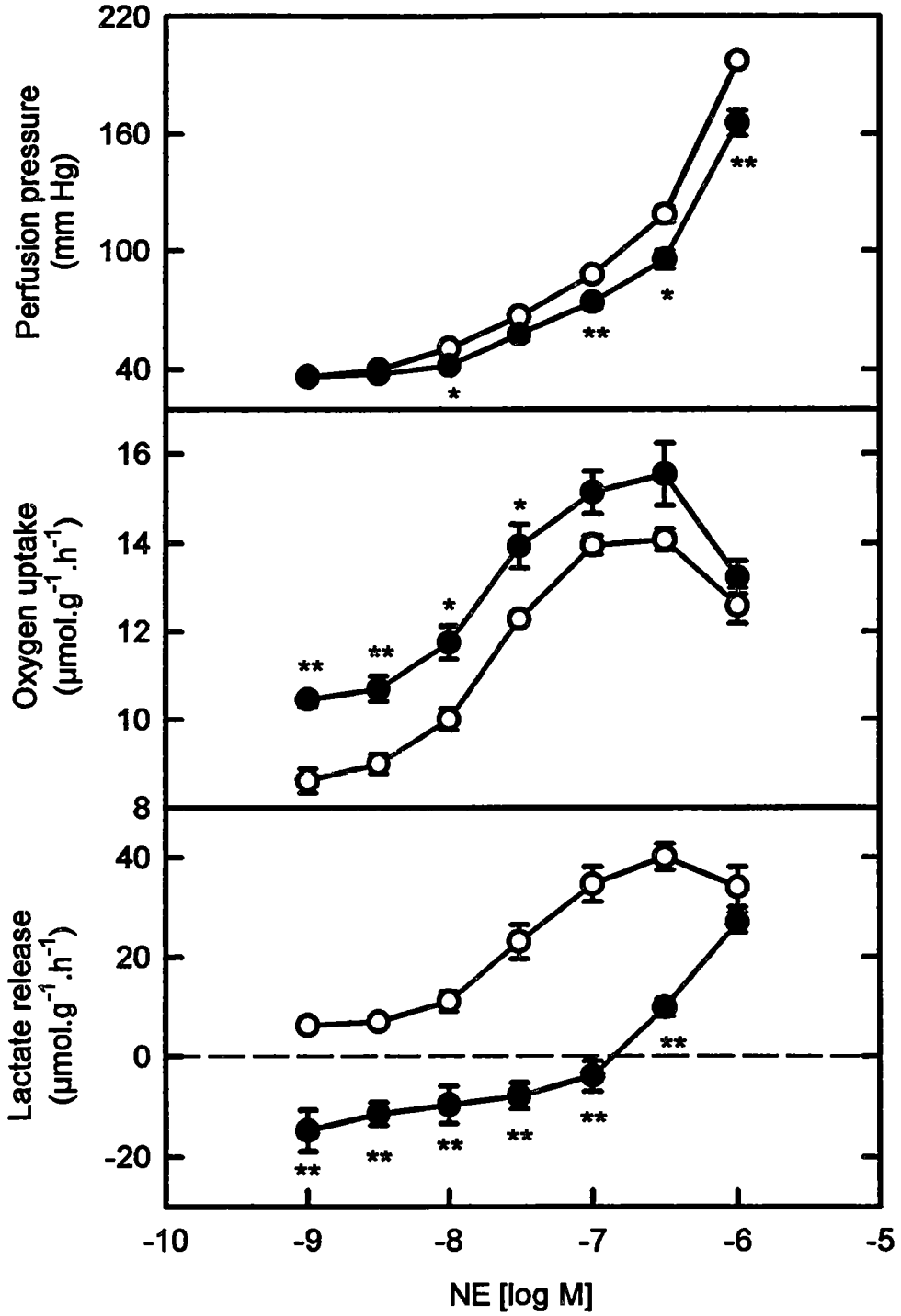

Figure 6.1. Dose-response curves for the effect of norepinephrine (NE) on perfusion pressure, oxygen uptake and lactate balance. The hindlimb was perfused either in the absence (○) or in the presence (●) of 10 mM L-(+)-lactate. Lactate uptake by the hindlimb is expressed as the negative value of release. Data were obtained from four perfusions for each group. When not visible, error bars are within the symbols. *P<0.05, **P<0.01 vs lactate-free perfusions.

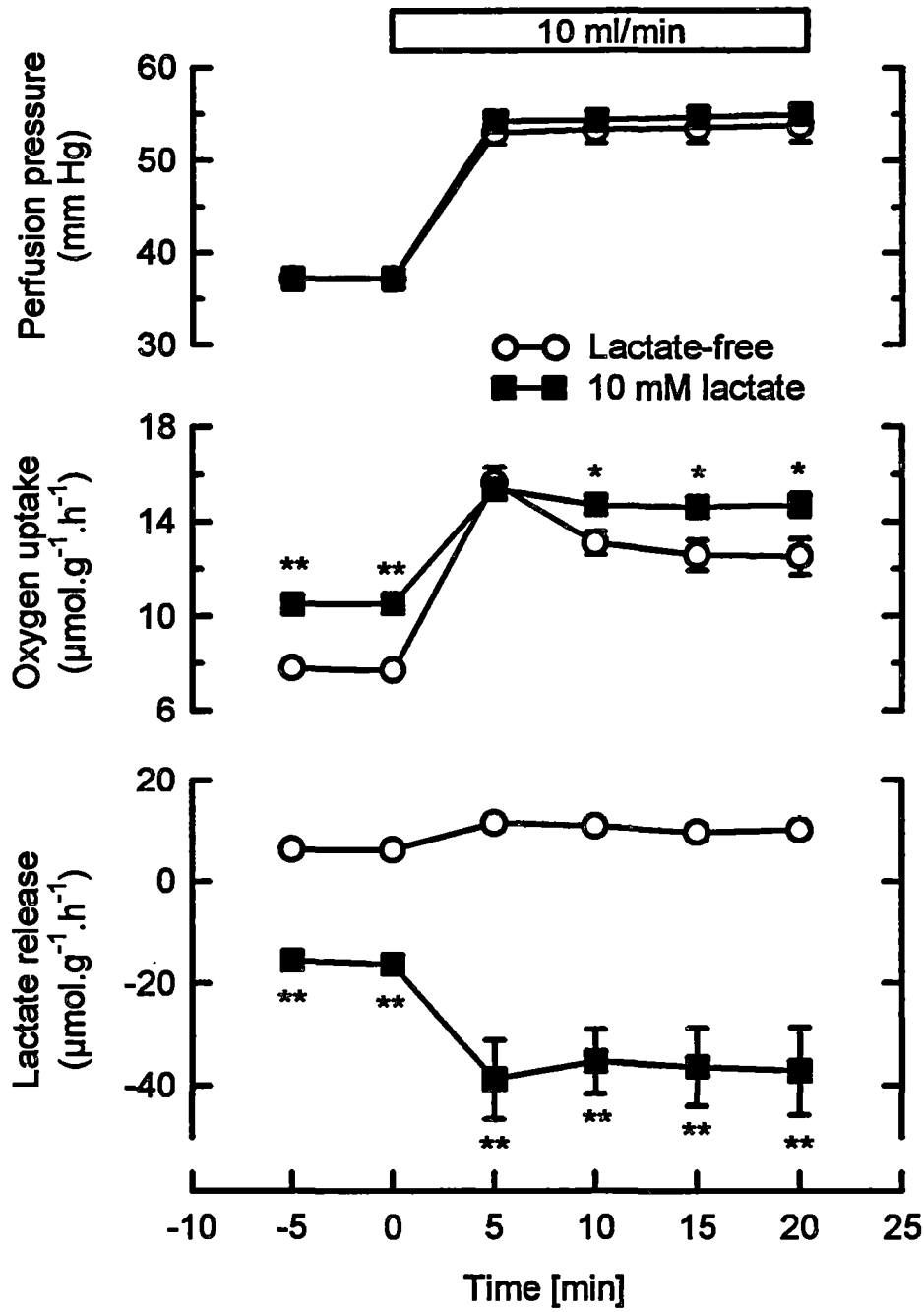

Figure 6.2. Effect of increasing flow on perfusion pressure, oxygen uptake and lactate balance. The hindlimb was perfused at 5 ml/min for 40 min (30 min equilibration and 10 min experimental) followed by a perfusion at an increased flow rate of 10 ml/min. Four experiments were conducted for each group for 20 min. O: Lactate-free, ■: 10 mM lactate. Lactate uptake is expressed as the negative value of release. When not visible, error bars are within the symbols. *P<0.05, **P<0.01 vs lactate-free perfusion.

