Nutritive and Non-Nutritive Blood Flow In Skeletal Muscle

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by

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I, Andrew James Hoy, declare that this thesis, submitted in partial fulfilment of the requirements for the award of Master of Science (Research), in the Department of Biomedical Science, University of Wollongong, is wholly my own work unless otherwise referenced or acknowledged. The document has not been submitted for qualifications at any other academic institution.

Andrew James Hoy BSc

8 November 2004

"The opposite of a correct statement is a false statement.
The opposite of a profound truth may well be another profound truth."

Niels Bohr
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ABSTRACT

The vascular structure of mammalian skeletal muscle has been intensively investigated for the last seventy years. Since the early work of Pappenheimer and Barlow, the existence of a parallel dual vascular pathway has been suggested to explain the differences between total flow and clearance rates of a variety of infused substances. Direct microscopy studies using superficial muscles have shown that the microvascular arterioles have very frequent connections with the capillary modules of the associated connective tissue and adipose tissue within skeletal muscle. In more recent times, Clark and colleagues have identified two vascular pathways according to the opposing actions of two groups of vasoconstricting agents. While all increase perfusion pressure, Type A vasoconstrictors (low dose noradrenaline (NAd), vasopressin, angiotensin II) increase oxygen uptake but Type B vasoconstrictors (serotonin, high dose noradrenaline) decrease hindlimb oxygen consumption. The opposing effects on oxygen consumption are thought to arise from selective vasoconstriction of the microvasculature. Type A vasoconstrictors redirect blood into muscle tissue capillary beds (termed nutritive bed) whilst Type B vasoconstrictors redirect blood into the associated connective tissue, adipose and septum capillary beds (termed non-nutritive bed). Many of the previous studies are based on variations of an in situ rat, isolated perfused hindlimb model, having low vascular tone and often with insufficient oxygen carrying capacity to support active metabolism. In vivo, skeletal intramuscular blood redistribution during exercise occurs principally via the release of vasodilatory metabolites and the nervous system. This thesis used a novel in vivo model to test the hypothesis that nutritive and non-nutritive blood flow distribution can still be observed under conditions of high vascular tone and oxygen delivery at rest and in metabolically active (contracting) muscle. Utilising the high vascular tone, it also tests the hypothesis that the vascular pathways can be differentiated using vasodilators.

Male Wistar rats were anaesthetised with sodium pentobarbital (6mg·100g⁻¹ i.p.). The right femoral artery was cannulated to supply blood to the left femoral artery (perfused) at a constant flow (basal 1ml·min⁻¹, contraction 2ml·min⁻¹) via a pump. Perfused hindlimb pressure was recorded distal to the pump and passive venous return occurred from the left femoral vein to the right external jugular vein. Systemic
blood pressure was recorded from the left common carotid artery. Polyethylene cannulae were filled with heparinized 0.9% saline containing 6% w/v dextran70. The left sciatic nerve was isolated and stimulated (5Hz) to produce twitch contraction in the lower hindlimb muscle bundle and developed tension was recorded. Vasoactive drugs (2 constrictor, 8 dilator) were prepared with saline and 0.01% ascorbic acid, and injected into the arterial loop. Blood was sampled from the venous and arterial loops and oxygen consumption determined using the Fick equation.

In the autoperfused rat hindlimb, the Type B vasoconstrictor increased perfusion pressure and caused a significant decrease in basal hindlimb oxygen consumption, however during muscle contraction this effect on oxygen consumption was diminished. The Type A vasoconstrictor had no significant effect on hindlimb oxygen consumption during significant increases in perfusion pressure. Eight vasodilators with a variety of mechanisms of action were screened at rest but none were observed to decreases hindlimb oxygen consumption in a fashion similar to Type B vasoconstrictors. Increases in oxygen availability at rest via increased nutritive flow by noradrenaline and vasodilator infusion had no effect upon basal metabolic rate. Therefore, during adequate oxygen delivery, increased availability has no effect upon metabolic demand. Isoprenaline and histamine significantly increased hindlimb oxygen consumption during the contraction protocol, whilst there was no significant effect observed at rest. It can be concluded that selective vasoconstriction occurs in vivo, however during muscle contraction, local release of vasodilatory metabolites can overcome exogenous vasoconstriction. These results confirm the possible existence of a dual vascular pathway however blood flow redistribution via vasodilation is likely determined by the locale of vasodilator release rather than differences in receptor distribution.
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ABBREVIATIONS

- approximate
+dT/dt_{\text{max}} maximum rate of tension development
-dT/dt_{\text{max}} maximum rate of relaxation
< less than
°C degrees celsius
µl microlitre
µmol micromoles
µM micromolar
µmol·min^{-1}·gww^{-1} micromoles per minute per gram of wet weight
(a-v)O\textsubscript{2}diff arterial-venous oxygen difference
cc/m cubic centimetre per metre
Cao\textsubscript{2} arterial oxygen content
[\text{Vo}_2] oxygen content
Cvo\textsubscript{2} venous oxygen content
Hb haemoglobin
Hct haematocrit
Hz hertz
IU international units
i.p. intraperitoneal
K\textsuperscript{+} potassium ion
min minute
ml millilitre
mm millimetre
mmHg millimetres of mercury
ml·min^{-1} millilitre per minute
mM millimolar
ms millisecond
Na\textsuperscript{+} sodium ion
nM nanomolar
N Newtons
<table>
<thead>
<tr>
<th>Symbol/Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>N·s⁻¹</td>
<td>Newtons per second</td>
</tr>
<tr>
<td>PcO₂</td>
<td>partial pressure of carbon dioxide</td>
</tr>
<tr>
<td>Po₂</td>
<td>partial pressure of oxygen</td>
</tr>
<tr>
<td>RBC</td>
<td>red blood cells</td>
</tr>
<tr>
<td>s</td>
<td>seconds</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>So₂</td>
<td>oxygen saturation of haemoglobin</td>
</tr>
<tr>
<td>T_max</td>
<td>maximal force developed</td>
</tr>
<tr>
<td>˙V O₂</td>
<td>oxygen consumption</td>
</tr>
<tr>
<td>w/w</td>
<td>wet weight</td>
</tr>
<tr>
<td>w/v</td>
<td>weight per volume</td>
</tr>
</tbody>
</table>
PUBLICATIONS AND ORAL PRESENTATIONS


Andrew J Hoy, Gregory E Peoples, Peter L McLennan (2003) The Reduced Effect of Serotonin on Oxygen Consumption during Muscle Consumption in the Autoperfused Rat Hindlimb. Exercise, Muscle and Metabolism, November 12th to 15th, Deakin University, Melbourne, Australia. Student poster presentation.
CHAPTER ONE

Literature Review
1.1 DUAL CIRCULATION IN SKELETAL MUSCLE

1.1.1 Introduction

The relationship between skeletal muscle metabolism and the distribution of blood throughout its vascular network has been a major focus of researchers for some time now. Since the work of Pappenheimer in the 1940’s, controversy has surrounded the existence of dual circulation in skeletal muscle. A lack of histological evidence for classical shunts such as large arterio-venous anastomoses (Barlow et al. 1958; Piiper and Rosell 1961) has tended to contradict physiological evidence supporting dual circulation. Redistribution of blood flow brought about by an increase in vascular resistance is thought to play an oxygen-saving role. However, this will not protect from oxygen consumption by the tissue at the peripheral level. Oxygen uptake is determined by tissue partial pressure of oxygen ($P_{O_2}$), capillary $P_{O_2}$, or by intercapillary distance (Proctor et al. 1981). It is subsequently thought that the existence of dual circulation, with one route having a low metabolic demand, could play a crucial role (Stainsby and Lambert 1979). It has been postulated that preferential tissue blood flow results from heterogeneity of perfusion, which occurs at the capillary level of the vascular network. Studies have shown that skeletal muscle blood flow shunting does not require specific anatomical structure (Hyman et al. 1959; Cieslicki and Przybyski 2000) such as anastomoses found in the lungs (Frazier et al. 2000; Do et al. 2001) and the digit (Burch and DePasquale 1962). Blood flow distribution within skeletal muscle has been defined as a tightly regulated process involving stimulation by tissue metabolites, integrated with the vascular mechanisms of neural, flow-dependent and myogenic responses (Rivers and Frame 1999).

1.1.2 Evidence For Dual Circulation

Evidence has been presented supporting dual circulation from studies where total blood flow into the muscle did not correlate with metabolic or heat transfer responses. These studies have termed the routes nutritive and non-nutritive. Pappenheimer (1941) studied neurohormonal effects on oxygen consumption in the perfused hindlimb and isolated dog gastrocnemius. Oxygen consumption decreased during stimulation of the sympathetic vasoconstrictor nerve whilst vasodilation, via adrenaline ($\alpha$- and $\beta$-adrenoceptor agonist) infusion, led to an increase in oxygen
consumption. Pappenheimer reported an increase in arterial-venous temperature difference during reduced perfusion pressure or adrenaline infusion and a decrease during vasoconstrictor nerve stimulation. These changes could not be accounted for by changes in metabolism. It was hypothesised that during vasoconstrictor nerve stimulation, blood flow is diverted into areas of the muscle where oxygen consumption and surfaces for heat loss are reduced.

Other experimental evidence has come from studies using clearance rates of various substances, including radioactive sodium ($^{24}$Na) (Walder 1953, 1955; Barlow et al. 1959, 1961), potassium ($^{42}$K) (Barlow et al. 1961), rubidium ($^{86}$Rb) (Renkin and Rosell 1962), xenon ($^{133}$Xe) (Sejrsen and Tönnesen 1968), iodine ($^{131}$I) (Rapaport et al. 1952; Hyman et al. 1959; Ballard et al. 1964), antipyrine (Renkin 1971), inert gases (Piiper et al. 1985), and hydrogen (Nakamura et al. 1972; Harrison et al. 1990), before and during infusion of adrenaline. Renkin (1955) described the vascular bed of perfused cat hindlimb as having well perfused and poorly perfused areas which are not identifiable with any particular compartmental tissue of the hindlimb, and which have differing responses to vasoactive agents such as adrenaline. The clearance rates of radioactive substances did not correlate with total blood flow within the perfused hindlimbs. The discrepancies between the infused substance washout and venous outflow could be explained by a dual vascular pathway, with a considerable fraction of total flow passing through the non-nutritive route (Piiper et al. 1985).

Increases in skeletal muscle blood flow during adrenaline infusion do not match with the clearance of arterially infused $^{24}$Na in various whole animal and isolated muscle models (Barlow et al. 1959, 1961). In these studies there was a strong correlation between $^{24}$Na clearance and total blood flow during sympathetic vasoconstrictor nerve stimulation. There was no change in $^{24}$Na clearance rates during cholinergic vasodilator nerve stimulation. It was suggested at the time that the vasodilator nerve acted on intramuscular shunts (non-nutritive route) to redistribute this flow (Hyman et al. 1959). Barlow et al. (1959, 1961) described the clearance of $^{24}$Na from skeletal muscle during adrenaline infusion as having two areas: fast and slow clearance areas. If the removal by the lymphatic system is considered negligible, then the rate of clearance of $^{24}$Na is dependent upon the number of capillaries perfused and/or surface area (Miller and Wilson 1951). Autoradiographs showed that periods of slow clearance was representative of the clearance of $^{24}$Na from the intramuscular septa and tendon, whereas the rapid clearance was attributed to the deposition amongst the muscle fibres. It was concluded that skeletal muscle
has two circulations, one being concerned with the delivery of nutrients to the muscle fibres (nutritive), and the other delivering nutrients to the intramuscular septa and tendons (non-nutritive). Further these circulations are affected differently by adrenaline (Barlow et al. 1959, 1961). It should be noted that the exchange in each compartment may be either flow limited or diffusion limited, depending on the perfused compartment and the substance under consideration (Renkin 1971).

In a following study, Renkin and Rosell (1962) showed that vascular resistance increased during sympathetic adrenergic stimulation, while both $^{86}$Rb and oxygen clearance decreased. This decrease in clearance was thought to occur through vasoconstriction of perfused nutritive capillaries resulting in redistribution of blood flow into the non-nutritive capillaries.

Isotopic xenon clearance from the perfused cat gastrocnemius was found to have deviations from a monoexponential curve, which was thought to be due to either uneven binding or uneven perfusion of the tissue (Kjellmer et al. 1967; Sejrsen and Tönnesen 1968). Sejrsen and Tönnesen (1968) concluded that there are no fixed in-parallel compartments within the muscle and that countercurrent exchange of $^{133}$Xe across the vessel wall explains the shape of the clearance curve. $^{133}$Xe had a uniform distribution within the skeletal muscle during rest. However, it has been reported that $^{133}$Xe has a ten times preferential binding affinity to adipose tissue compared to muscle tissue although the fat content of cat muscle is very low (1%) (Kjellmer et al. 1967).

Further evidence of dual circulation came from Harrison et al. (1990), who concluded that there was local redistribution of capillary flow after stimulation of the rabbit sciatic nerve to contract the vastus medialis. At low frequencies (< 2Hz) there was an increase in capillary flow without an increase in arterial flow whilst a clear redistribution of flow was observed. There was no correlation between capillary flow and femoral arterial flow, and nor between oxygen consumption and femoral arterial flow during hydrogen clearance from the perfused rabbit hindlimb at rest. Harrison et al. proposed a model for oxygen-dependent regulation of capillary blood flow involving two compartments, one with high-flow and the other with normal-flow.

### 1.1.1.1 Direct Observation Studies

Further supporting evidence for the existence of a non-nutritive pathway was obtained through the use of direct intravital microscopy techniques in a variety of animals (rabbit (Branemark and Eriksson 1972; Borgström et al. 1988), and rat
One of the first studies to describe the perfusion of the non-nutritive vessels following vasoactive agonist infusion was conducted by Grant and Payling Wright (1970). They observed the vasculature in the tibial tendon of the rat biceps femoris muscle and infused a variety of vasoactive agents. Acetylcholine and histamine dilated arterio-venous (non-nutritive) vessels in the tendon whilst noradrenaline and adrenaline both vasoconstricted the same vessels.