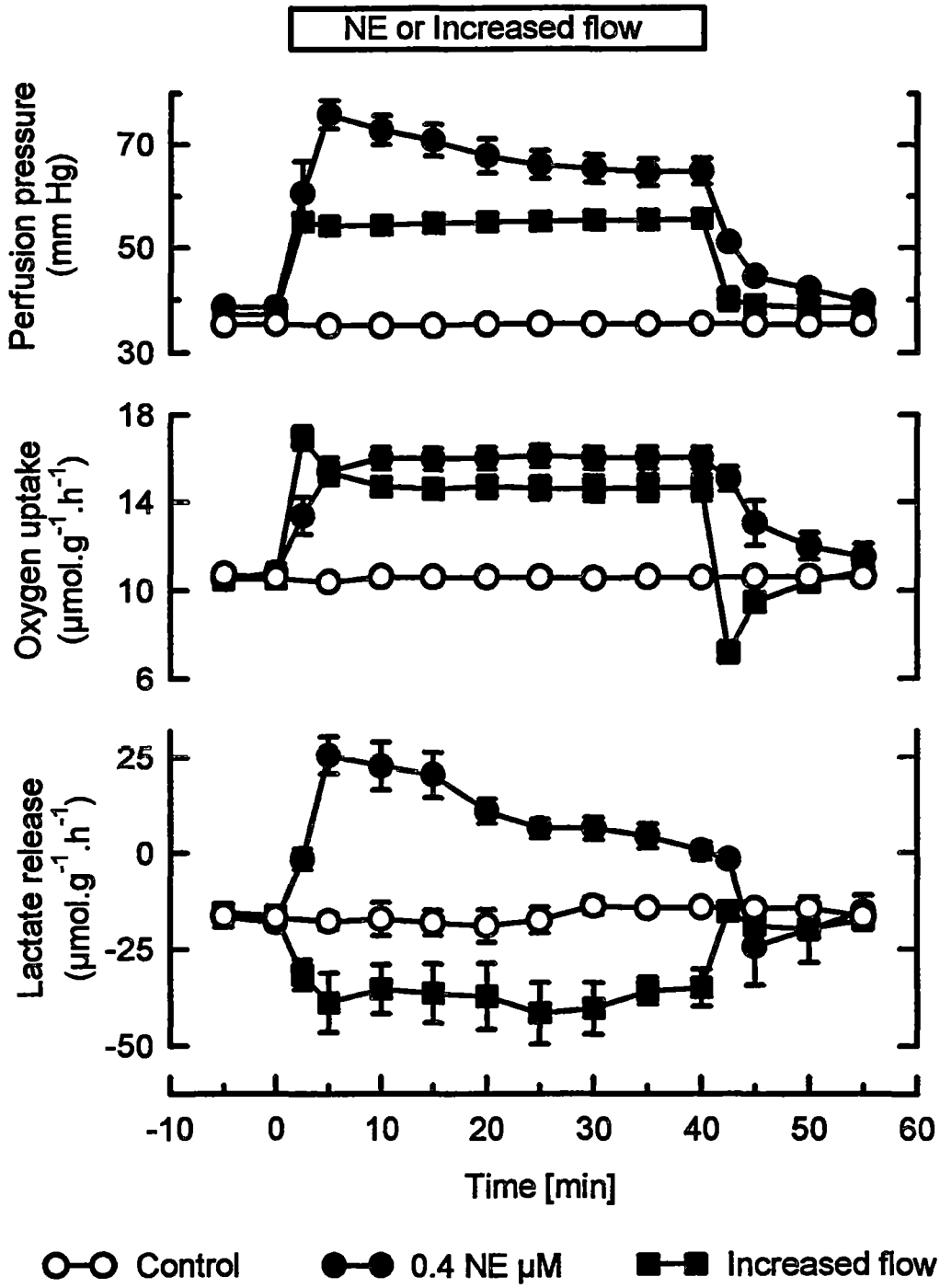

Figure 6.3. Time course for the changes induced by norepinephrine and increased flow. All perfusions were conducted with 10 mM lactate presence at 5 ml/min for the control (O) and norepinephrine (0.4 µM NE ●) groups. Other perfusions were conducted at 10 ml/min (■). Lactate uptake is expressed as the negative value of release. Four or five perfusions were performed for each group. The results of statistical tests are not shown in the figure.

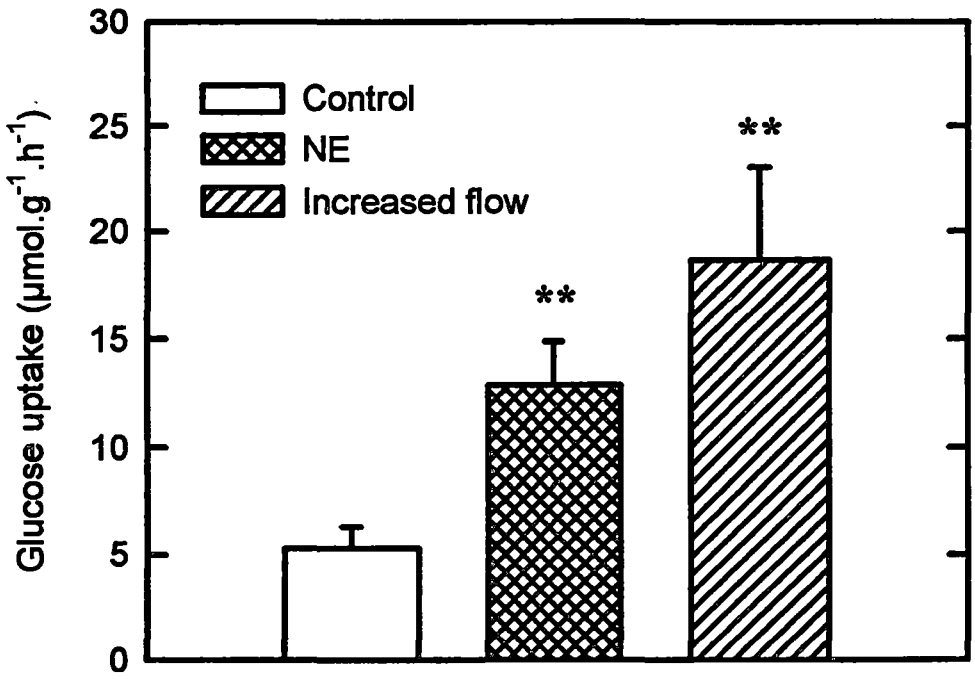

Figure 6.4. Effect of norepinephrine and increased flow on glucose uptake by the hindlimb perfused in the presence of 10 mM L-(+)-lactate. The perfusion flow rate was set at 5 ml/min for the control and NE groups, other perfusions were at 10 ml/min and denoted "increased flow". Nine perfusions were performed for the control and five perfusions each for norepinehrine (0.4 µM NE) and increased flow. **P<0.01 vs control.