Figure 1.1: Taken from Clark et al. (2000). Schematic illustration of vasculature in the tenuissimus muscle of rabbits. Transverse arteriole supplies both capillaries in the muscle tissue and the adjacent tissue.

A large body of supporting evidence has come from the use of the tenuissimus muscle of the rabbit, due to the accessibility and transparency of the muscle, which allows it to be viewed under a microscope. The tenuissimus muscle originates at the sacral bone, runs down the crural fascia and inserts into the coccygeal bone (Branemark and Eriksson 1972; Eriksson and Myrhage 1972). This muscle is supplied by vasculature comprised of a transverse arteriole that supplies both the muscle fibres and associated connective tissue (Figure 1.1). An increase in oxygen availability by elevated ambient oxygen levels reduces blood flow to the muscle with no concomitant change in flow to the connective tissue. A decrease in oxygen availability led to similar changes in both muscle and connective tissue flow. This was attributed to proportionate resistance regulation in the transverse and
terminal arteriole in view of the lack of evidence for precapillary sphincters (Lindbom and Arfors 1984).

Borgström et al. (1988) indicated that blood flow could be controlled between the two vascular routes (nutritive and non-nutritive) by vasoactive agents. Their study used isoprenaline (β-adrenoceptor agonist), adrenaline and adrenaline with propranolol (β-adrenoceptor antagonist). During isoprenaline infusion there was a fractional redistribution of flow from the muscle tissue to the adjacent connective tissue (nutritive to non-nutritive) via dilation of the transverse arteriole, as well as a slight constriction of the terminal arteriole. Adrenaline infusion induced constriction of both the transverse and terminal arterioles, with a minimal blockage of this constriction during propranolol infusion. Adrenaline over the dose range decreased total flow to the muscle and connective tissue without affecting the proportion of distribution between either tissue. They concluded that β-adrenergic control of skeletal blood flow is via distribution between the transverse arteriole (connective tissue) and the terminal arteriole (muscle).

Borgström et al. (1990) also observed microvascular regulation in rabbit skeletal muscle after a 30% haemorrhage. During the 30 minute post-haemorrhage period, the transverse arteriole gradually constricted to 75% of the control diameter. The terminal arteriole constricted to 65% within 10 minutes and then gradually returned to 80% of the prehaemorrhage diameter. During the early post-haemorrhage phase, the reduced total flow was diverted into the connective tissue (non-nutritive) at the expense of nutritive flow to the muscle tissue to retain nutrient levels for the vital organs such as the brain and heart. This flow redistribution was gradually reversed to restore nutritive flow to the muscle tissue. It has been shown that during times of hypovolaemia, this expansion of intravascular volume is primarily mediated through β₂-adrenoceptor activity (Lundvall and Hillman 1978; Hillman et al. 1982).

The capillary network is arranged into fundamental units termed modules (Figure 1.2). The number of capillaries per module is dependent upon age and other factors (Berg and Sarelius 1995). These modules have interconnections with transverse arterioles and venules but lack connections with other modules (Skalak and Schmid-Schönbein 1986). Berg et al. (1997) observed that discrete stimulation of a small bundle of muscle fibres produced an increase in erythrocyte flux and velocity only in the capillary module supplying the contracting muscle fibres and not the feeding arteriole (Gorczynski et al. 1978; Berg et al. 1997). No change was seen in
the erythrocyte flux in the other capillary module arising from the same terminal arteriole. Furthermore, dilation was observed in the associated transverse arteriole but not in other transverse arterioles belonging to other unstimulated modules from the same network. This study and other similar studies have identified a mechanism connecting increases in skeletal muscle metabolism during contraction to local capillary recruitment via the terminal arteriole control of capillary modules (Delashaw and Duling 1988; Berg et al. 1997). There are multiple signalling pathways by which conducted vasodilation can be initiated in microvessels underlying contracting muscle fibres (discussed below) (Cohen et al. 2000). The spread of a vasodilatory signal which is initiated by muscle contraction at the arteriolar level seems likely to have the capacity to aid in the coordination of muscle metabolism and blood flow during exercise (Murrant and Sarelius 2000b).

Figure 1.2: Adapted from Berg et al. (1997). Simplified schematic diagram (not to scale) showing the relationships between the distal ramifications of the arteriolar microvasculature. Feed arteries give rise to transverse arterioles and these give rise to terminal arterioles. Downstream of each terminal arteriole lie a capillary network which consists of a number of distinct capillary module or units.

Although the majority of direct observation studies have been performed in the tenuissimus muscle (a non weight bearing muscle) the outcomes can be extrapolated to the regulation of larger muscles. The vascular arrangements of the
tenuissimus, the thicker biceps femoris, lateral and medial heads of the gastrocnemius and the soleus of the cat have very similar patterns. The tenuissimus muscle has a vascular arrangement like that of a singular basic unit of a thicker muscle (Myrhage and Eriksson 1980). Lund et al. (1987) determined that capillaries are arranged into units or modules fed by a single arteriole and largely drained by a single venule in the tibialis anterior muscle.

1.1.1.2 Pharmacological Studies

The majority of physiological evidence for a dual circulation has arisen from pharmacological studies in various animal models (Table 1.1). The relationship between blood flow and twitch development has been demonstrated by a number of studies (Sonnenchein and Hirvonen 1961; Hirvonen and Sonnenschein 1962; Hirvonen et al. 1964; Peoples 2004). These studies have concluded that contractile force is reduced in a dose-dependent manner during vasodilator infusion into the constant flow hindlimb (Hirvonen et al. 1964). It is possible that this depression could be overcome by a specific antagonist of the vasodilator, or by an increase in blood flow rate, although there was no evidence of neuromuscular blockade. Hirvonen and Sonnenschein (1962) concluded that a redistribution of blood flow within the muscle and associated connective tissue could account for these results. Generally these studies did not report any metabolic measures such oxygen consumption or lactate efflux.

Acetylcholine is thought to be released locally from vascular endothelium in response to increased shear stress within the arterioles resulting in vasodilation (Brock et al. 1998). At rest, acetylcholine increases regional blood volume and mean hydrostatic capillary pressure, resulting in net fluid movement from the blood into the tissue (Kjellmer and Odelram 1965). It is thought that this effect is consequent upon the greater dilatation of precapillary arterioles than postcapillary venules (Ablad and Mellander 1963; Mellander 1966). Nutritive flow at rest is increased by acetylcholine through vasodilation resulting in increased heterogeneity (Vetterlein et al. 1977). In contracting muscle, acetylcholine decreases nutritive or increases non-nutritive flow in the perfused cat gastrocnemius-soleus muscles. Acetylcholine delays sodium iodide (NaI) washout, and has been shown to either increase total flow without a change in force or decrease force without a change in total flow (Sonnenchein and Hirvonen 1961; Hirvonen et al. 1964).
Table 1.1: Adapted from Altura (1971). Summary table of the vascular actions of humoral and chemical substance that have implications in the regulation of skeletal muscle blood flow.

<table>
<thead>
<tr>
<th>Humoral (blood-borne) substance response</th>
<th>Chemical (locally produced-metabolic) agent response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catecholamines:</td>
<td>Adenosine and Adenine nucleotides</td>
</tr>
<tr>
<td>Adrenaline</td>
<td>VD</td>
</tr>
<tr>
<td>Noradrenaline</td>
<td>VC</td>
</tr>
<tr>
<td>Dopamine</td>
<td>VC, VC</td>
</tr>
<tr>
<td>Amines:</td>
<td>Inorganic Phosphate</td>
</tr>
<tr>
<td>Serotonin</td>
<td>VC, VD</td>
</tr>
<tr>
<td>Histamine</td>
<td>VD</td>
</tr>
<tr>
<td>Acetylcholine</td>
<td>VD</td>
</tr>
<tr>
<td>Polypeptides:</td>
<td>G-I Tract polypeptides:</td>
</tr>
<tr>
<td>Angiotensin</td>
<td>VC</td>
</tr>
<tr>
<td>Kinins</td>
<td>VC</td>
</tr>
<tr>
<td>Vasopressin</td>
<td>VC</td>
</tr>
<tr>
<td>Oxytocin</td>
<td>VC, VD</td>
</tr>
<tr>
<td>Hyperosmolarity</td>
<td>VD</td>
</tr>
</tbody>
</table>

VD: Vasodilation; VC: Vasoconstriction.

The focus on skeletal muscle flow distribution has been revived in recent years through the interest in dual circulation and its potential role in muscle metabolism (Clark et al. 1995). The constant flow perfused rat hindlimb has been the main method used by Clark and co-workers investigating haemodynamic effects on skeletal muscle metabolism. Vasoactive agents can regulate skeletal muscle metabolism and contribute to whole body thermogenesis through regulation of the microvasculature (Clark et al. 1994; Clark et al. 1995). One of the first studies by this group showed that physiological doses of vasopressin (V1-receptor agonist) and angiotensin II (AT1-receptor agonist) can increase oxygen uptake by up to 70% of basal uptake (Colquhoun et al. 1988). In Colquhoun et al. (1988) studies there was no effect on vasopressin and angiotensin II induced increase in oxygen uptake during α- and β-adrenoceptor blockade by the infusion of phentolamine and propranolol respectively. Angiotensin II administration increases tetanic force development but
not twitch force development in the perfused rat hindlimb (Rattigan et al. 1996). The
vasodilators, nifedipine (Ca\textsuperscript{2+} channel blocker), nitroprusside (cyclic GMP and nitric
oxide donor) and isoprenaline infused together blocked the effect of vasopressin and
angiotensin II on oxygen uptake and perfusion pressure (Colquhoun et al. 1988;
Colquhoun et al. 1990) while nitroprusside alone was shown not to inhibit either
oxygen uptake or force development during hindlimb stimulation in the same
preparation (Ye et al. 1990).

The effects of various vasoconstricting agents on perfusion pressure and
oxygen uptake in the constant flow non-recirculating perfused rat hindlimb show
differing results (Table 1.2). While all increase perfusion pressure, some increase
oxygen consumption whilst others reduce it. Clark et al have grouped
vasoconstrictors that increase perfusion pressure in the hindlimb into two categories
depending on their effects on metabolic activity (Clark et al. 1994; Clark et al. 1995).
Type A vasoconstrictors increase oxygen uptake whilst type B vasoconstrictors
decrease oxygen uptake. Those Type A vasoconstrictors studied have shown
changes consistent with an increase in muscle metabolism (Table 1.3). The effect of
the vasoactive agents on oxygen uptake could be due to: a) receptor location on the
resistance arterioles, which redirect the perfusate into previously under-perfused
regions of the hindlimb; b) receptor location is not only on the vasculature but also on
the skeletal muscle to directly increase oxygen uptake; or c) oxygen uptake by the
vascular smooth muscle to maintain vasoconstriction (Côté et al. 1985).
Table 1.2: Adapted from Clark et al. (1995) Vasoconstrictor stimuli that increase (Type A) or decrease (Type B) oxygen uptake in perfused rat hindlimb.

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Change From Control at Maximum Dose of Agonist %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Perfusion Pressure</td>
</tr>
<tr>
<td><strong>Type A</strong></td>
<td></td>
</tr>
<tr>
<td>Noradrenaline</td>
<td>52, 130</td>
</tr>
<tr>
<td>Adrenaline</td>
<td>67</td>
</tr>
<tr>
<td>Phenylephrine</td>
<td>160</td>
</tr>
<tr>
<td>Methoxamine</td>
<td>167</td>
</tr>
<tr>
<td>Amidephrine</td>
<td>24</td>
</tr>
<tr>
<td>Ephedrine</td>
<td>70</td>
</tr>
<tr>
<td>Norephedrine</td>
<td>57</td>
</tr>
<tr>
<td>Angiotensin II</td>
<td>133</td>
</tr>
<tr>
<td>Vasopressin</td>
<td>121</td>
</tr>
<tr>
<td>Capsaicin</td>
<td>54.5</td>
</tr>
<tr>
<td>Dihydrocapsaicin</td>
<td>49</td>
</tr>
<tr>
<td>[6]-Gingerol</td>
<td>30</td>
</tr>
<tr>
<td>[6]-Shogaol</td>
<td>30</td>
</tr>
<tr>
<td>Low frequency sympathetic nerve stimulation (0.5-4Hz)</td>
<td>6</td>
</tr>
<tr>
<td><strong>Type B</strong></td>
<td></td>
</tr>
<tr>
<td>Noradrenaline (≥ 1µM)</td>
<td>700, 200, 380</td>
</tr>
<tr>
<td>Serotonin</td>
<td>76</td>
</tr>
<tr>
<td>Capsaicin (&gt; 1µM)</td>
<td>110</td>
</tr>
<tr>
<td>Dihydrocapsaicin (&gt; 1µM)</td>
<td>↑</td>
</tr>
<tr>
<td>[6]-Gingerol (≥ 20µM)</td>
<td>96</td>
</tr>
<tr>
<td>High-frequency sympathetic nerve stimulation (&gt; 4Hz)</td>
<td>37</td>
</tr>
</tbody>
</table>

Hindlimbs were perfused at 25°C with constant flow. ↑ and ↓, indicative of an increase or decrease, respectively, when compared with control (vehicle only) perfusion.

Two vasoactive agents that have been the primary focus of the Clark group are noradrenaline (Type A) and serotonin (Type B) due to their opposing effects on the perfused hindlimb oxygen consumption despite similar effects on perfusion pressure (Table 1.3). Noradrenaline is both a neurotransmitter and a mitogenic hormone (Buu et al. 1993) which acts on both $\alpha_1$- and $\alpha_2$-adrenoceptors (Gardiner and Peters 1982). Decreases in capillary surface area associated with increasing doses of noradrenaline and serotonin infusion are reflected in a progressive decrease in both capillary diffusion capacity and capillary filtration capacity. These changes are associated with increases in both pre- and post-capillary resistance, which cause an increase in both arterial and capillary resistance via vasoconstriction (Rippe and Folkow 1980). During local application of noradrenaline, a temporary reduction of capillary flow occurs via arteriolar constriction (Dietrich and Tyml 1992b).
Table 1.3: Adapted from Clark et al (1994). Vasomodulator effects on perfused hindlimb.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Vasoconstrictors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Perfusion pressure</td>
<td>↑</td>
</tr>
<tr>
<td>Oxygen uptake</td>
<td>↑MENTS[^134]</td>
</tr>
<tr>
<td>Lactate efflux</td>
<td>↑</td>
</tr>
<tr>
<td>Fatty Acid efflux</td>
<td>↑</td>
</tr>
<tr>
<td>Glycerol efflux</td>
<td>↑</td>
</tr>
<tr>
<td>Urate efflux</td>
<td>↑</td>
</tr>
<tr>
<td>Insulin mediated glucose uptake</td>
<td>Not Tested</td>
</tr>
<tr>
<td>Skeletal muscle contraction</td>
<td>Not Tested</td>
</tr>
<tr>
<td>Perfusate distribution volume</td>
<td>↑</td>
</tr>
<tr>
<td>Vascular space using fluorescein-labelled dextran</td>
<td>↑</td>
</tr>
<tr>
<td>Corrosion cast volume</td>
<td>↑</td>
</tr>
<tr>
<td>Erythrocyte flux from previously perfused areas</td>
<td>↑</td>
</tr>
<tr>
<td>1-MX metabolism</td>
<td>↑</td>
</tr>
</tbody>
</table>

**Effect of the following on vasoconstriction and associated changes:**

<table>
<thead>
<tr>
<th></th>
<th>Type A</th>
<th>Type B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Removal of external Ca^{2+}</td>
<td>Blocked</td>
<td>Not Blocked</td>
</tr>
<tr>
<td>Replacement of O_{2} with N_{2}</td>
<td>Blocked</td>
<td>Not Blocked</td>
</tr>
<tr>
<td>Addition of N_{3}^{-}, CN^{-}</td>
<td>Blocked</td>
<td>Not Blocked</td>
</tr>
<tr>
<td>Addition of vasodilator</td>
<td>Blocked[^134]</td>
<td>Blocked</td>
</tr>
</tbody>
</table>

[^134]: Hindlimbs were perfused at 25°C with constant flow. 1. Includes nitroprusside, nifedipine, isoprenaline, adenosine, AMP, ADP, ATP and UTP. 2. Includes nitroprusside, carbamyl choline and isoprenaline (partial blockade).

While noradrenaline normally increases both perfusion pressure and oxygen uptake, it has been observed to decrease hindlimb oxygen uptake at supraphysiological levels (> 1µM) (Dora et al. 1992). Increases in perfusion pressure occur through constriction of both pre- and postcapillary vessels (Haddy et al. 1957; Hudlicka 1973) which result in a temporary reduction of flow in the perfused capillary (Dietrich and Tyml 1992b). During hypoxia or the infusion of sodium cyanide or azide (both uncouple mitochondrial oxidative phosphorylation), noradrenaline-induced
vasoconstriction is almost totally abolished (Dora et al. 1992) and the effect on perfusion pressure and oxygen uptake is also inhibited by the combined infusion of nifedipine, nitroprusside and isoprenaline (Colquhoun et al. 1990). It has been speculated that at lower doses, noradrenaline-induced vasoconstriction is dependent upon oxidative metabolism, whilst during higher doses it is dependent on anaerobic metabolism (Dora et al. 1992).

Serotonin is found in the central nervous system and platelets, and is a potent skeletal muscle vasoconstrictor (Rang et al. 1995). Serotonin does not significantly change total resistance in the perfused dog hindlimb. However, serotonin does increase large artery and vein resistance (5-HT2a mediated) and decreases small vessel resistance (5-HT1 mediated) (Haddy et al. 1957). The response to locally infused serotonin is dependent upon the initial level of vascular resistance (Dietrich and Tyml 1992b). Serotonin decreases muscle vascular resistance when initial resistance is high and increases resistance if initial resistance is low (Cheng and Shibata 1980). This variable response occurs when the initial resistance is manipulated neurogenically or via metabolically induced vasodilation (Emerson et al. 1973). Maximal serotonin induced vasodilation occurs during sympathetic stimulation in vivo, which may be caused by inhibiting the release of noradrenaline (Haddy et al. 1959; McCubbin et al. 1962; Emerson et al. 1973; Blackshear et al. 1985). Alternatively, serotonin at high concentrations has a direct vasodilator effect in vitro that can only be observed when the initial resistance is high or selectively blocked by ketanserin (5-HT2 – antagonist) (McLennan and Taylor 1984). Serotonin has been shown to decrease oxygen uptake and lactate production in rat skeletal muscle which is blocked via ketanserin (Dora et al. 1991). During hypoxia or the infusion of cyanide or azide there is no effect on serotonin-induced vasoconstriction or decreased oxygen uptake. It has been speculated that serotonin vasoconstriction is capable of anaerobic metabolism through the use of exogenous glucose when glycogen stores are exhausted (Dora et al. 1992). Aerobic contractility is inhibited during serotonin infusion which is thought to occur through selective vasoconstriction resulting in diminished nutrient delivery through redirection of blood flow. In the study conducted by Dora et al, the vasoconstrictor action of serotonin was not over come by local vasoactive metabolites released during skeletal muscle contraction of the perfused rat hindlimb (Dora et al. 1994). Serotonin has also been shown to inhibit insulin-mediated glucose uptake, especially in muscles that are rich in fast-twitch glycolytic fibres and reduce free fatty acid uptake across the hindlimb by selective vasoconstriction (Rattigan et al. 1993; Baron and Clark 1997; Clerk et al. 2003).
Using three different experimental protocols, Newman et al. (1996) studied the differing effects of low dose noradrenaline (Type A) and serotonin (Type B) on the vascular routes in the rat hindlimb. One method evaluated the release of entrapped erythrocytes from previously equilibrated hindlimbs into a perfusate medium that did not contain erythrocytes. Low dose noradrenaline markedly increased the erythrocyte efflux levels whilst serotonin showed no change in erythrocyte washout.

The vascular entrapment of fluorescein-labelled dextran showed that low dose noradrenaline recruits a new vascular space (most likely nutritive vessels) without closing off any pre-existing perfused areas. Access to the new space could be re-accessed only with a second infusion of low dose noradrenaline. In contrast, serotonin was found to close off previously perfused areas, (most likely nutritive vessels) and did not recruit any new regions, this effect being reversible only by the removal of serotonin (Newman et al. 1996).

Corrosion of vascular casts of the rat hindlimb vasculature using 12µm microspheres resulted in low dose noradrenaline significantly increasing the filling of smaller vessels but had no effect on the volume of the arterial tree compared to the basal castings. Serotonin and high dose noradrenaline (> 1µM) decreased the total extent of arterial vascular filling and cast weight (Newman et al. 1996).

The metabolic changes induced during either noradrenaline or serotonin infusion can be reversed by the infusion of microspheres (< 15µm). It was proposed that during microsphere infusion, the route receiving most flow would be blocked first, and thus flow will be redirected to the more constricted route. The end result in studies by Vincent et al. showed that while perfusion pressure further increases, the metabolic effect of the vasoconstrictor is reversed (Vincent et al. 2001a; Vincent et al. 2001b). However, as the distribution patterns of the microspheres could not differentiate between noradrenaline and serotonin vasoconstriction sites, it was concluded that the nutritive and non-nutritive routes are distributed evenly throughout the muscle (Vincent et al. 2001b).

Clark et al. has attempted to use the endothelial cell metabolism of 1-methyxanthine as a marker of capillary flow (Rattigan et al. 1997a). 1-Methyxanthine (1-MX) is converted solely to 1-methylurate (1-MU) via xanthine oxidase (XO), which is a capillary endothelial derived enzyme. The recoveries of 1-MX and 1-MU are quantitative and 1-MX has no effect on vascular resistance in perfused rat hindlimb studies. The conversion of 1-MX to 1-MU is inhibited by allopurinol (an XO-specific
inhibitor) and xanthine. The rationale for this is that if Type A and B vasoconstrictors act by altering the pattern of perfusate flow within muscle there would be an associated change in the exposure of exogenous substrate to enzymes located in the vasculature (Clark et al. 1997). Changes in 1-MX metabolism were found to positively correlate with changes in nutritive flow in perfused rat hindlimb muscle. The lowest production of 1-MU was found during serotonin induced vasoconstriction, and the highest during muscle contraction (Clark et al. 2000). Serotonin had no effect on the distribution of 15µm fluorescent microspheres and was concluded that 1-MX metabolism is a potential marker for the decrease in muscle nutritive flow or nutrient access during serotonin vasoconstriction.

Activation of the sympathetic nervous system increases perfusion pressure through vasoconstriction (Hall et al. 1997). The sympathetic nervous system also has a biphasic effect on oxygen consumption (Table 1.2) which together with the vasoconstrictor effect is though to act via the $\alpha_1$-adrenoceptor (discussed below).

The vascular network in the septa (connective tissue such as perimysium, endomysium and epimysium) and tendon could serve as a functional shunt for the skeletal muscle blood supply (Barlow et al. 1959, 1961; Grant and Payling Wright 1970; Newman et al. 1997; Clark et al. 2000). Vasoactive agents known to regulate oxygen uptake in the constant flow perfused hindlimb were also found to regulate blood flow in the associated connective tissue. An inverse relationship between tendon vessel blood flow and oxygen uptake suggests that the amount of perfusion in the tendon and surrounding connective tissue plays a major regulatory role of total hindlimb metabolism by acting as a functional shunt to reciprocally control nutritive flow (Newman et al. 1997).

Therefore, noradrenaline and serotonin may act at different sites in the vasculature to distribute blood to different areas in the hindlimb supporting the earlier hypothesis of Barlow and his colleagues. It has been suggested that Type A and B vasoconstrictors act at different sites in the hindlimb vasculature (Figure 1.3) to influence muscle metabolism by either increasing or decreasing nutrient access to muscle by controlling different capillary flow routes (Newman et al. 1996). It is suggested that: a) Type A vasoconstrictors increase nutritive flow (that is provide an increase in oxygen to highly metabolic tissues) by constricting points on the transverse arteriole distal to the muscle but proximal to the connective tissue; and b) Type B vasoconstrictors increase non-nutritive flow by constricting at branch points leading from the transverse arteriole such as the terminal arterioles supplying the muscle cells thereby directing blood flow away from the muscle.
1.1.1.3 Measurement of Nutritive and Non-Nutritive Flow Distribution

As evidence arose to support the existence of dual circulation in skeletal muscle, the question was raised as to the distribution of blood flow between the two routes. Friedman (1966) first estimated that 75% of total flow was non-nutritive in an isolated constant flow perfused dog hindlimb that was denervated and resting. Radioactive albumin was used to measure total blood volume and $^{86}\text{Rb}$ to measure non-nutritional volume. It was assumed that the ability of $^{86}\text{Rb}$ to diffuse into the tissue was a result of passing through well-perfused channels and being limited at the capillary wall. $^{86}\text{Rb}$ has an extraction similar to potassium, where there is complete capillary extraction in the nutritive pathway with no extraction occurring during times of high non-nutritive flow (Friedman 1965, 1968, 1971). Therefore the extraction of $^{86}\text{Rb}$ by skeletal muscle has an inverse relationship to total blood flow (Renkin and Rosell 1962; Friedman 1969).

Using direct observation of the rabbit tenuissimus muscle it was estimated that around 33% of the flow entering the transverse arteriole from the central artery was distributed into the connective tissue at rest (Lindbom and Arfors 1984). Because the tenuissimus muscle mass is not evenly distributed, but rather concentrated in the middle and decreases towards the edge, it was re-estimated that the connective tissue flow fraction was closer to 20-25% of total flow. In a similar study measuring skeletal muscle capillary haematocrit, it was calculated that 20% of
transverse arterial blood flow passes through the non-nutritive route (associated connective tissue) of the rabbit tenuissimus muscle (Ley et al. 1988). Connective tissue microcirculation represents a significant functional erythrocyte shunt under normal resting condition and has a haematocrit discharge 30% greater than systemic haematocrit (Ley et al. 1988) due to structural factors (see structure of skeletal muscle vasculature).

Clearance rates of iodine from the skinned leg of the anaesthetised cat during hyperaemia following arterial occlusion were higher than pre-occlusion levels. It was suggested that redistribution of blood flow from non-nutritive into the nutritive route could explain this increase in clearance following occlusion (Ballard et al. 1964). From the difference between pre-occlusion and post-occlusion clearance rates it was calculated that at rest 40% of total muscle blood flow passes through the non-nutritive route.

Using clearance of hydrogen ions, Harrison et al. (1990) reported two areas of clearance similar to earlier studies. One area contained high flow capillaries where mean flow is six-times higher than the normal flow capillaries in the other area. Using Poiseuille’s law (flow = 4th power of the radius), Harrison et al calculated that if just 13% (high flow) of all capillaries within the skeletal muscle had a diameter of 7.5µm (Potter and Groom 1983) then these capillaries could carry 71% of total blood flow. Therefore if the remaining 87% (normal flow) had a mean diameter of 5.5µm (Potter and Groom 1983) then these would carry the remaining 29% resulting in a 4:21 ratio of nutritive to non-nutritive flow.