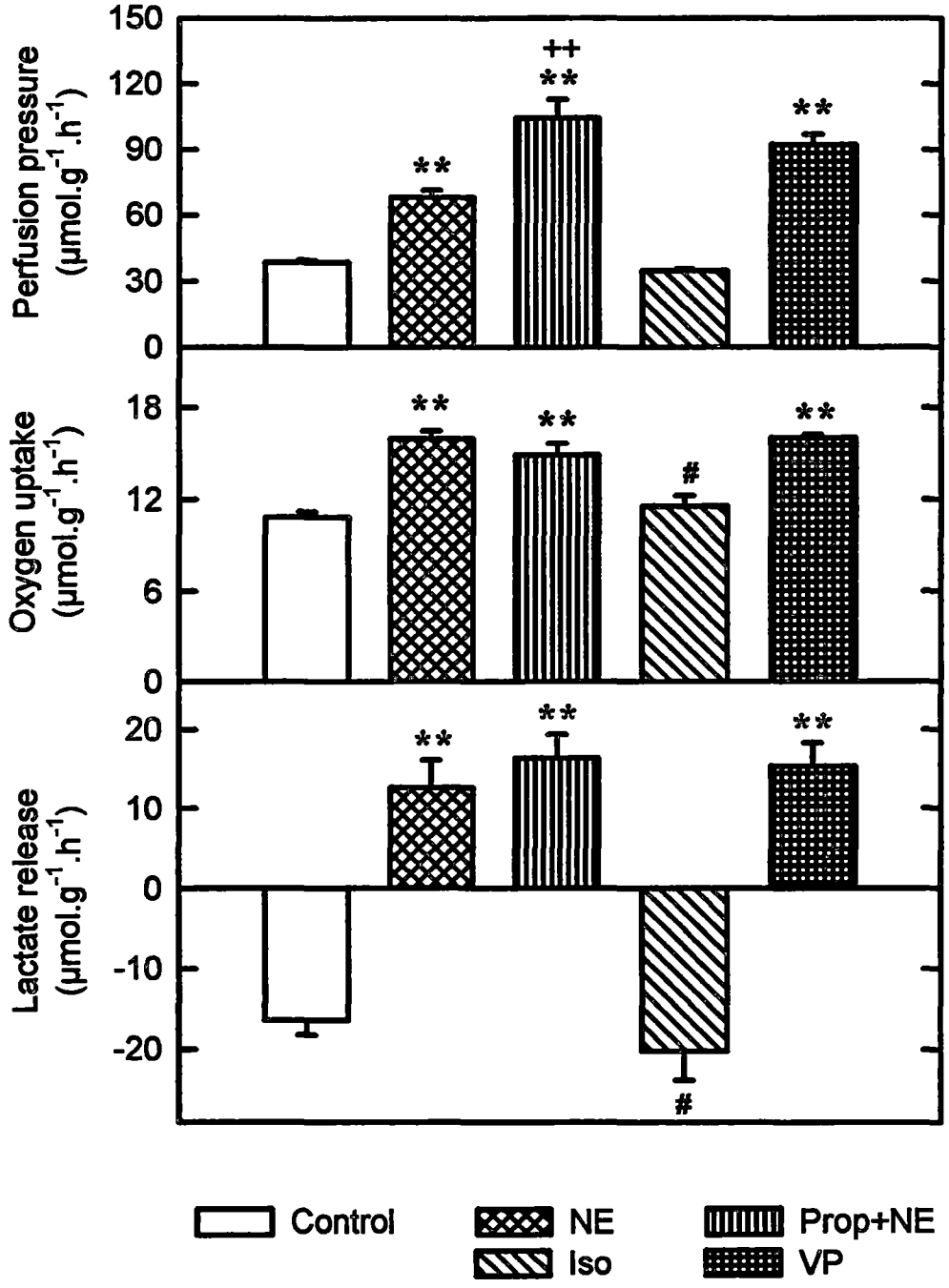

Figure 6.5. Effect of adrenergic agents and vasopressin on perfusion pressure, oxygen uptake and lactate balance. The hindlimb was perfused at 5 ml/min in the presence of 10 mM L-(+)-lactate. Lactate uptake is expressed as the negative value of release. Control (n=5), NE (0.4 µM, n=4) and 0.4 µM NE in the presence of 10 µM (+/-)-propranolol (Prop n=4), Iso (1 µM isoproterenol, n=4), VP (1 nM vasopressin, n=4). **P<0.01 vs control, ++P<0.01 vs NE alone, #P<0.05 vs control (Paired *t*-test).

Table 6.3 shows the effect of 0.4 μM NE and increased flow on muscle lactate content. In the absence of arterial lactate, NE decreased muscle lactate. Increasing the flow rate caused a significant rise in muscle lactate. In the presence of arterial lactate (10 mM), muscle lactate content tended to rise during increased flow but was not altered by NE or a combination of propranolol and NE. In the presence of isoproterenol, muscle lactate content was significantly increased.

Table 6.3 Effects of norepinephrine and increasing flow on muscle lactate content

	n	Control	NE	Prop+NE	Iso	Flow↑
		μmol/g wet				
Lactate-free	5-6	1.22 ± 0.14	0.66 ± 0.07 [a]	nd	nd	2.04 ± 0.24 [a b]
10 mM lactate	4	6.96 ± 0.22	7.28 ± 0.28	7.19 ± 0.28	8.56 ± 0.48 [a]	7.86 ± 0.31 [+]

Lactate was assayed from the gastrocnemius-plantaris-soleus muscle freeze-clamped under liquid N_2. Details of the assay was described in Chapter 2. For the lactate-free perfusions, the control muscle was taken from the hindlimb perfused at 5 ml/min, other groups were from hindlimbs perfused at 8 ml/min. All perfusions in the presence of 10 mM lactate were conducted at 5 ml/min except for increasing flow group (10 ml/min). NE: 0.4 μM norepinephrine. Prop: 10 μM *dl*-propranolol. Iso: 1.0 μM. a, $P<0.05$, vs control, b, $P<0.01$ vs NE, +, $P=0.0557$ vs control. nd: not detected.

6.4 Discussion

The major finding of this investigation is that in the presence of 10 mM lactate, increased flow enhanced, whereas NE reversed, the lactate uptake by the perfused rat hindlimb despite their similar effects on perfusion pressure and oxygen uptake. These results differ from those in the lactate-free perfusions where both NE and increased flow increased lactate release in parallel with their stimulatory effects on oxygen uptake (Ye *et al.* 1990, Hettiarachchi *et al.* 1992, Clark *et al.* 1995). Although flow induced lactate influx into skeletal muscle has been reported by others (Watt *et al.* 1994) in rat hindlimb perfused with low arterial lactate concentration (1 mM), the effects of NE and flow on muscle lactate balance in the presence of high concentration lactate have not been reported.

Previous studies in lactate-free hindlimb perfusion have revealed that NE stimulates lactate release and oxygen uptake in association with an α-adrenoceptor-mediated increase in perfusion pressure (Ye *et al.* 1990, Hettiarachchi *et al.* 1992, Clark *et al.* 1995). These changes may result from vasoconstriction at discrete sites on the vascular network to divert more perfusate through nutritive capillary routes in skeletal muscle (Clark *et al.* 1995, Newman *et al.* 1996). Therefore, an improved nutritive flow leading to increased lactate clearance may account for the increased lactate release associated with lower muscle lactate content obtained during NE infusion in the present study.

When 10 mM lactate is present the gradient of lactate across the muscle membrane would appear to favor uptake (Pagliassotti and Donovan, 1990, McDermott and Bonen, 1992). Increased flow, by increasing the rate of lactate exchange with skeletal muscle (Watt *et al.* 1994) should increase lactate uptake. This is true for the effect of increased flow on lactate balance. As suggested by Figs. 3-4, the improved exchange was a result of the unchanged arteriovenous lactate difference multiplied by a doubled flow rate.

The basal perfusate flow rate of 5 ml/min (0.33 $ml \cdot min^{-1} \cdot g^{-1}$) used in these experiments is higher than the estimated blood flow rates *in vivo* (0.27 $ml \cdot min^{-1} \cdot g^{-1}$, Armstrong and Laughlin 1985). Despite the absence of erythrocytes, such a flow rate is able to provide sufficient nutrients and oxygen under the present conditions at 25°C (Colquhoun *et al.* 1990, Ye *et al.* 1990). This conclusion is strengthened in the present chapter by the low muscle lactate content and a ratio of phosphocreatine to creatine comparable to *in vivo* values. These data together with those obtained in the absence of arterial lactate indicate that flow modulates hindlimb metabolism without altering the directional balance of lactate determined by the concentration of arterial lactate.

In contrast to increased flow rate, NE reversed lactate balance from negative (uptake) to positive (release) in the presence of 10 mM lactate even though perfusion pressure and oxygen uptake were increased in the same way as when flow was increased. These changes were entirely due to the changes in lactate extraction by the hindlimb because the perfusion flow rate remained constant. Clearly, the mechanism

underlying this is different from that induced by increasing flow from 5 to 10 ml/min in the presence of 10 mM arterial lactate.

Theoretically, NE-reversed negative lactate balance in the present study could be accounted for by a direct blockade of lactate influx. Further studies are required to examine whether NE has any effect directly or indirectly on the lactate transport system in skeletal muscle membranes. If a receptor mediated event does control the lactate transporter then it is unlikely that the β-adrenergic receptor is involved. In the present study the β-adrenergic blocker propranolol did not significantly affect the effect of NE on lactate balance, suggesting that NE induced vasoconstriction-associated changes in lactate balance were mediated by α-adrenoceptors. Furthermore, the β-adrenergic agonist, isoproterenol, did not inhibit lactate uptake even though it elevated the muscle lactate content.

A previous study using perifused (not perfused) rat skeletal muscle has revealed that vasoconstrictors including NE and vasopressin do not exert direct stimulation on muscle lactate production despite their stimulatory effects on lactate release in the perfused hindlimb during vasoconstriction (Hettiarachchi *et al.* 1992). As discussed in the Introduction (3.1), NE-induced vasoconstriction in the hindlimb can direct flow to more nutritive capillaries (Newman *et al.* 1996). Given the heterogeneity of the hindlimb skeletal muscle fiber types, it is possible NE-induced increase in nutritive flow occurs with redistribution of perfusate between different muscle fiber types. For example, Pagliassotti and Donovan (1990) have shown that lactate removal rates of oxidative fibers were significantly higher than the glycolytic fibers at 8.6 mM extracellular lactate. Based on studies of lactate transporters in skeletal muscle in different fiber types, Bonen and colleagues have recently hypothesized that the monocarboxylate transporter 1 (MCT1) which exits mainly in the cell membrane of oxidative muscle fibers is kinetically adapted to favor the uptake of lactate into the myocyte (McCullagh *et al.* 1996). These authors also postulated that there might be another MCT in glycolytic muscle to facilitate the extrusion of lactate when rates of glycolysis are high. Thus, a decreased lactate uptake could be expected if NE-increased nutritive flow also involves a general shift from oxidative to glycolytic fibers. Such an explanation appears to be consistent with the finding that the α_1-adrenoceptor density is much higher in soleus muscle compared with other muscles of

the gastrocnemius-plantaris group (Rattigan *et al.* 1986). Furthermore, the distribution of the α-receptor has been shown to be predominantly on small arterioles rather than skeletal muscle *per se,* suggesting that α-adrenergic agonists act via a vascular mechanism (Martin *et al.* 1990).

Although it was not the prime purpose of this chapter to examine the fate of lactate in skeletal muscle, a direct stimulatory effect of lactate on muscle oxygen consumption was noted when lactate was taken up by muscle. This effect was additive to the changes produced by either increased flow or NE infusion. These results were consistent with the observations by others when 1 mM L-lactate was added to perfused muscle (Watt *et al.* 1994). Despite the dominance of glycolysis, resting skeletal is also capable of limited glyconeogenesis from lactate that is taken up (Pagliassotti and Donovan 1990, McDermott and Bonen 1992, Guttierrez *et al.* 1994). Hence, lactate-stimulated oxygen consumption may be due to a substrate futile cycle between glycolysis and glyconeogensis.

In conclusion, it appears that NE can control lactate balance, in the perfused hindlimb, by a vascular mechanism rather than by a direct effect on the muscle cells such as occurs on parenchymal tissue in liver (Ciprés *et al.* 1995). This finding may have particular relevance to muscle exercise performance. As lactate must be transported out of the myocytes if fatigue is to be avoided (Poole and Halestrap 1993), the present data suggest that part of the NE-improved muscle contraction previously noted in the perfused rat hindlimb (Clark *et al.* 1995) may be related to its effect of promoting lactate release to avoid the inhibition by accumulated intracellular lactate during exercise.

Chapter 7

Influence of vasoconstrictors on fat metabolism of perfused hindlimb.

7.1 Introduction

The metabolism of fatty acids and triglyceride is an important source of reducing equivalents for muscle. It has recently been demonstrated that hormone sensitive lipase (HSL) mRNA is expressed in skeletal muscle tissue (Holme *et al.* 1987) and may be associated with triglyceride droplets within the cell (Reitman *et al.* 1973, Egan *et al.* 1992). Another important source of triglycerides and fatty acids comes from the circulating plasma. Lipoprotein lipase is expressed by muscle and resides outside endothelial cells (reviewed in Oscai *et al.* 1990). Lipoprotein lipase induction and HSL activity appear to be regulated by the same cAMP dependent phosphorylation enzymes (Small *et al.* 1989, Oscai *et al.* 1990).

The initial characterization of the perfused rat hindlimb by Ruderman demonstrated the importance of lipid as a substrate for resting metabolism (Ruderman *et al.* 1971). That study found that during a typical 30 minute perfusion 300-400 μmol of O_2 was consumed but only 1.3 μmol of glucose was oxidized to CO_2. This implies that exogenous glucose accounted for less than 4% of the oxidative fuel of the resting hindquarter. Further evidence that lipid is a major fuel for resting muscle metabolism comes from *in vivo* studies which describe respiratory quotients close to 0.7 (Dagenais *et al.* 1976). The respiratory quotient (RQ) is often used as a non-invasive indicator of the substrate that is being used for metabolism (Frayn 1983). Theoretically, by measuring the ratio of oxygen consumed to CO2 produced it is possible to determine if the tissue is catabolising fat or carbohydrate or protein (Lusk 1923).

Fat metabolism has a strong association with thermogenesis. Fatty acids are known to be both the signal and the fuel for β-mediated thermogenesis in brown fat cells (Nichols and Locke 1984). Furthermore, animals without brown fat, such as adult humans, are known to possess β-mediated thermogenic mechanisms in skeletal muscle (Blaak *et al.* 1994). This is significant because β-agonists are known to

mobilize muscle and adipose tissue fatty acids (Blaak *et al.* 1994). Several hypotheses exist to explain how fat mobilization could induce thermogenesis. One possibility is the fatty acid reesterification cycle whereby free fatty acids produced by lipase action are recycled using glycerol-3-phosphate. The production of glycerol-3-phosphate by glycolysis requires ATP so this cycle consumes energy (Myrmel *et al.* 1992). Another possibility is that fatty acids may act as proton carriers across the inner mitochondrial thus increasing the permeability of protons to the membrane, causing uncoupling of oxidative phosphorylation (Schonfeld 1992). During vasoconstrictor mediated thermogenesis in the perfused rat hindlimb there is a close association between glycerol release and oxygen consumption (Clark *et al.* 1994). A causative role for fatty acids in this observation is a plausible hypothesis. The origin of glycerol release from the hindlimb is uncertain (Ruderman *et al.* 1970). There are some significant fat deposits in the hindlimb, particularly subcutaneous fat which may be accessed by flow under the influence of Type A vasoconstrictors. Furthermore it has been suggested that glycerol release from tissues may be a direct result of dephosphorylation of glycerol-3-PO_4, an intermediate closely associated with glycolysis (Riol-Cimas and Melendez-Hevia 1986, Wardle *et al.* 1994). A glycolytic origin of glycerol from the perfused rat hindlimb is also a possibility considering the close relationship between glycerol and lactate release from the perfused rat hindlimb (Clark *et al.* 1994)

The aim of the study described in this chapter was to investigate the influence of vasoconstrictors on hindlimb fat metabolism using three indicators of lipid metabolism. Namely, glycerol release, respiratory quotient and release of free fatty acids into the venous effluent. The origin of glycerol released from the hindlimb was also considered. Since lipase activity is regulated by the cAMP cascade the activation of glycogen phosphorylase was also meaured.

7.2 Materials and Methods

7.2.1 Respiratory quotient measurements

The surgery for the perfused rat hindlimb has been described in chapter 3. Some modification of the perfusion medium was needed to measure CO_2 production and arterio-venous fatty acid production. The quantity of CO_2 produced by the hindlimb is small compared with the concentration of bicarbonate in the Krebs

medium. It was therefore necessary to replace the bicarbonate with another suitable buffer. A buffer commonly used in cell culture is HEPES which is non toxic and suitable for the pH of a perfusion buffer. Two different HEPES (10 mM) buffered perfusion media were used. One of these had no bicarbonate while the other had 2 mM bicarbonate. Apart from the modification to the bicarbonate the concentration of electrolytes in the perfusion medium was identical to that described in the General Materials and Methods. The only exception to this was that the bicarbonate-free preparation contained an extra 25 mM of NaCl whilst the 2 mM bicarbonate medium contained an additional 23 mM NaCl to compensate for the removal of $NaHCO_3$.The medium was gassed with oxygen rather than 95% O_2:5% CO_2 to facilitate the accurate measurement of CO_2. Total CO_2 was measured using a CO_2 coulombeter (UIC inc). Samples for total CO_2 were collected from the venous effluent of the perfusion apparatus using a 1ml Hamilton syringe before the perfusate came into contact with air. The coulombeter was not capable of analyzing liquid samples so it was necessary to "strip" the CO_2 from the perfusate by injecting the perfusate (500 µl) into a small chamber containing 200µl phosphoric acid (8.8% v/v). The chamber was continuously stirred with a magnetic flea to ensure that the entire sample was acidified. High purity nitrogen gas was passed through the chamber to carry the evolved CO_2 to the coulombeter. Recovery of 0.5 µmols of $NaHCO_3$ standard solution, which was at the lower range of perfusate CO_2 concentration, was 98 ± 1.8 % (n=3).

7.2.2 Measurement of perfusate glycerol

The measurement of perfusate glycerol was adapted from an enzymatic assay for glycerol described by Bergmeyer (1974). Briefly, the assay involves coupling glycerol phosphorylation and dehydrogenation, by glycerol kinase and glycerol phosphate dehydrogenase, to NADH formation. The resulting NADH is measured by fluorometry at an excitation wavelength of 340 nM and an emission wavelength of 460 nM.