However, a major obstacle in measuring non-nutritive flow has been the lack of knowledge of specific characteristics of the non-nutritive vessels that would assist in devising an accurate method. The endothelial cell metabolism of 1-methyxanthine (1-MX) has been utilised as a marker of nutritive capillary flow (Clark et al. 1997; Rattigan et al. 1997a). The changes in 1-MX metabolism were found to positively correlate with changes in nutritive flow in perfused muscle.

Table 1.4 shows the summary of the calculated distribution of flow from a variety of models. The majority of these studies concluded that there is substantial non-nutritive flow in skeletal muscle during resting conditions.

It has been postulated that non-nutritive capillary beds could act as a flow reserve and amplify nutrient delivery during exercise (Clark et al. 1998a). If the ratio of nutritive to non-nutritive flow is 4:21 as suggested by Harrison et al. (1990), exercise could result in a six fold increase alone through redistribution from non-
nutritive to nutritive vessels. Also a three-fold increase in cardiac output would result in an overall eighteen fold increase in nutritive flow during exercise (Clark et al. 1998a). Similarly, post-exercise blood flow would remain elevated for a considerable time, where the balance between nutritive and non-nutritive flow may be important in allowing muscle to recover through removal of lactate and restoring nutrient supplies to energy stores (Clark et al. 1998b).

**Table 1.4:** Summary table of the distribution of flow through the skeletal muscle vasculature at rest.

<table>
<thead>
<tr>
<th>Model</th>
<th>Method</th>
<th>Nutritive Flow</th>
<th>Non-Nutritive Flow</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolated perfused dog hindlimb</td>
<td>Radioactive rubidium</td>
<td>25%</td>
<td>75%</td>
</tr>
<tr>
<td>Rabbit tenuissimus muscle</td>
<td>Direct observation</td>
<td>75 - 80%</td>
<td>20 - 25%</td>
</tr>
<tr>
<td>Rabbit tenuissimus muscle</td>
<td>Direct observation</td>
<td>80%</td>
<td>20%</td>
</tr>
<tr>
<td>Perfused cat hindlimb</td>
<td>Radioactive Iodine</td>
<td>60%</td>
<td>40%</td>
</tr>
<tr>
<td>Perfused rabbit hindlimb</td>
<td>Hydrogen</td>
<td>29%</td>
<td>71%</td>
</tr>
</tbody>
</table>
1.2 **PERFUSED HINDLIMB METHODOLOGY**

The nutritive/non-nutritive hypothesis has been based most recently on studies using a constant flow, non-recirculating *in situ* perfused hindlimb, or isolated muscle preparations using either cell free, human erythrocyte or bovine erythrocyte perfusates. Non-physiological conditions, including low oxygen consumption and low perfusion pressure are the norm for these preparations. Shiota and Sugano (1986) concluded that the use of an erythrocyte- and albumin-free perfusate in the isolated perfused rat hindlimb is a useful tool for biochemical investigation into skeletal muscle function. However, the importance and physiological relevance of using blood in perfusion when investigating oxygen consumption, tissue function and metabolic response has been demonstrated in earlier work involving the heart (Topping and Trimble 1985; Pepe and McLennan 1993) and the liver (Storer *et al.* 1980) perfusion systems.

The low perfusion pressure in some models is due to the absence of vascular tone and therefore they are incapable of vasodilation to increase blood flow. As this nutritive/non-nutritive theory is related to the provision of oxygen and nutrients for metabolism, the capacity to vasodilate is important under conditions of high metabolic rate (that is in contracting muscle). Most of these studies have been conducted in resting state with no muscle contraction and it is not surprising that when the hindlimb has been stimulated to contract, the ability of the muscle to perform over a long duration is poor.

A majority of studies have been performed at 25°C under conditions of constant flow with a 95% oxygen-5% carbon dioxide gas phase. This type of solution has low oxygen content (Pepe and McLennan 1993) and leads to oxygen delivery to the muscle well below that occurring *in vivo*. With increases in flow rate (decrease in RBC transit time) it has been shown that hindlimb oxygen consumption increases reflecting increased oxygen delivery (Figure 1.4) (Ye *et al.* 1990). It has been suggested that qualitatively similar changes occur in other studies when the hindlimb is perfused at 37°C under constant flow with a perfusate using bovine red blood cells with the same gas phase (Clark *et al.* 1995).
Figure 1.4: Adapted from Ye et al. (1990). The effect of flow rate on oxygen consumption and perfusion pressure in the isolated perfused hindlimb.

At 25°C cell-free perfusion studies using a perfusion rate of 2 ml·min⁻¹ (Table 1.5) produce hindlimb perfusion pressures of 21mmHg, with a basal oxygen consumption of 4.8 µmol·g⁻¹·hr⁻¹ (0.08µmol·min⁻¹·gww⁻¹) (Colquhoun et al. 1988; Ye et al. 1990). Higher perfusion rates produce higher perfusion pressures and increase oxygen consumption (Colquhoun et al. 1988; Colquhoun et al. 1990; Ye et al. 1990; Dora et al. 1991; Dora et al. 1992; Rattigan et al. 1993; Newman et al. 1996; Newman et al. 1997; Tong et al. 1998). At 8ml·min⁻¹ and 37°C, cell-free perfusate perfusion pressure was 37.5mmHg and oxygen consumption was 0.39µmol·min⁻¹·gww⁻¹ (Vincent et al. 2001b). Using bovine RBC perfusate at 37°C, a perfusion rate of 4ml·min⁻¹ produced a resting perfusion pressure of 49.5mmHg and an oxygen consumption of 0.62µmol·min⁻¹·gww⁻¹ (Rattigan et al. 1996; Clark et al. 2001b). All of these perfusion pressures are well below normal in vivo blood pressure. To achieve physiological perfusion pressure, flow rates of 15-20ml·min⁻¹ have been required (Ye et al. 1990). Blood flow through the iliac artery in vivo is approximately 1.5 ml·min⁻¹ per vessel (Janiak et al. 2002). Furthermore, increases in
temperature and oxygen uptake kinetic changes result in a greater rate of diffusion across the cell membrane. Therefore, the temperature and the presence of erythrocytes in a perfusate or whole blood may be crucial to the study of in vivo conditions.

Table 1.5: A comparison of hindlimb flow rates, pressure and oxygen consumption responses from varying models based upon Ruderman et al. (1971). This table represents a range of studies that have used variations in the rat hindlimb perfusion method. It is by no means completely comprehensive.

<table>
<thead>
<tr>
<th>Perfusate</th>
<th>Flow Rate</th>
<th>Per fusate Temperature</th>
<th>Perfusion Pressure</th>
<th>( \dot{V}_{O_2} )</th>
<th>Reference</th>
</tr>
</thead>
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<tr>
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<td>4</td>
<td>37</td>
<td>49</td>
<td>0.62</td>
<td>5</td>
</tr>
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</table>

Perfusate: perfusion medium used during the perfusion of the hindlimb. Flow Rate: Hindlimb flow expressed as \( \text{ml} \cdot \text{min}^{-1} \). Perfusate Temperature: expressed in degrees celsius. Perfusion Pressure: Hindlimb perfusion pressure expressed as mmHg. \( \dot{V}_{O_2} \): Hindlimb oxygen consumption expressed as \( \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{gww}^{-1} \). Reference: 1. (Colquhoun et al. 1988; Ye et al. 1990), 2. (Colquhoun et al. 1988; Colquhoun et al. 1990; Dora et al. 1991; Rattigan et al. 1993; Newman et al. 1997; Tong et al. 1998; Ye et al. 1998), 3. (Colquhoun et al. 1988; Newman et al. 1996), 4. (Vincent et al. 2001b; Clerk et al. 2003), 5. (Rattigan et al. 1996; Clark et al. 2001a; Newman et al. 2001; Newman et al. 2002)

This thesis uses an autoperfused rat hindlimb with controlled perfusion using the animal’s own blood and allows for sampling of both arterial and venous blood (Peoples and McLennan 2001b, a; Peoples 2004). The animal remains alive throughout and with intact autonomic nervous system ensuring vascular tone and maintained perfusion pressure at low perfusion rates. It is believed that this model is an improved method to provide optimal physiological tissue oxygenation during times of increased metabolic demand (exercise metabolism) under close to in vivo conditions.
1.3 **STRUCTURE OF SKELETAL MUSCLE VASCULATURE**

1.3.1 **Site of Control of Skeletal Muscle Blood Flow**

The structure of skeletal muscle vasculature has a vital role in providing evidence of skeletal muscle dual circulation. It has been shown that no precapillary sphincters are present in skeletal muscle for the control of capillary perfusion (Eriksson and Lisander 1972; Eriksson and Myrhage 1972; Lindbom *et al.* 1980; Lindbom and Arfors 1985; Ohlen *et al.* 1988). Skeletal muscle capillary flow is regulated by the transverse and terminal arterioles and overall muscle blood flow, and resistance is controlled by large resistance arterioles and small arteries (Figure 1.5) (Lindbom 1983; Sweeney and Sarelius 1989; Anderson and Faber 1991). Vasoconstriction of the arteriolar vessels during low oxygen tension only extends down to the first part of the terminal arteriole, which is still a considerable distance from the branching points of the capillary network (Lindbom *et al.* 1980). Zweifach and Metz (1955) defined terminal arterioles as vessels that only have a single layer of smooth muscle, regardless of the arteriole diameter, in which supporting connecting tissue elements are almost completely lacking.

Acceptance of non-nutritive flow in skeletal muscle was largely prevented by the lack of evidence for the location of a discrete route associated with muscle. Such a route would have the ability to accept high flow and deny nutrient delivery to the muscle cells but cannot be discriminated by microsphere infusion. The debate over the existence of true arterio-venous shunts or anastomoses was added to by Hammersen (1970) who believed that the partial opening and closing (flow heterogeneity) of “capillary units” (modules) could explain the fast and slow compartments in skeletal muscle. Hammersen and his colleagues were not convinced that the vessels in the intramuscular connective tissue could act as true a-v shunts, so they proposed that these vessels be termed “Bügelkapillaren” or arc-capillaries, due to their morphological appearance, rather than their functional characteristics. These capillaries are usually very long, unramified and extend along the external surface of the muscle fibre bundles to often join to distal capillary beds or venules (Hammersen 1968). It was also argued that since these arc-capillaries only represent 5% of the capillaries studied in the connective tissue sheath they could not act as true shunts under normal conditions. When fully opened at hyper-perfusion pressures beyond the normal physiological range, these arc-capillaries could explain
the circulatory phenomena described under the conditions of previous studies (Hammersen 1970). A variation in length and degree of branching, different flow velocities within the capillary, a minor exchange function of non-nutritive vessels or a shift in perfusion from superficial vessels to deep vessels of the muscle may also explain the discrepancies Hammersen raised (Vetterlein and Schmidt 1980). From studies discussed above however, the non-nutritive vessels have a lower resistance and only 5% of skeletal muscle capillaries would therefore be capable to carry substantial proportions of total flow.

Figure 1.5: Adapted from Borgström et al. (1990) showing a simplified diagram of the microvasculature of the rabbit tenuissimus muscle illustrating the dual vasculature, muscle and connective tissue. TR: Transverse Arteriole; TE: Terminal Arteriole; CAP: Capillary Bed.

1.3.2 Capillary Functional Arrangement

Large arterio-venous anastomoses (larger than 20µm) that serve as functional shunts do not appear to exist in mammalian skeletal muscle (Barlow et al. 1958; Piiper and Rosell 1961). Corrosion casts of the rat gastrocnemius by Potter and Groom (1983) revealed that capillaries of the rat hindlimb are either 5.5µm or 7.5µm in diameter, with larger diameter capillaries constituting 17% of total capillaries. Zweifach and Metz (1955) observed what they termed “metarterioles”
(non-nutritive routes) along the free edge of the rat spinotrapezius muscle. These vessels act as shunts to convey the most rapid stream of blood in this muscle. Within this muscle, capillaries interconnected freely within the connective tissue. In the rabbit tenuissimus muscle, vascular connections between the transverse arteriole of the muscle and the associated connective tissue capillaries are a regular and frequent phenomenon (Lindbom 1983). The connective tissue surrounding the muscle has vessels ~ 12µm wide and ~ 1000µm long running directly from the transverse arteriole to the transverse venule. These vessels often supply nutrients to associated adipose tissue but are not arterio-venous anastomoses (Eriksson and Myrhage 1972). Lindbom and Arfors (1984) reported that 67% of the transverse arterioles in the tenuissimus muscle of the rat supply the adjacent connective tissue as well as the muscle tissue proper (Figure 1.5). Lindbom (1986) reported that the continuation of the transverse arteriole into the connective tissue had only small luminal changes after vasoactive agent infusion and that this tissue appears to have little capacity to regulate its own blood supply. In essence, these studies concluded that non-nutritive vessels have a larger diameter compared to the nutritive vessels resulting in a resting lower resistance.

1.3.3 Vascular Innervation

Adrenergic innervation of the skeletal muscle microvasculature is largely limited to the arterial/arteriolar side in close contact with the vessel wall, although substantial variations of innervation of the precapillary vessels do occur (Baez et al. 1977; Marshall 1982; Saltzman et al. 1992). Nerve plexus density of noradrenergic and neuropeptide Y neurons (sympathetic neurons) in the spinotrapezius and cremaster muscles of the rat and tenuissimus muscle of the rabbit are highest in the transverse and terminal arterioles and are not found in capillaries or venules (Ohlen et al. 1988; Fleming et al. 1989). Substance P and calcitonin gene-related peptide containing perivascular axons innervate large arterioles and arteries, and are likely to originate from sensory neurons (Fleming et al. 1989; Saltzman et al. 1992). Furthermore, substance P neurons innervate the transverse arteriole in the tenuissimus muscle of the rabbit and have been shown to increase blood flow in a dose dependent manner (Ohlen et al. 1988), whilst there is an inverse relationship between arteriolar diameter and sympathetic adrenergic stimulation (Fleming et al. 1987).
1.3.4 Receptor Location

Skeletal muscle has both $\alpha$- and $\beta$-adrenoceptors which are thought to regulate metabolism in the cell (Ye et al. 1995). There are two subtypes of $\alpha$-adrenoceptor ($\alpha_1$- and $\alpha_2$-adrenoceptors) and three $\beta$-adrenoceptors ($\beta_1$, $\beta_2$ and $\beta_3$- or atopic-adrenoceptor) (Bowman et al. 1971; Goodman Gilman et al. 1975). The relationship between skeletal muscle blood flow and $\alpha$- and $\beta$-adrenoceptor regulation is thought to be brought about by their distribution in the vasculature. Belfrage (1978) speculated that vascular $\alpha$-adrenoceptors are primarily located near nerve endings and $\beta$-adrenoceptors (humoral receptor) are situated near the lumen at some distance from the nerve endings. This would tend to suggest that $\alpha$-adrenoceptors are largely responsive to nervously-released noradrenaline, while the $\beta$-adrenoceptors are mostly influenced by circulating adrenaline.