7.2.3 Measurement of Fatty Acids released into the perfusate

To accurately measure fatty acid release from the perfused hindlimb it was necessary to use an alternative perfusion colloid. Commercial bovine serum albumin contains fatty acids, so measuring an arterio-venous difference becomes difficult when

very small quantities of fatty acids are released. Fatty-acid free BSA is available but would be economically unviable for use in a perfusion medium. One commonly used alternative is dextran 40 (polysucrose, MW 40 000). Unfortunately Dextran induces anaphylaxis resulting in the release of serotonin and histamines from mast cells and consequent vasoconstriction (Perez-Tripichio *et al.* 1991). Ficoll, another carbohydrate polymer does not have these problems and was used as the perfusion colloid in these experiments. The buffer was therefore a modified Krebs' buffer with 4% Ficoll 70 (Pharmacia) and 0.2% fatty acid free BSA (Sigma) to carry the fatty acids released from the hindlimb in the perfusate. Released fatty acids were measured colorimetrically with an enzymatic kit for non-esterified fatty acids (NEFA C by Wako Pure Chemical Industries Ltd.).

7.2.4 Glycogen phosphorylase Activity Ratio.

Glycogen phosphorylase assays were conducted on freeze clamped gastrocnemius-soleus-plantaris muscle groups after 30 min of 1μM isoproterenol (β-agonist), 5 nM Angiotensin II (Type A vasoconstrictor) or control perfusion. The phosphorylase assay is described by Birnbaum and Fain (1977). The activity ratio is defined as the ratio of activated (phosphorylated) enzyme to total enzyme. Thus a fully activated muscle will have an activity ratio of 1.

7.3 Results

7.3.1 Respiratory quotient in the perfused rat hindlimb.

The results obtained from measurement of respiratory quotient in the absence of perfusate bicarbonate are shown in Figure 7.1. It can be seen that when venous PO_2 (venous oxygen tension) was lowered by NE there was a corresponding increase in CO_2 production. However, the respiratory quotient remained above the theoretical upper limit of 1 despite an apparent drifting baseline towards an RQ of around 1. Further experiments using 1mM cyanide to block respiration demonstrated that cyanide raised the RQ from 1.28± 0.08 to 2.02±0.08 (n=2, data not shown). The non-respiratorily associated CO_2 production suggested that some carbonate was tightly bound within the hindlimb and was gradually released during perfusion resulting in the observed downward drifting RQ baseline. To overcome this problem, successive hindlimb perfusions measuring respiratory quotient contained 2 mM bicarbonate in the buffer to facilitate CO_2 equilibration with the tightly bound CO_2. Because of the

increased error associated with the measurement of CO_2 production against a background of bicarbonate it was not possible to obtain a time course. Instead, three measurements of the respiratory quotient at each steady state in each experiment were made. The respiratory quotient at the steady state was an average of the three measurements. The results of this experiment are shown in Figure 7.2. The initial respiratory quotient was above 1 and was not significantly altered by the addition of a stimulatory dose of norepinephrine. After norepinephrine was removed the respiratory quotient fell to 0.88.

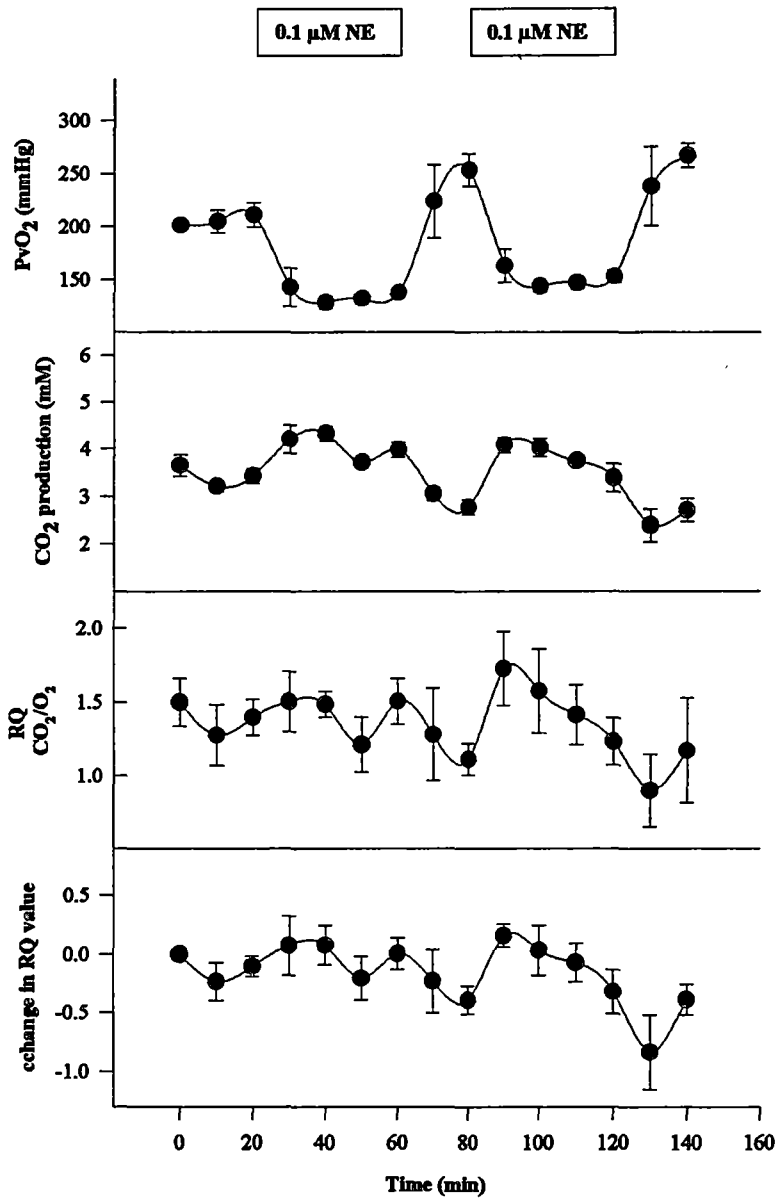

Figure 7.1 Respiratory quotient time course for repeated doses of norepinephrine (0.1 µM). Perfusions were conducted at 25°C at a flow rate of 4 ml/min. The bicarbonate in the Krebs buffer was replaced with 10 mM HEPES. Means ± SE are shown for 4 separate perfusions. When not visible error bars are within the symbol.

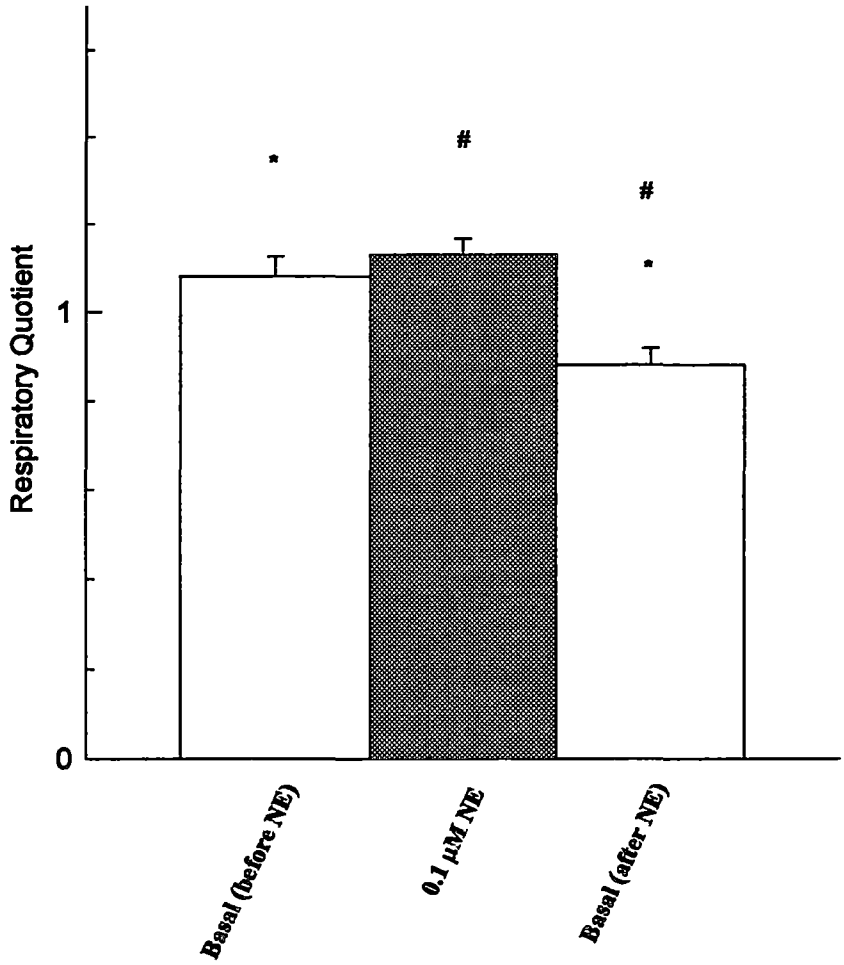

Figure 7.2 Respiratory quotients before during and after the infusion of norepinephrine (0.1 μM). Within each experiment 3 measurements of total CO2 output from the hindlimb were made at each steady state for oxygen consumption. The RQ for each steady state within each perfusion was the average of the three assays. The perfusion medium was buffered with 2 mM bicarbonate and 10 mM HEPES. Means ± SE for 5 separate perfusions are shown. (*,#, $P<0.05$ compared with the same symbol).