1.4 BLOOD FLOW REGULATION DURING EXERCISE

1.4.1 General Exercise Blood Flow Phenomenon

Exercise is accompanied by general increases in muscle blood flow and there is evidence to suggest that this is metabolically regulated at the level of individual muscle fibres (Berg et al. 1997; Delp and Laughlin 1998). Blood flow to active skeletal muscle increases in a linear fashion with the increase in oxygen uptake by muscle cells. This is thought to occur (collectively) through arteriolar vasodilation, the removal of vasomotion, capillary module recruitment, an increase in capillary diameter due to elevated transmural pressure and decreased $P_{O_2}$ (Renkin et al. 1966; Klitzman et al. 1982; Laughlin and Korzick 2001; Slaaf and Oude Egbrink 2002). Arteriolar (resistance vessels) and venular (capacitance vessels) diameter and capillary perfusion have a positive proportional relationship to the mass of muscle stimulated and the intensity of the exercise (Kjellmer 1964; Gorczynski et al. 1978). The increase in muscle blood flow at the onset of exercise occurs biphasically (Kindig et al. 2002). The first phase, hyperaemia, is the mechanical effect of the muscle pump and vasodilation. The second phase has characteristics of feedback regulation to match the metabolic demand (Hughson and Tschakovsky 1999). The purpose of the first phase is to enhance the coupling of muscle perfusion to muscle contraction. The recruitment of muscle fibres during the transition into exercise would necessarily have a relationship with the rapid dilation, and this would be under local control rather than a change in systemic sympathetic activity.
Mohrman and Regal (1988) showed that Po$_2$ and Pco$_2$ are key variables in the local control of muscle blood flow during steady state exercise. They concluded that whilst they did not demonstrate a causal relationship there could be a strong synergistic effect of Po$_2$ and Pco$_2$ that is similar to those found in coronary blood flow control.

Vascular dependent differences have been found between fast and slow twitch muscle fibres. At rest, nutritive blood flow can be up to three to six times higher in soleus muscle than in white gastrocnemius of the cat (Hilton et al. 1970). Glucose consumption at rest is up to ten times higher in soleus muscle when compared to the gastrocnemius but decreases in soleus and slightly increases in the gastrocnemius during contraction (Hudlicka 1975). This is most likely due to differences in metabolic demand, with the aerobically metabolising red muscle fibres having a higher capillary density and nutritive flow compared to the anaerobically metabolising white muscle fibres (Reis et al. 1967).

It is generally assumed that, because arteriolar vasodilation is required to increase capillary perfusion, and since blood flow and metabolic rate are closely related and locally regulated (Berg et al. 1997), the relevant mediators of signalling between contracting skeletal muscle fibres and the microvasculature will be vasodilatory metabolites acting directly on small arterioles (Murrant and Sarelius 2000a).

1.4.1.1 Neural Regulation

A classic study by Barcroft et al. (1952) showed that during rhythmic contractions of the human forearm it was unlikely that the local circulatory response was facilitated by the inhibition of sympathetic vasoconstrictor activity. In more recent studies, sympathetic nervous system-mediated vasoconstriction of low metabolic demand tissue (such as digestive organs) has been characterised as a feature of exercise whilst acting as a stimulator at other sites (such as the heart). It has been shown that this sympathetic vasoconstriction can be overcome at the local arteriolar level through release of vasoactive metabolites such as nitric oxide in both rodents and awake humans (Chavoshan et al. 2002). Neurally derived nitric oxide plays a crucial part in vascular changes during mouse hindlimb contraction (Thomas and Victor 1997; Thomas et al. 1998). Sympathetic nerve activity has a local reflex response to exercise and metabolic products such as H$^+$ (Hansen et al. 1996). These metabolic products activate chemically sensitive afferent neurons to increase
efferent-sympathetic vasoconstrictor activity as a reflex. It is thought that this reflex
(called the muscle metaboreflex) offsets the local metabolic vasodilation to maintain
systemic blood pressure and to limit the reflex increase in blood pressure of
augmented blood flow to the working muscle (Hansen et al. 1994). This sympathetic
vasoconstriction can be negated by metabolic vasodilation thereby increasing oxygen
delivery to active muscle fibres and has recently been termed “functional
sympatholysis” (Segal and Kurjiaka 1995; Hansen et al. 1996).

Sympathetic vasodilation can be induced by either blockade of
vasoconstrictor activity or through cholinergic vasodilator neurons in rats, dogs and
cats (Mauskopf et al. 1969). Cholinergic vasodilation has been reported to reduce
oxygen consumption through redistribution of blood into the non-nutritive route
(Rosell and Uvnas 1960). This was contested by Folkow et al. (1961) who reported
that cholinergic vasodilator nerves do not open a non-nutritive route (>20μm),
although in some experimental conditions “functional shunting” could occur through
uneven perfusion of capillaries (Piiper and Rosell 1961). In humans it appears that
sympathetic vasodilator responses could lie with circulating adrenaline or local
cholinergic mechanisms, which stimulate the release of vascular endothelial-derived
nitric oxide (Joyner and Dietz 2003).

1.4.1.2 Vasoactive Metabolites

Vasoactive metabolites, as well as endothelial-derived dilating factors such as
prostaglandins and endothelial-derived relaxing factor (which is believed to be nitric
oxide), are believed to play the major regulatory role in the second phase of
increased skeletal muscle blood flow during exercise. Nitric oxide has been shown to
activate soluble guanylyl cyclase which may contribute to relaxation of the vascular
smooth muscle via cGMP-myosin regulatory light chain cascade (Moncada et al.
1991; Lau et al. 1998). Endothelial-derived dilating factors are released following an
increase in blood velocity and shear stress (Shoemaker and Hughson 1999). Other
endothelial-derived dilating factors such as endothelial-derived hyperpolarising factor
also contribute to endothelium-mediated vasodilation (Laughlin and Korzick 2001).

1.4.2 Mechanisms for Communication of Metabolic Demand

The communication of metabolic demand from the tissue to the sites of
regulation, the transverse and terminal arterioles, has been intensively investigated.
The site for regulating blood flow at metabolically active muscle must be directly
upstream from the active cells because metabolite release into the circulation would produce a generalised whole body response. The main mechanism for blood flow regulation is produced by the release of vasodilatory agents. Evidence suggests that capillaries can act as a communicating medium that can sense and integrate biological signals but the mechanism for this upstream communication has yet to be defined (Song and Tyml 1993). So it has been hypothesised that upstream communication via cell-to-cell coupling, or periarteriolar neural pathways collectively called “conducted vasomotor response”, could be the possible mechanisms by which metabolites released from the active skeletal muscle cells might selectively influence blood flow to those same cells (Gustafsson and Holstein-Rathlou 1999). Although calcium-dependent spikes can mediate neural conduction, periarteriolar neural pathways were ruled out as a possible mechanism when tetrodotoxin (TTX, a selective Na⁺ channel blocker), papaverine (phosphodiesterase inhibitor) and various calcium antagonists were infused failed to modify propagated upstream vasodilation (Ohlen et al. 1988; Fleming et al. 1989; Segal and Duling 1989; Dietrich and Tyml 1992b).

In short, it appears that skeletal muscle blood flow is regulated by multiple mechanisms. During exercise, metabolic vasodilation and increased vascular conductance through the skeletal muscle pump seem to be the primary mechanisms. Modulation of these mechanisms could be dependent on other factors such as endothelial derived nitric oxide and PGI₂ (Delp and Laughlin 1998).
1.5 **AIMS & HYPOTHESES**

This thesis aimed to test the hypothesis that skeletal muscle beds contain alternate parallel vascular pathways that can be controlled to redistribute blood flow during active metabolism.

We aimed to:

(a) Establish selective vasoconstriction (seen before in *in-vitro*),

(b) Establish selective vasodilation (vasodilator metabolites most likely to influence local blood flow with increased metabolic activities),

(c) Establish that distribution is altered during active metabolism.

It has been proposed that there are alternative vascular pathways within skeletal muscle that enable high blood flow to be directed to the muscle cells when they are active (so called nutritive pathway) and to bypass the muscle cells when they are inactive (non-nutritive pathway).

While the nutritive/non-nutritive circulation is an attractive hypothesis to explain control of blood flow to metabolising tissue, it has been largely defined in terms of vasoconstrictors. In contrast, local control of blood flow during increased metabolism has been largely described in terms of vasodilatory metabolites released from the active tissue. That is, an active muscle cell can produce metabolites that will selectively increase perfusion to that muscle.

Furthermore, while purporting to explain metabolically regulated blood flow, the nutritive/non-nutritive hypothesis is based entirely on studies using non-contracting skeletal muscle (tissues of low metabolic demand). These perfused hindlimb or isolated muscle preparations exhibit low vascular tone, and being incapable of vasodilation to increase blood flow and inevitably produce extremely low blood pressure. They are also limited by their low oxygen delivery and are unable to provide data on the relationship of oxygen consumption to muscle activity. Since this is a theory relating to the provision of oxygen for metabolism, the capacity to vasodilate is important under conditions of high metabolic rate, that is, in contracting muscle. We proposed to use an autoperfused hindlimb method. This technique uses an anaesthetised animal, with its own blood allowing for high oxygen delivery
provided to the hindlimb skeletal muscle. This method further allows for the retention of vascular tone aiding in the study of muscle contraction and oxygen use and the influence of drugs that affect the blood vessels supplying the muscle.
CHAPTER TWO

Methods and Materials
2.1 ANIMALS

Male Hooded Wistar rats (weight 450–785g) were obtained from Gore Hill Animal Research Laboratory (Royal North Shore Hospital, Sydney) and were housed (2 per cage) in the University of Wollongong animal house. Animal house environmental conditions were controlled with room temperature maintained at 23°C-25°C and a 12-hour light/dark cycle. Animals were used and cared for according to the National Health and Medical Research Council guidelines for the care and maintenance of laboratory animals and were fed on a commercial chow-diet ad-libitum with water freely available. All experimental procedures were approved by the Animal Ethics Committee of the University of Wollongong.

2.2 ANIMAL ANAESTHESIA

Animals were anaesthetised before the commencement of surgical procedures with sodium pentobarbital (6 mg·100g⁻¹ body weight i.p.). Anaesthesia was maintained with supplementary injections (2 ml·100g⁻¹ i.p.) as required during surgery and protocols. Experiments were performed in a heated perspex chamber, with animal body temperature maintained with the aid of a heating lamp placed above the rat.

2.3 CALIBRATION OF EQUIPMENT

Pressure and force transducers were calibrated immediately prior to each hindlimb perfusion. Pressure transducers were calibrated against a mercury sphygmomanometer in the range of 0-300mmHg. The force transducer was calibrated using standard calibration weights in the range of 0-200g (HB Selby and Co Ltd. Sydney). An amp meter (Testmate 308) was used to read voltage output for calibration of computer data acquisition.
*Figure 2.1:* Surgical cannulations redirect and regulate arterial blood flow in the rat from the control to the perfused hindlimb. Venous blood flow is returned passively to the heart via the jugular vein for re-oxygenation by the lungs. Stimulator attached to the sciatic nerve. Gastrocnemius-plantaris-soleus muscle bundle attached to the Grass force transducer.

1. Perfusion pump  
2. Pressure transducers  
3. Femoral Artery, Control  
4. Femoral Artery, Perfused  
5. External Jugular Vein  
6. Common Carotid Artery  
7. Femoral Vein, Perfused  
8. Rodent Ventilator  
9. Stimulator  
10. Force Transducer  

→ Arrow indicate direction of blood flow
2.4 **SURGICAL PREPARATION FOR BLOOD FLOW, VENTILATION AND MUSCLE STIMULATION.**

2.4.1 Ventilation and Blood Flow

2.4.1.1 Ventilation and Systemic Blood Pressure

The animal was shaved across both hindlimbs, the abdominal region and anterior neck. A tracheotomy was performed using a polyethylene tube allowing the animal’s breathing to be maintained by artificial ventilation, thereby ensuring that the level of oxygen in the arterial blood remained high and constant during each experiment (Figure 2.1). The animal was connected to a rodent ventilator (7025, Ugo Basile, Italy) and the stroke volume was set according to animal body weight \((1\text{ml} \cdot 200\text{g}^{-1})\).

A cannula was inserted into the left common carotid artery and connected to a pressure transducer (Argon CDXIII, Maxxim Medical, USA) for direct measurement of systemic blood pressure. All cannulae (Dural Plastics, Internal Diameter 0.58mm Outside Diameter 0.96mm) were fluid filled with isotonic saline containing 6% Dextran 70 (w/v) and Heparin 5000 IU.100ml⁻¹.