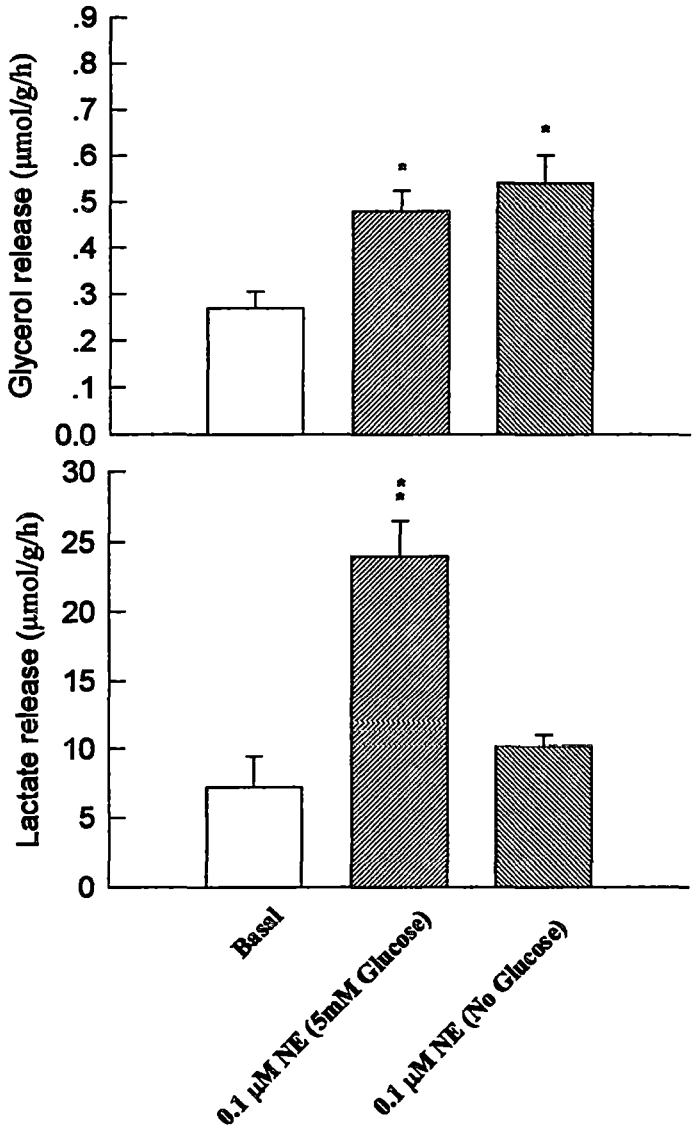

Figure 7.3 Norepinephrine (0.1 µM) mediated glycerol and lactate efflux from the perfused rat hindlimb in the presence or absence of perfusate glucose. Perfusions were conducted at 25°C at 4 ml/min using standard Krebs-bicarbonate buffer (n=3). *P<0.05 vs control.

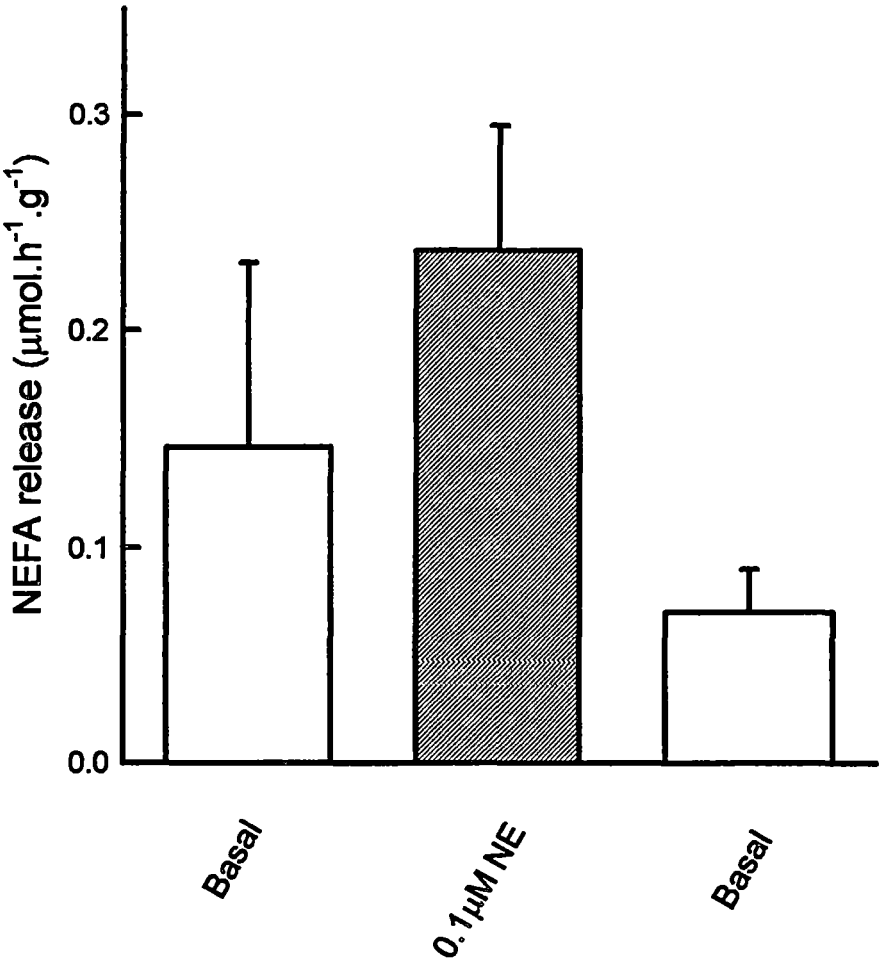

Figure 7.4 Steady state release of free fatty acids from the perfused rat hindlimb. The protocol for the experiment was 20 min basal, 20 min NE (0.1 μM) and 30 min basal. Perfusions were conducted at 25°C at 4 ml/min using Krebs buffer with 4% Ficoll/0.2% fatty acid free BSA instead of albumin. Means ± S.E for 3 experiments are shown. No differences were significant, statistically $P<0.05$

7.3.2 Glycerol efflux during glucose free perfusion.

The removal of glucose from the perfusion medium did not prevent the increased release of glycerol from the hindlimb during Type A stimulation by 0.1μM norepinephrine (Fig. 7.3). Norepinephrine (0.1 μM) increased the rat of glycerol production from around 0.4 to 0.7 μmol/g/h. However, the release of lactate during Type A stimulation was significantly inhibited by the removal of glucose to an extent

where the lactate release was not statistically different from basal. Thus the removal of perfusate glucose separated the release of glycerol and lactate from the perfused hindlimb.

7.3.3 Colourimetric assay of perfusate fatty acids.

The colourimetric assay is typically used to measure fatty acids in serum where the concentration of fatty acids is greater than 0.1 mM The concentration of fatty acids in this experiment was on the limit of reliable detection. This is reflected in the large error bars in Figure 4.4. Despite this, there is a similarity between the pattern of fatty acid release and the change in respiratory quotient shown in Figure 7.2. However, no differences in NEFA were statistically significant ($P<0.05$).

7.3.4 Glycogen phosphorylase activity ratio and glycerol release.

Basal glycerol release from the hindlimb perfused at 25°C at a flow rate of 4 ml/min was 0.23 ± 0.02 μmol/g/h (Table 7.1). Infusion of 5 nM Angiotensin (a Type A vasoconstrictor) or 1 μM Isoproterenol (a β-adrenergic agonist) increased the rate of glycerol release to 0.53 ± 0.02 and 0.46 ± 0.02 μmol/g/h, respectively. The unstimulated activity ratio of glycogen phosphorlyase in the gastrocnemius/soleus/plantaris muscle group was 0.32 ± 0.04. This ratio was not significantly altered by 5 nM Angiotensin II despite the observed increase in glycerol release. However, the β-adrenergic agonist, Isoproterenol raised the activity ratio to 1.04 ± 0.07, indicating total activation (Table 7.1).