2.4.1.2 Arterial blood flow

A cannula was inserted into the right femoral artery (non-perfused leg) towards the heart for direct access to oxygenated arterial blood from the descending aorta. This cannula was connected to peristaltic pump tubing (Gilson, France, 2.50cc/m), passed through a peristaltic roller pump (Miniplus 3, Gilson, France) and connected to a cannula that was inserted into the left femoral artery (perfused leg) towards the foot. The cannula was passed well down the femoral artery towards the knee to close off unwanted side branches and restrict perfusion to the muscle beds of the lower hindlimb. Other side branches were ligated. A T-junction inserted in the blood flow line on the perfused hindlimb side of the pump was connected to a pressure transducer for measurement of hindlimb perfusion pressure. A Windkessel pressure dampener was inserted into the blood flow line to reduce large pulsatile changes in pressure, such as that occurring as the pump was started.
2.4.1.3 Venous blood flow

A fluid filled cannula (Internal Diameter 0.58mm Outside Diameter 0.96mm) was inserted into the left femoral vein towards the foot of the perfused leg with the other end inserted in the right external jugular vein for passive venous return to the heart and lungs for re-oxygenation.

When all cannulae were in place, passive flow was allowed through the pump from the right femoral artery of the non-perfused hindlimb to the left femoral artery of the perfused hindlimb. When all tubes had filled with the animal’s own blood (≈1 minute) the pump was engaged to provide a constant flow rate of 1 ml·min\(^{-1}\) through the hindlimb vasculature. All hindlimbs were then perfused for 30 minutes at 1 ml·min\(^{-1}\) to allow for mean perfusion pressure to reach steady state (≈100mmHg). During these setting up procedures the perfused hindlimb was hypoxic for less than 2 minutes. Previous studies have demonstrated that the catheter diameter has no adverse affect upon venous pressure as measured by foot volume (Peoples 2004).

2.4.1.4 Nerve and muscle isolation

The rat was turned onto its right side and a small incision was made in the skin, 1cm below the iliac crest. The gluteal muscles were separated to expose the sciatic nerve trunk. A bipolar electrode (Grass Instrument Division, USA) was placed under the nerves for direct electrical stimulation of the trunk supplying the muscles of the gastrocnemius-plantaris-soleus muscle bundle. Saline-soaked gauze was placed over the incision to prevent drying of the nerve.

The animal was then turned onto its back and the left leg was secured at the knee and foot to prevent movement during stimulation. The gastrocnemius-plantaris-soleus muscle group tendons were tied with non-stretch silk and connected to a force transducer (FT03C, Grass Instrument Division, USA). Saline-soaked gauze was placed over the muscle tendons to prevent them from drying.

2.4.2 Muscle Stimulation

For muscle contraction protocols, after the initial 30 minutes equilibrium, the peristaltic pump speed was increased to 2 ml·min\(^{-1}\) and stimulation of sciatic nerve of the perfused hindlimb commenced at 5Hz for 3 minutes.
2.5 COLLECTION OF BLOOD PRESSURE AND TWITCH FORCE

Data was referenced to ground and amplified (Onspot Australia). Data acquisition software, Labview for Windows (National Instruments), was used to collect both pressure and twitch force simultaneously during the perfusion protocols at a sampling rate of 200Hz. The data was displayed in real time and stored on computer for later analysis.

2.5.1 Calculation of Oxygen Consumption

Oxygen consumption was assessed according to the Fick equation and adjusted for muscle mass:

\[
\text{Oxygen consumption} = \frac{(a-\bar{v})O_2\text{diff} \times (\text{hindlimb flow rate})}{\text{Weight of perfused muscle}}
\]

Where:

- \(O_2\) consumption expressed in \(\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{gww}^{-1}\).
- \((a-\bar{v})O_2\text{diff}\): the arterial – venous oxygen difference expressed in \(\mu\text{mol} \cdot \text{ml}^{-1}\).
- Hindlimb flow rate: expressed in \(\text{ml} \cdot \text{min}^{-1}\).
- Weight of contracting muscle: expressed in grams wet weight (gww).

Arterial and venous oxygen content (ml.100ml\(^{-1}\)) was calculated using:

\[
\text{Oxygen content} = (1.39 \times \text{Hb} \times \text{RBC saturation (\%)})+(0.003 \times \text{Po}_2)
\]

Where:

- 1.39 = millilitres of oxygen bound to 1 gram haemoglobin.
Hb: blood haemoglobin concentration expressed in g.100ml⁻¹.

RBC saturation (%): percentage of the haemoglobin that is saturated with oxygen calculated using measured Po₂, Pco₂ and pH for blood specific P₅₀.

Po₂: partial pressure of oxygen in whole blood expressed in mmHg

0.003 = constant for dissolved oxygen in plasma at 38°C.

2.5.2 Collection of Blood Samples and Muscle Tissue

2.5.2.1 Blood

Arterial and venous blood was sampled (100µl) from the arterial input and venous output lines of the hindlimb through in-line, thick walled, self-sealing silicone tubing. Measurement of pH, Po₂, Pco₂, haematocrit (Hct), K⁺, and haemoglobin were performed using a blood gas and electrolyte analyser (ABL77 Radiometer, Copenhagen) using 70µl of sampled blood. The remaining sample volume was re-suspended in an equal volume of heparinised isotonic saline (1:1 heparin to saline) and re-injected into the venous side of the perfusion so as to maintain haemoglobin levels above 12.5g.100ml⁻¹ whole blood throughout the whole experiment.

2.5.2.2 Muscle

At the completion of each experiment, muscles from the perfused hindlimb were collected to establish total weight of the muscle involved in the perfusion. Muscles sampled were gastrocnemius, plantaris, soleus, deep posterior tibial, extensor digitorum longus, and tibialis anterior.

2.6 PROTOCOLS

2.6.1 Infusion of Drugs

Agonist drugs were infused in a constant volume (0.2ml) into the arterial perfusion through the self-sealing silicon tubing. Drugs were infused in an increasing molar dose sequence with a 10 minute rest period in between doses to allow for washout (natural clearance or uptake processes) to occur.
2.6.2 Basal Protocol
2.6.2.1 Resting

One arterial and two venous blood samples were taken at the end of the 30 minute equilibrium perfusion to determine resting hindlimb oxygen consumption. To test the effects of the vehicle on hindlimb pressure and oxygen consumption, 0.2ml saline was infused in the arterial perfusion with venous sampling as described below.

2.6.2.2 Arterial

Arterial sampling occurred an average of 3-4 times during the course of an experiment. With the aid of the rodent ventilator, arterial haemoglobin saturation and oxygen content remained consistent throughout the experiment.

2.6.2.3 Venous

During the resting protocols, venous sampling occurred at 2, 3, 4 and 5 minutes after infusion of an agonist. Without continuous recordings of oxygen consumption, it was anticipated that maximal change would be seen in one of the four samples.

2.6.3 Contraction Protocol
2.6.3.1 Contracting muscle

At the end of the 30 minute equilibrium perfusion muscle stimulation commenced as described above (see 2.4.2 muscle stimulation). One arterial and two venous blood samples were taken to determine contracting hindlimb oxygen consumption. To test the effects of the vehicle on hindlimb pressure and oxygen consumption, 0.2ml saline was infused in the arterial perfusion with venous sampling as described below. After 3 minutes, pump speed was returned to $1\text{ml} \cdot \text{min}^{-1}$ and muscle stimulation terminated. A minimum of 10 minutes of resting perfusion was allowed between protocols to reduce the likelihood of fatigue and to allow for drug washout. Figure 2.2 shows a time line for events during this protocol.
2.6.3.2 Venous

The agonist drug was infused 10 seconds after the commencement of the hindlimb stimulation and after achievement of maximum peak tension. Venous sampling occurred 2.5 minutes and 3.5 minutes after infusion of a drug.

![Figure 2.2: Time line of events for the contraction protocol. Arrow represents venous blood sampling (100 µl).](image)

2.7 STATISTICAL ANALYSIS

Results are expressed as mean ± SEM. All statistics were performed using paired Student’s t-test in Microsoft Excel (Microsoft Corporation, USA). A probability value of $P < 0.05$ was considered to be significant. Mean perfusion pressure was determine by the mean of 3 pump cycles.
2.8 **DRUGS AND CHEMICALS**

Saline was made up in stock solution (0.9% NaCl) and used at room temperature. Dextran 70 (Sigma-Aldrich Diagnostics, Sydney) was dissolved in the prepared saline (6% by weight) at room temperature 24 hours prior to use. Heparin was dissolved fresh daily (5000IU·100ml⁻¹) to solution.

All drugs were prepared at a 10mM stock with isotonic saline and 0.1% ascorbic acid (Sigma-Aldrich Diagnostics, Sydney), and stored at −18°C in 1ml eppendorf tubes. Drugs were prepared fresh before each protocol using defrosted stock and isotonic saline.

Drugs used were (-+)-Arterenol Hydrochloride, 5-Hydroxytryptamine Hydrochloride, (+-)-Isoproterenol Hydrochloride, Adenosine Hemisulphate, Nifedipine, Bradykinin Acetate, Hydralazine Hydrochloride, Sodium Nitroprusside Dihydrate, Histamine Diphosphate, and Acetylcholine Chloride. These were all supplied by Sigma-Aldrich, Sydney.
CHAPTER THREE

Results
3.1 MODEL AND VEHICLE

3.1.1 Model

3.1.1.1 Blood Profile

The autoperfused rat hindlimb was developed as a model that could provide oxygen delivery within an in vivo range at in vivo vascular resistance in both resting and contracting muscle for the study of skeletal muscle function and metabolism. Arterial blood pH (7.3 to 7.5) and haemoglobin (14 to 16 g·100ml⁻¹) were maintained within the normal range both under basal conditions and during muscle contraction (Table 3.1) (Spector 1956; Altman and Dittmer 1974). Arterial blood oxygen tension (Po₂) (90 to 100mmHg) and arterial blood carbon dioxide tension (Pco₂) (35 to 45mmHg) were also maintained in the normal range. Venous Po₂ was significantly lower than arterial Po₂ both during contraction and at rest. Venous Po₂ (40 to 50mmHg) and venous Pco₂ (45 to 55mmHg) were also within the normal range (Spector 1956; Tuttle and Schottelius 1969; Altman and Dittmer 1974), as was arterial oxygen saturation of haemoglobin (So₂) (92.5-98.5%) and venous So₂ (65 to 75%) (Spector 1956; Marshall et al. 1962; Altman and Dittmer 1974). The haematocrit and Po₂ maintaining high haemoglobin O₂ saturation ensuring that arterial oxygen content ([O₂_v]a) was also maintained within the normal range of 18.8 to 23 ml·100ml⁻¹ as was the venous Q₉ content ([O₂_v]v,15.9 to 17.5 ml·100ml⁻¹) (Spector 1956; Altman and Dittmer 1974; Pepe and McLennan 1993). The resulting basal (a-‾v)O₂diff was 4.98 ± 0.41 ml·100ml⁻¹ (n = 75) whilst the contraction (a-‾v)O₂diff was 3.99 ± 1.00 ml·100ml⁻¹ (n = 20).
Table 3.1: Blood profile of experimental animals for both arterial and venous blood circulated to and from the perfused hindlimb.

<table>
<thead>
<tr>
<th></th>
<th>Arterial Basal</th>
<th>Arterial Contraction</th>
<th>Venous Basal</th>
<th>Venous Contraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>55</td>
<td>10</td>
<td>75</td>
<td>20</td>
</tr>
<tr>
<td>pH</td>
<td>7.37 ± 0.01</td>
<td>7.39 ± 0.01</td>
<td>7.27 ± 0.01 *</td>
<td>7.29 ± 0.01 *</td>
</tr>
<tr>
<td>Pco₂ (mmHg)</td>
<td>34.7 ± 0.8</td>
<td>34.4 ± 1.1</td>
<td>51.4 ± 1.1 *</td>
<td>50.6 ± 1.5 *</td>
</tr>
<tr>
<td>Po₂ (mmHg)</td>
<td>102.1 ± 1.6</td>
<td>88.7 ± 5.2</td>
<td>46.3 ± 1.5 *</td>
<td>45.4 ± 2.5 *</td>
</tr>
<tr>
<td>Hct (%)</td>
<td>45.4 ± 0.5</td>
<td>47.5 ± 1.3</td>
<td>47.1 ± 0.4</td>
<td>49.8 ± 0.9</td>
</tr>
<tr>
<td>Hb (g·100ml⁻¹)</td>
<td>14.8 ± 0.2</td>
<td>15.5 ± 0.4</td>
<td>15.4 ± 0.1</td>
<td>16.3 ± 0.3</td>
</tr>
<tr>
<td>So₂ (%)</td>
<td>97.6 ± 0.1</td>
<td>95.9 ± 1.1</td>
<td>71.2 ± 1.8 *</td>
<td>71.7 ± 3.7 *</td>
</tr>
<tr>
<td>[ollow o₂]   (ml·100ml⁻¹)</td>
<td>20.4 ± 0.2</td>
<td>20.8 ± 0.4</td>
<td>15.4 ± 0.4</td>
<td>16.3 ± 0.7</td>
</tr>
<tr>
<td>K⁺ (mmol·l⁻¹)</td>
<td>3.7 ± 0.1</td>
<td>3.8 ± 0.1</td>
<td>3.3 ± 0.1</td>
<td>3.7 ± 0.1</td>
</tr>
</tbody>
</table>

Mean ± SEM Significant from arterial represented by * (P < 0.05)

3.1.1.2 Perfusion Pressure

The autoperfused hindlimb had no significant effect on mean systemic blood pressure during both protocols. The mean systemic blood pressure at a hindlimb perfusion rate of 1 ml·min⁻¹ for the basal protocol was 85 ± 2 mmHg (n = 44) and when the flow rate was increased to 2 ml·min⁻¹ during the contraction protocol, mean systemic blood pressure remained stable at 79 ± 6 mmHg (n = 8) (Figure 3.1). At a basal flow rate of 1 ml·min⁻¹ the mean hindlimb perfusion pressure was 92 ± 3 mmHg which was increased to 166 ± 6 mmHg when the flow rate was increased to 2 ml·min⁻¹ for the contraction protocol.
3.1.1.3 Oxygen Consumption

The mean basal oxygen consumption ($\dot{V}\text{O}_2$) was $0.33 \pm 0.02 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{gww}^{-1}$ ($n = 74$) (Figure 3.2). Mean $\dot{V}\text{O}_2$ was significantly reduced to $0.19 \pm 0.03 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{gww}^{-1}$ ($n = 20$) when flow rate increased to 2 ml min$^{-1}$ without contraction. However, this was significantly increased to $0.57 \pm 0.08 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{gww}^{-1}$ ($n = 20$) during the contraction protocol (2 ml min$^{-1}$ with hindlimb stimulation).

Figure 3.2: The effect of flow and hindlimb muscle contraction upon oxygen consumption in the autoperfused hindlimb. Significance from basal at 1 ml min$^{-1}$ flow rate represented by * ($P < 0.05$).
3.1.1.4 Force of Contraction

The maximal rate of force development (+dT/dt$_{\text{max}}$) and maximal rate of relaxation (-dT/dt$_{\text{max}}$) was recorded to observe any changes after agonist infusion that would limit substrate delivery through blood flow redistribution within the muscle during the contraction protocol. The mean force production was 0.15 ± 0.01N (14.8 ± 2.1g) and a +dT/dt$_{\text{max}}$ of 10.2 ± 1.3N·s$^{-1}$ and a -dT/dt$_{\text{max}}$ of -9.4 ± 1.4 N·s$^{-1}$ (n = 8). The mean peak force production (T$_{\text{max}}$) was 0.24 ± 0.02N (24.2 ± 3.4g, n = 8). Figure 3.3 shows an example trace of the hindlimb force developed during the contraction protocol.

Figure 3.3: An example trace of a single twitch during the contraction protocol. Sample rate was 200Hz. +dT/dt$_{\text{max}}$: maximal rate of force development; -dT/dt$_{\text{max}}$: maximal rate of relaxation; T$_{\text{max}}$: mean peak force production

3.1.2 Vehicle

3.1.2.1 Perfusion Pressure

Basal mean pre-infusion hindlimb perfusion pressure was 98 ± 9mmHg (1 ml·min$^{-1}$) and contraction was 168 ± 9mmHg (2 ml·min$^{-1}$). Hindlimb perfusion pressure was significantly decreased following the infusion of the saline vehicle under basal (1 ml·min$^{-1}$ hindlimb flow, see Figure 3.5) or contraction (2 ml·min$^{-1}$, see Figure 3.7) flow rates. The effect was short lived and by the time of peak drug effect (90s, see Figure 3.4 and 3.6) had returned to baseline (Figure 3.4). There was no significant effect on mean systemic blood pressure after the infusion of the vehicle.
Figure 3.4: Summary of the maximal effect and the effect 90 seconds post-infusion of the vehicle saline on mean hindlimb perfusion pressure during basal (filled bars, n = 6) and contraction (clear bars, n = 8) protocols. Significance from pre-infusion perfusion pressure represented by * (P < 0.05).

Figure 3.5: An example trace of hindlimb perfusion pressure and systemic blood pressure after saline infusion. The black arrow indicates the saline effect.
3.1.2.2 Oxygen Consumption

Prior to vehicle or agonist infusion the mean basal hindlimb $\dot{V}O_2$ was $0.50 \pm 0.06 \mu mol \cdot min^{-1} \cdot gww^{-1}$ ($n = 6$), whilst mean contraction hindlimb $\dot{V}O_2$ was $0.49 \pm 0.09 \mu mol \cdot min^{-1} \cdot gww^{-1}$ ($n = 17$). Basal $\dot{V}O_2$ was not significantly affected following infusion of saline vehicle, although $\dot{V}O_2$ was significantly decreased during the contraction protocol (Figure 3.6).

At all perfusion rates the effect of the vehicle (saline) on all parameters was nullified by recording the effects of the drug after the effect of the vehicle had been completed (see Figures 3.8, 3.9, 3.10 and 3.11).

**Figure 3.6:** Summary of the effect of the vehicle saline on oxygen consumption during basal (filled bars, $n = 6$) and contraction (clear bars, $n = 17$) protocols. Significance from pre-infusion oxygen consumption represented by * ($P < 0.05$).
**Figure 3.7:** An example trace of hindlimb twitch; hindlimb perfusion pressure and systemic blood pressure during saline infusion. The shaded section indicates when the pump speed was $2 \text{ ml} \cdot \text{min}^{-1}$. The black arrow indicates the saline effect.
The following figures (3.8, 3.9, 3.10, and 3.11) show typical basal and contraction pressure recordings of both systemic blood pressure and hindlimb perfusion pressure during agonist infusion. The injection artefact of the saline vehicle and the measurement of mean perfusion pressure are shown.

**Figure 3.8:** An example trace of hindlimb perfusion and systemic blood pressure during Noradrenaline infusion. The white arrow indicates the time when the drug effect was sampled.
Figure 3.9: An example trace of hindlimb twitch; hindlimb perfusion pressure and systemic blood pressure during Serotonin infusion. The shaded section indicates when the pump speed was 2 ml · min⁻¹. The black arrow indicates the saline effect and the white arrow indicates the time when the drug effect was sampled.
Figure 3.10: An example trace of hindlimb perfusion pressure and systemic blood pressure during Histamine infusion at rest. The black arrow indicates the saline effect and the white arrow indicates the time when the drug effect was sampled.

It is important to note that the vasoconstrictor-induced increase in mean hindlimb perfusion pressure (Figure 3.8 and 3.9) could possibly have been greater without the injection artefact of the saline vehicle. There was no significant effect by the infusion of the agonists or vehicle alone on systemic blood pressure.
Figure 3.11: An example trace of hindlimb twitch; hindlimb perfusion pressure and systemic blood pressure during Isoprenaline infusion. The shaded section indicates when the pump speed was $2 \text{ ml} \cdot \text{min}^{-1}$. The black arrow indicates the saline effect and the white arrow indicates the time when the drug effect was sampled.
3.2 VASOCONSTRICTORS

3.2.1 Basal Protocol
3.2.1.1 Perfusion Pressure

The Type A (noradrenaline, NAd) and Type B (serotonin, 5-HT) vasoconstrictors were studied in this model, in resting and contracting hindlimb. Under resting conditions, there was a threshold observed with noradrenaline, with dose-dependent effects seen above 16 µM (Figure 3.12). Mean pre-infusion hindlimb perfusion pressure for the low dose noradrenaline (100nM – 1.6 µM) was 80 ± 5 mmHg (n = 3) and for the high dose noradrenaline (1 µM – 256 µM) was 87 ± 2 mmHg (n = 6). Low dose noradrenaline and two high dose noradrenaline doses (1 µM and 4 µM) had no significant effect on mean hindlimb perfusion pressure.

There was a dose-dependent increase in mean hindlimb perfusion pressure after serotonin administration (Figure 3.13). Mean pre-infusion hindlimb perfusion pressure for serotonin was 80 ± 2 mmHg (n = 6) and was significantly increased for all doses screened. There was a linear relationship between serotonin and perfusion pressure until 50 µM after which the vasoconstrictor effect on perfusion pressure was reduced. 50 µM serotonin maximally increased mean hindlimb perfusion pressure by 171 ± 21 mmHg whilst at 100 µM serotonin increased perfusion pressure was 3% less than 50 µM during basal conditions.
3.2.1.2 Oxygen Consumption

Changes in hindlimb oxygen consumption are thought to be associated with redistribution of hindlimb muscle blood flow. The mean pre-infusion $\dot{V}O_2$ for low dose noradrenaline was $0.47 \pm 0.04 \mu$mol$\cdot$min$^{-1} \cdot$gww$^{-1}$ ($n = 3$) and high dose noradrenaline was $0.48 \pm 0.06 \mu$mol$\cdot$min$^{-1} \cdot$gww$^{-1}$ ($n = 6$). There was no significant effect observed for all doses of noradrenaline on hindlimb $\dot{V}O_2$ during the basal protocol (Figure 3.12).
After serotonin infusion, there was a significant dose-dependent effect on hindlimb oxygen consumption (Figure 3.13). The mean pre-infusion hindlimb $\dot{V}O_2$ for serotonin was $0.57 \pm 0.06\mu mol\cdot min^{-1}\cdot gww^{-1}$ ($n = 6$). Serotonin significantly decreased hindlimb $\dot{V}O_2$ for all doses screened (12.5 – 100$\mu$M) but the magnitude of the effect did not increase with increasing dose. Maximal effect of serotonin on hindlimb $\dot{V}O_2$ was to reduce it by $0.40 \pm 0.09\mu mol\cdot min^{-1}\cdot gww^{-1}$ at 25$\mu$M, but at 100$\mu$M $\dot{V}O_2$ was reduced by $0.35\pm 0.08\mu mol\cdot min^{-1}\cdot gww^{-1}$.

Figure 3.13: The effect of Serotonin on mean hindlimb perfusion pressure and oxygen consumption during basal (●) and contraction (○) protocols. Significance from pre-infusion represented by * ($P < 0.05$). $n = 6$. 
3.2.2 Contraction Protocol

3.2.2.1 Perfusion Pressure

Mean pre-infusion hindlimb perfusion pressure during muscle contraction for low dose noradrenaline was 150 ± 5mmHg (n = 6) and for high dose noradrenaline was 202 ± 2mmHg (n = 3). After noradrenaline infusion during the contraction protocol, a threshold was observed similar to the basal threshold on mean hindlimb perfusion pressure (Figure 3.12). Low dose noradrenaline had no significant effect, except for 1.6µM, whilst high dose noradrenaline significantly increased hindlimb perfusion pressure at 16µM and 64µM only.

Serotonin infusion significantly increased perfusion pressure for all doses with a similar dose-dependent trend as seen during the basal protocol (Figure 3.13). The mean pre-infusion hindlimb perfusion pressure for serotonin was 148± 4mmHg (n = 6). Serotonin maximally increased perfusion pressure by 163 ± 15mmHg at 50µM with higher doses producing a slightly diminished effect upon hindlimb perfusion pressure.

3.2.2.2 Oxygen Consumption

Low dose noradrenaline infusion had no significant effect on $\dot{V}O_2$ during the contraction protocol whilst high dose noradrenaline significantly decreased $\dot{V}O_2$ (Figure 3.12). Mean pre-infusion $\dot{V}O_2$ for low dose noradrenaline was 0.52 ± 0.07µmol·min⁻¹·gww⁻¹ (n = 6) and for high dose noradrenaline was 1.14 ± 0.38µmol·min⁻¹·gww⁻¹ (n = 3), which was 3-fold higher than the mean contracting $\dot{V}O_2$ of 0.57 ± 0.08µmol·min⁻¹·gww⁻¹ (n = 20).

Serotonin significantly decreased $\dot{V}O_2$ in a dose-dependent manner during the contraction protocol with a similar trend to that observed during the basal protocol (Figure 3.13). Pre-infusion hindlimb $\dot{V}O_2$ for serotonin was 0.59 ± 0.11µmol·min⁻¹·gww⁻¹ (n = 6). The maximal change observed in hindlimb $\dot{V}O_2$ was 0.30 ± 0.11µmol·min⁻¹·gww⁻¹ (46%) at 25µM (n = 6). This was the only dose of serotonin to have a significant effect on hindlimb $\dot{V}O_2$ during the contraction protocol. During the basal protocol, 25µM serotonin induced a 67% decrease in $\dot{V}O_2$ compared to only a 46% decrease during the contraction protocol (Figure 3.14). During
contraction 100 μM 5-HT produced a 64% ($P = 0.054$) reduction in $\dot{V}o_2$ from $\dot{V}o_2$ at rest (Figure 3.15).

**Figure 3.14:** The percentage difference of the effect of Serotonin on oxygen consumption during basal (filled) and contraction (clear). Significance from pre-infusion oxygen consumption represented by * ($P < 0.05$). $n = 6$

**Figure 3.15:** The percentage difference between contraction and basal protocols during Serotonin infusion on oxygen consumption. $n = 6$
3.2.2.3 Force of Contraction

Skeletal muscle contraction could be diminished if vasoconstrictor infusion limited substrate delivery through blood flow redistribution within the muscle. No significant effects were observed on either +dT/dt\text{max} or -dT/dt\text{max} after either low dose or high dose noradrenaline infusion (Figure 3.16). Mean pre-infusion +dT/dt\text{max} was $13.3 \pm 0.7 \text{N} \cdot \text{s}^{-1}$ for low dose noradrenaline ($n = 6$) and $9.6 \pm 0.6 \text{N} \cdot \text{s}^{-1}$ for high dose noradrenaline ($n = 6$). Low dose noradrenaline had a mean pre-infusion -dT/dt\text{max} of $-8.0 \pm 0.5 \text{N} \cdot \text{s}^{-1}$ and high dose noradrenaline was $-7.4 \pm 0.5 \text{N} \cdot \text{s}^{-1}$.

**Figure 3.16:** The effect of Noradrenaline on maximal rate of contraction (low dose: $\square$, $n = 3$; high dose: $\triangle$, $n = 6$) and relaxation (low dose: ■; high dose: ▲) during muscle contraction. The dashed line indicates pre-infusion maximal rate of contraction and relaxation. Significance from pre-infusion represented by * ($P < 0.05$).