	Basal	5 nM Angiotensin II	1 μM Isoproterenol
Glycerol Release (μmol/g/h)	0.23 ± 0.02	0.53 ± 0.02*	0.46 ± 0.02*
Phosphorylase Activity Ratio	0.32 ± 0.04	0.26 ± 0.04	1.04 ± 0.07*

Table 7.1 Phosphorylase activity ratio and glycerol release from the perfused hindlimb during Angiotensin II and Isoproterenol stimulation (n=3) (* $P< 0.05$ compared to "Basal")

7.4 Discussion

The experiments conducted in this chapter do not imply a causative role for glycerol or fatty acid release in the metabolism observed in stimulatory

vasoconstriction. Firstly, the addition of Type A norepinephrine (0.1 μM) elevated the respiratory quotient. If fat metabolism had been predominant during vasoconstriction then the RQ should have been lowered. To the author's knowledge this is the first attempt at measuring the respiratory quotient of the perfused rat hindlimb. This has been made possible by advances in the technology of CO_2 measurement. While the CO_2 measurement may be precise using the coulombeter method, some caution is required when interpreting these RQ data. The problem of slow release of CO_2 not evolved from metabolism was highlighted in the perfusions where bicarbonate was omitted from the perfusion medium. Estimates of the respiratory quotient in these experiments were unreasonably high and can only be explained by a net synthesis of protein or fat, or by removal of tightly bound carbon dioxide and carbonate (Frayn 1983).The fact that this CO_2 production was insensitive to cyanide (see 7.3.1) suggests that tightly bound carbon dioxide was being slowly removed. Cyanide would have stopped the synthesis of protein and fat because these synthetic pathways require ATP (Stryer 1988). The inclusion of 2 mM bicarbonate to facilitate the equilibration lowered the respiratory quotient measurement to around 1. Norepinephrine does appear to raise the RQ, especially compared with the post norepinephrine RQ, but there is an important consideration to be made here. If low dose norepinephrine does indeed access an unperfused space in the constant flow perfused rat hindlimb as suggested by Newman *et al.* (1996) then it is possible that the increased respiratory quotient is cased by the removal of bound bicarbonate from the newly accessed space. Estimations of diffusion coefficients of CO_2 and O_2 have shown that CO_2 diffuses through tissue more than 20 times faster than O_2 (Kawashiro *et al.* 1975). It is therefore more probable that removal of bicarbonate causes the increased RQ rather than the removal of CO_2 gas. Despite these technical problems the post-norepinephrine estimation, in the presence of 2 mM bicarbonate, is probably an accurate estimation of the respiratory quotient. The obtained value of 0.88 agrees with measurements from incubated muscle (0.85, Kawashiro *et al.* 1975) but is higher than a figure obtained from muscle *in vivo* (0.76, Dagenais *et al.* 1976). Because of the problems associated with measuring the respiratory quotient of unequilibrated tissue it is unlikely that any meaningful RQ results can be obtained when new spaces are accessed. This does not rule out the use of this technique to investigate changes in

metabolism that are not associated with changes in perfusion pressure and flow redistribution.

One possible interpretation of the release of fatty acids associated with the increase of respiratory quotient is that Type A vasoconstriction initiates a net synthesis of fatty acid which is supported by increased oxygen consumption. Theoretically, the RQ during lipogenesis could be as high as 5.6 (Frayn *et al.* 1983). It is possible to calculate the amount of oxygen required to synthesize 1 mol of fatty acid. Assuming that the fatty acid is synthesized from glucose, about 1.6 mols of O_2 are required to synthesize 1 mol of fatty acid (calculated from Frayn *et al.* 1983). If we assume that the increased rate of lipid release during norepinephrine stimulation is in fact lipid synthesis supported by oxygen consumption, then the 0.07 μmol/g/h increase in fatty acid production would account for a 0.11 μmol/g/h increase in oxygen consumption. This is an extremely small fraction of the observed increase in oxygen consumption. Therefore, the observed increase of both the respiratory quotient and the fatty acid release probably reflects the clearance of fatty acids and bound bicarbonate from the newly accessed perfusion space.

Glycerol production from fatty acid reesterification is also an ATP consuming process and will therefore use oxygen. The *de novo* synthesis of glycerol-3-PO_4 from glucose requires 2 ATP and 1 NADH (Stryer 1988). This roughly equates to 0.8 mols of O_2 for every mol of glycerol-3-PO_4 produced, assuming that the ATP for synthesis is derived from oxidative phosphorylation. The observed increase in glycerol release during Type A vasoconstriction is only 0.3 μmol/g/h so the fatty acid reesterification cycle will consume about 0.24 μmol/g/h of O_2. This is a comparatively minor component of the observed increase in oxygen consumption.

The metabolic and cellular origin of glycerol released from the perfused hindlimb is unclear. During the initial hindlimb charaterisation by Ruderman it was suggested that there may be enough fat tissue in the hindlimb to account for the glycerol release (Ruderman *et al.* 1971). Furthermore, smaller intermuscular fat deposits may be numerous enough to contribute significantly to the production of glycerol. This notion is further supported by the recent finding of adipsin mRNA, a marker for fat cells, in rat soleus muscle (Evans *et al.* 1996).

The appearance of glycerol is often regarded to be indicative of lipolysis (eg Ruderman *et al.* 1971). However this dogma has been occasionally disputed. Some researchers have found that the production of glycerol is associated with an increase in glycerol-3-PO_4. Such an association has been found in the perfused heart during hypoxia (Wardle *et al.* 1994) and in post exercise equine muscle (Snow *et al.* 1985). Both of these investigations concluded that the increase in glycerol may not come from lipolysis but rather from the hydrolysis of glycerol-3-PO_4, originating from glycolysis. Wardle *et al.* noted a correlation between glycerol and lactate production which has been also noted in ischaemic muscle (Ye *et al.* 1996). It is therefore possible that glycerol release may simply reflect glycolytic flux. However, the results obtained in the present study do not support the notion of glycerol release being related to glycolytic flux. The release of lactate from the perfused rat hindlimb during type A vasoconstriction is dependent on extracellular glucose. In comparison, glycerol release during Type A stimulation occurs independently of extracellular glucose. It therefore seems most likely that glycerol release is indeed reflecting the rate of fatty acid reesterification. However, it is a possibility that glycerol release in the absence of extracellular glucose may have glycogenolytic origins.

If glycerol was a "dead end metabolite" from the reesterification cycle in skeletal muscle then a stoicheometric relationship between glycerol release and lipolysis should be evident. However, significant activities of glycerol kinase have been found in rat skeletal muscle. For example, the activity of glycerol kinase in red muscle may be as high as 1.2 μmol/g/h (Newsholme and Taylor 1969). Considering that glycerol efflux from the perfused hindlimb is about 0.3 μmol/g/h it would seem quite likely that a competition exists between glycerol salvage and glycerol efflux. If this is indeed the case then a stoicheometric relationship between glycerol release and lipolysis would seem unlikely. A possible explanation for glycerol release during type A stimulation would therefore be increased nutritive flow favoring the efflux pathway for glycerol. Recent measurements of glycerol concentrations in human quadriceps muscle support this idea. For example, interstitial glycerol concentrations in muscle may be as high as 3.7 mM compared with plasma concentrations of 87 μM (Maggs *et al.* 1995). It is therefore plausible that alterations in nutritive flow would alter the steepness of this gradient and increase the rate of glycerol efflux.

Chapter 8

Conclusions

The results of the present thesis suggest that heterogeneity of flow in the perfused rat hindlimb may be an important factor in the regulation of perfused rat hindlimb metabolic rate. Fluorometry data measuring flow through connective tissue of the biceps-femoris tendon suggests that blood vessels in connective tissue can act as functional shunts (Chapter 5) during Type B mediated non-nutritive perfusion (Chapter 4). Thus, at constant rate arterial perfusion vasoconstrictors can control the rate of muscle perfusion by directing blood flow to or away from connective tissue.

Results from Chapter 6 suggest that a different form of heterogeneity may also be important in the perfused hindlimb. This chapter raised the possibility that type A vasoconstrictors, particularly norepinephrine, could divert perfusion from areas of red muscle to regions of white muscle. It has been known for some time that in the resting state, red muscle such as the soleus received more blood flow than required for resting metabolism, particularly in the dilated state (Folkow and Halicka 1968). If this is indeed the case in the hindlimb then type A could divert flow away from red muscle to white muscle without compromising basal metabolism in the red fibers. This unequal distribution of flow between red and white fibers in the hindlimb deserves further investigation.

Whilst these heterogeneities may explain vasoconstrictor control of muscle perfusion they do not explain vasoconstrictor regulation of hindlimb metabolism. At present there appear to be two main possibilities

Vasoconstrictor-controlled metabolism (possibility 1).

The two heterogeneities (outlined above) acting in tandem can alter perfusion of muscle to such an extent that the perfused hindlimb may be supplied with a large quantity of substrate but this may not be delivered effectively to the muscle (see Fig. 2.2, Piiper 1992). If this is indeed the case then substrate limitation of the perfused rat hindlimb could explain the metabolic effects of Type A and Type B vasoconstriction.

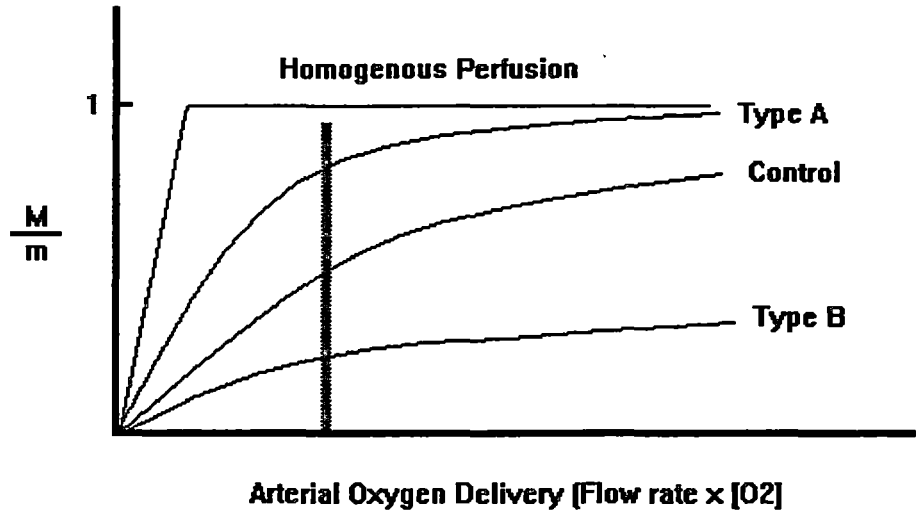

Figure 8.1 Model of vasoconstrictor-mediated oxygen delivery in the constant flow perfused rat hindlimb. The Y-axis, (M/m), represents an hypothetical O2 uptake/O2 requirement ratio. The typical arterial oxygen delivery of 95% O2 at 5 ml/min is indicated by the vertical bar.

Using the model shown in Fig. 8.1 it can be seen that critical oxygen delivery, where the oxygen requirement of the muscle is met by a discrete rate of oxygen delivery, will only exist under homogenous perfusion conditions (Top curve). Under "normal conditions" of hindlimb perfusion in the absence of any vasoconstrictors the relationship between arterial oxygen content and oxygen delivery to muscle becomes a curve with no apparent rate of critical oxygen delivery. During Type A vasoconstriction shunting and heterogeneity have a smaller influence on the curve so that the "Type A" curve approaches the curve for homogenous perfusion. However, during Type B vasoconstriction shunting will dominate the shape of the curve resulting in a less efficient delivery of oxygen and consequently flatter curve will be observed. After generating these curves we can see that at a given rate of oxygen delivery (Flow rate x [O2]) such as 95% O2 and 5 ml/min, indicated by the vertical bar there can be a range of M/m (O2 uptake/O2 requirement) ratios depending on whether the hindlimb is dilated ("Control") or under Type A or Type B vasoconstriction. Thus a substrate regulation of oxygen consumption is quite plausible in the constant flow perfused hindlimb.

If a "systems biochemistry" approach (Connett *et al.* 1990, Brand 1996) is applied to the constant-flow perfused rat hindlimb then the diagram shown in figure 8.2 may provide a simple model of vascular control of perfused muscle metabolism. It is axiomatic that muscle metabolism is intrinsically connected to perfusion. However the present thesis extends this idea and suggests that perfusion can regulate metabolism. Both lactate transport and removal and cytosolic pH can strongly influence glycolytic control as well as mitochondrial-glycolytic interactions (Connett *et al.* 1990) and can therefore be considered as a component of the "substrate oxidation" group of reactions that are under vascular control.

Brand and colleagues (Brand 1996) have applied metabolic control analysis to systems biochemistry in order to obtain a quantitative description of cellular metabolism. In a recent application of this method to the resting perfused hindlimb it was found that both producers and consumers (see Fig 8.2) of $\Delta\Psi m$ controlled the metabolic rate of perfused skeletal muscle (Rolfe and Brand 1996b). The conclusion drawn from their study was that '... the idea that muscle respiration is controlled solely by ATP demand does not apply to resting skeletal muscle. The data show that it is possible to stimulate skeletal muscle respiration by increasing the activity of either the substrate oxidation system, the proton leak or the reactions involved in ATP turnover.' Developing this notion a little further, it would seem reasonable that a tissue where substrate oxidation exerts control over respiration is a metabolically conforming tissue (see discussion in Chapter 2). Studies of metabolic control analysis on metabolic regulators such as brain cells may have completely different control patterns. Future applications of metabolic control analysis may provide further insight into metabolic regulation in muscle.

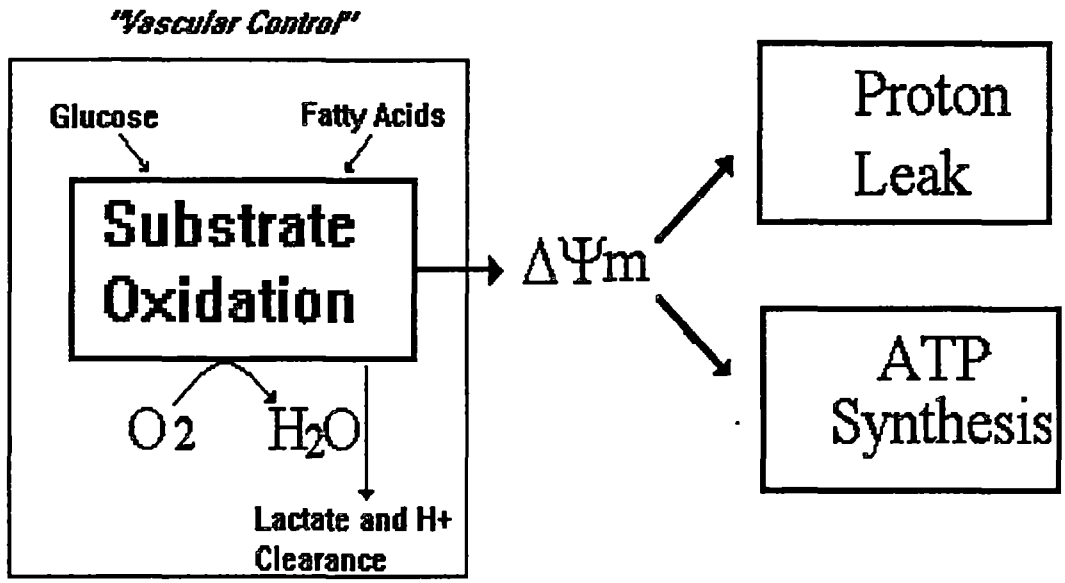

Figure 8.2 Systems biochemistry diagram showing vascular control of hindlimb metabolism.

Vasoconstrictor-controlled metabolism (possibility 2)

If the hindlimb is truly saturated with substrate under basal conditions then the delivery of more substrate during Type A vasoconstriction will not stimulate metabolism. It is therefore possible that complex signaling systems may exist in the perfused hindlimb to integrate metabolic rate with flow and oxygen delivery. Whilst many possibilities exist, it has been suggested that, 'the most parsimonious model to accommodate such striking and often-observed dependence of ATP-turnover on O_2 availability is that of a single O_2 sensor, which displays a much lower apparent O_2 affinity than typical of mitochondria and which modulates both ATP demand and ATP supply pathways in a simultaneous coordinated way' (Hochachka 1994). One possible O_2 sensor is an oxygen-sensitive K^+ channel (Lopez-Barneo *et al.* 1993). Recent experiments by Tong and Clark (unpublished) have shown that membrane stabilizing agents and ouabain may block the type A induced metabolic increase whilst retaining the vasoconstriction. These findings suggest that plasma membrane potential may play an important role in the "sensing" of perfusion. Once perfusion is sensed then signal transaction would be activated to stimulate other metabolic pathways such

as triglyceride turnover, respiration (and proton leak) and glycolysis. This model would account for the metabolic effects observed during vasoconstriction.

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