Serotonin infusion had no significant effect on either +dT/dt\text{max} or -dT/dt\text{max} in the autoperfused hindlimb (Figure 3.17). Mean pre-infusion +dT/dt\text{max} was $12.4 \pm 1.8 \text{N} \cdot \text{s}^{-1}$ with a mean pre-infusion -dT/dt\text{max} of $-8.7 \pm 0.8 \text{N} \cdot \text{s}^{-1}$ ($n = 4$). The maximal effect of serotonin was observed at $50\mu\text{M}$, which resulted in +dT/dt\text{max} increasing to $22.3 \pm 3.3 \text{N} \cdot \text{s}^{-1}$ and -dT/dt\text{max} to $-18.9 \pm 4.1 \text{N} \cdot \text{s}^{-1}$.
Figure 3.17: The effect of Serotonin on maximal rate of contraction (□) and relaxation (■) during muscle contraction. The dashed line indicates pre-infusion maximal rate of contraction and relaxation. Significance from pre-infusion represented by * (P < 0.05). n = 4

Generally noradrenaline had a dose-dependent threshold of 16µM on perfusion pressure during both basal and contraction protocols (Figure 3.18). Noradrenaline had no significant effect on oxygen consumption during basal conditions whilst hindlimb oxygen consumption decreased during hindlimb stimulation (Figure 3.19). The pre-infused oxygen consumption during the contraction protocol was two times greater than the mean contraction hindlimb oxygen consumption (Table 3.2).

Table 3.2: Summary of the greatest effects of vasoconstrictors on perfusion pressure and oxygen consumption in the autoperfused rat hindlimb.

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Protocol</th>
<th>n</th>
<th>Perfusion Pressure (mmHg)</th>
<th>$\dot{V}O_2$ (µmol·min$^{-1}$·gww$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L.D Noradrenaline</td>
<td>Basal</td>
<td>3</td>
<td>$\downarrow$ 4 ± 2</td>
<td>$\downarrow$ 0.13 ± 0.06</td>
</tr>
<tr>
<td>H.D. Noradrenaline</td>
<td>Basal</td>
<td>6</td>
<td>$\uparrow$ 216 ± 6</td>
<td>$\downarrow$ 0.23 ± 0.17</td>
</tr>
<tr>
<td>Serotonin</td>
<td>Basal</td>
<td>6</td>
<td>$\uparrow$ 171 ± 21</td>
<td>$\downarrow$ 0.40 ± 0.09</td>
</tr>
<tr>
<td>L.D Noradrenaline</td>
<td>Contraction</td>
<td>6</td>
<td>$\uparrow$ 14 ± 6</td>
<td>$\uparrow$ 0.33 ± 0.18</td>
</tr>
<tr>
<td>H.D. Noradrenaline</td>
<td>Contraction</td>
<td>3</td>
<td>$\uparrow$ 198 ± 15</td>
<td>$\ast \downarrow$ 1.13 ± 0.47</td>
</tr>
<tr>
<td>Serotonin</td>
<td>Contraction</td>
<td>6</td>
<td>$\uparrow$ 163 ± 15</td>
<td>$\downarrow$ 0.30 ± 0.11</td>
</tr>
</tbody>
</table>

Mean ± SEM. $\uparrow \downarrow$ denotes trend. Significance from pre-infusion represented by $\uparrow \downarrow \ast$ (P < 0.05). L.D. represents low dose; H.D. represents high dose.
Serotonin increased perfusion pressure significantly during both basal and contraction protocols in a similar manner (Figure 3.18). It was found that serotonin reduces oxygen consumption in both resting and contracting skeletal muscle, however muscle contraction diminished this effect (Figure 3.19). With higher doses of serotonin there was a diminished effect upon both perfusion pressure and oxygen consumption.

**Figure 3.18:** Summary of the maximal effect of low dose and high dose Noradrenaline and Serotonin on mean hindlimb perfusion pressure during basal (filled bars) and contraction (clear bars) protocols. Significance from pre-infusion perfusion pressure represented by * ($P < 0.05$).

**Figure 3.19:** Summary of the maximal effect of low dose and high dose Noradrenaline and Serotonin on oxygen consumption during basal (filled bars) and contraction (clear bars) protocols. Significance from pre-infusion oxygen consumption represented by * ($P < 0.05$).
3.3 **VASODILATORS**

3.3.1 **Basal Protocol**

3.3.1.1 **Perfusion Pressure**

After the initial screening of the eight vasodilators (n = 3) to identify a vasodilator that decreases basal oxygen consumption in similar fashion serotonin, isoprenaline and histamine were chosen for further analysis (n = 6). Mean pre-infusion hindlimb perfusion pressure was 89 ± 3mmHg (n = 6). There was a significant dose-dependent decrease of mean hindlimb perfusion pressure after isoprenaline infusion (Figure 3.20). Isoprenaline significantly decreased hindlimb perfusion pressure at all doses (100nM – 100µM) with a maximal decrease of 31 ± 2mmHg observed at the highest dose.

*Figure 3.20:* The effect of Isoprenaline on mean perfusion pressure and oxygen consumption during basal (●) and contraction (○) protocols. Significance from pre-infusion represented by * (P < 0.05). n = 6
Histamine infusion during basal conditions significantly decreased mean hindlimb perfusion pressure with increasing dose (Figure 3.21). The pre-infusion hindlimb perfusion pressure was $78 \pm 3$mmHg ($n = 6$). After infusion of $100\mu$M histamine, mean hindlimb perfusion pressure was significantly decreased by $7 \pm 3$mmHg (9%) whilst at the maximal dose of $1$mM maximal decrease in perfusion pressure of $22 \pm 2$mmHg (25%) was observed.

![Figure 3.21: The effect of Histamine on mean perfusion pressure and oxygen consumption during basal (●) and contraction (○) protocols. Significance from pre-infusion represented by * ($P < 0.05$). $n = 6$](image)

Acetylcholine had no significant effect on mean hindlimb perfusion pressure for doses between $100\text{nM} - 1\text{mM}$ that were screened (Figure 3.22). Pre-infusion mean hindlimb perfusion pressure for animals infused with acetylcholine was $81 \pm 1$mmHg ($n = 3$). There was a dose-dependent decrease in mean hindlimb perfusion pressure during nitroprusside administration (Figure 3.22). Resting mean hindlimb perfusion pressure was $123 \pm 1$mmHg ($n = 3$) and was significantly decreased by 36% after $100\mu$M infusion and maximally by 49% after $1$mM infusion. Hydralazine had no significant effect on mean hindlimb perfusion pressure for all doses screened.
between 100nM – 1mM (Figure 3.22). Pre-infusion mean hindlimb perfusion pressure
was 105 ± 3mmHg (n = 3).

Figure 3.22: The effect of Acetylcholine (■), Nitroprusside (▲) and Hydralazine (▼) on mean perfusion
pressure and oxygen consumption during basal protocol. Significance from pre-infusion represented by * (P < 0.05). n = 3

There was a dose-dependent decrease in mean hindlimb perfusion pressure
after bradykinin infusion (Figure 3.23). Pre-infusion hindlimb perfusion pressure was
89 ± 10mmHg for bradykinin-screened animals (n = 3). After 10µM bradykinin
infusion, mean hindlimb perfusion pressure was significantly decreased by 9%.
However, 100µM maximally decreased perfusion pressure by 15% (P = 0.05). There
was no significant effect observed after nifedipine administration on mean hindlimb
perfusion pressure (Figure 3.23) with a pre-infusion perfusion pressure of 108 ± 29mmHg (n = 3). Adenosine had no significant effect on mean hindlimb perfusion
pressure (Figure 3.23). Adenosine mean pre-infusion hindlimb perfusion pressure
was 105 ± 9mmHg (n = 3) which was maximally decreased after 10µM infusion by
16%. After 1mM adenosine infusion, mean hindlimb perfusion pressure was
increased by 8% (n = 3).
Figure 3.23: The effect of Bradykinin (■), Nifedipine (▲) and Adenosine (▼) on mean perfusion pressure and oxygen consumption during basal protocol. Significance from pre-infusion represented by * (P < 0.05). n = 3.

3.3.1.2 Oxygen Consumption

Isoprenaline had a positive dose-dependent relationship with hindlimb oxygen consumption during the basal protocol (Figure 3.20). Mean pre-infusion $\bar{V}O_2$ was 0.40 ± $0.10 \mu$mol·min⁻¹·gww⁻¹ ($n = 6$) which was significantly increased after 100nM infusion.

There was no significant effect on basal hindlimb oxygen consumption after histamine infusion (Figure 3.21). Mean pre-infusion $\bar{V}O_2$ was 0.34 ± $0.06 \mu$mol·min⁻¹·gww⁻¹ ($n = 6$).

Mean pre-infusion $\bar{V}O_2$ for acetylcholine was $0.32 \pm 0.11 \mu$mol·min⁻¹·gww⁻¹ ($n = 3$) and was maximally increased after 1mM acetylcholine infusion (Figure 3.22). Nitroprusside had a positive dose-dependent response on hindlimb oxygen consumption (Figure 3.22). Mean pre-infusion $\bar{V}O_2$ for nitroprusside was $0.22 \pm 0.03 \mu$mol·min⁻¹·gww⁻¹ ($n = 3$) which was significantly increased after 1μM (67%)
and 10 µM (78%) infusion. Hydralazine had a positive relationship with oxygen consumption during the basal protocol (Figure 3.22). Hydralazine had a mean pre-infusion $\dot{V}O_2$ of $0.33 \pm 0.07 \mu mol \cdot min^{-1} \cdot gww^{-1}$ (n = 3) which was significantly increased after 10 µM infusion.

Mean pre-infusion hindlimb $\dot{V}O_2$ was $0.47 \pm 0.07 \mu mol \cdot min^{-1} \cdot gww^{-1}$ (n = 3) which was significantly increased after 1 µM (20%) and 100 µM (28%) bradykinin infusion (Figure 3.23). Nifedipine significantly increased mean hindlimb $\dot{V}O_2$ during the basal protocol (Figure 3.23). Mean pre-infusion hindlimb $\dot{V}O_2$ was $0.38 \pm 0.09 \mu mol \cdot min^{-1} \cdot gww^{-1}$ (n = 3) which was maximally increased by 35% ($P = 0.08$) after 100nM administration, however 1 µM significantly increased $\dot{V}O_2$ by 27%. Adenosine had a basal $\dot{V}O_2$ of $0.23 \pm 0.04 \mu mol \cdot min^{-1} \cdot gww^{-1}$ (n = 3) and 100nM adenosine significantly increased $\dot{V}O_2$ by 75% whilst 1 µM significantly increased by 69% (Figure 3.23).

Table 3.3: Summary of the greatest effect of vasodilators screened on mean hindlimb perfusion pressure and oxygen consumption during the basal protocol.

<table>
<thead>
<tr>
<th>Drugs</th>
<th>n</th>
<th>Perfusion Pressure (mmHg)</th>
<th>$\dot{V}O_2$ ($\mu mol \cdot min^{-1} \cdot gww^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetylcholine</td>
<td>3</td>
<td>↓ 18 ± 7</td>
<td>$\uparrow 0.19 \pm 0.14$</td>
</tr>
<tr>
<td>Nitroprusside</td>
<td>3</td>
<td>*↓ 61 ± 3</td>
<td>*$\uparrow 0.16 \pm 0.02$</td>
</tr>
<tr>
<td>Hydralazine</td>
<td>3</td>
<td>↓ 11 ± 6</td>
<td>*$\uparrow 0.21 \pm 0.03$</td>
</tr>
<tr>
<td>Bradykinin</td>
<td>3</td>
<td>↓ 13 ± 2</td>
<td>$\uparrow 0.13 \pm 0.06$</td>
</tr>
<tr>
<td>Nifedipine</td>
<td>3</td>
<td>↓ 8 ± 6</td>
<td>$\uparrow 0.12 \pm 0.04$</td>
</tr>
<tr>
<td>Adenosine</td>
<td>3</td>
<td>↓ 17 ± 8</td>
<td>$\uparrow 0.18 \pm 0.07$</td>
</tr>
</tbody>
</table>

Mean ± SEM. $\uparrow \downarrow$ denotes trend, Significance from pre-infusion represented by $*^{\uparrow \downarrow}$ ($P < 0.05$).

The screening of eight vasodilators with a variety of mechanism in the autoperfused rat hindlimb resulted in the inability to identify a vasodilator that decreases hindlimb oxygen consumption in a similar fashion to a Type B vasoconstrictor. Table 3.3 shows the summary of the maximal effect on perfusion pressure (Figure 3.24) and oxygen consumption (Figure 3.25) after the administration of the six vasodilators screened.
**Figure 3.24:** Summary of the maximal effect of Acetylcholine (ACh), Sodium Nitroprusside (NP), Hydralazine (HDZ), Bradykinin (BKN), Nifedipine (NFP) and Adenosine (ADN) on mean hindlimb perfusion pressure. Significance from pre-infusion hindlimb perfusion pressure represented by * ($P < 0.05$).

**Figure 3.25:** Summary of the maximal effect of Acetylcholine (ACh), Sodium Nitroprusside (NP), Hydralazine (HDZ), Bradykinin (BKN), Nifedipine (NFP) and Adenosine (ADN) on oxygen consumption. Significance from pre-infusion oxygen consumption represented by * ($P < 0.05$).
3.3.2 Contraction Protocol

3.3.2.1 Perfusion Pressure

Isoprenaline and histamine were chosen from the eight vasodilators screened to observe their effects during the contraction protocol (2 ml·min\(^{-1}\) flow rate). There was a dose-dependent reduction in mean hindlimb perfusion pressure with isoprenaline administration (Figure 3.20). Mean pre-infusion perfusion pressure was 180 ± 9mmHg (n = 6). During the contraction protocol, isoprenaline infusion significantly decreased mean hindlimb perfusion pressure after the infusion of 1 µM to 100µM.

Histamine infusion during the contraction protocol resulted in a dose-dependent reduction in mean hindlimb perfusion pressure for all doses (Figure 3.21). Pre-infusion perfusion pressure was 182 ± 4mmHg (n = 6). 10µM histamine administration resulted in a reduction of perfusion pressure by 5% whilst 100µM infusion maximally reduced perfusion pressure by 9%.

3.3.2.2 Oxygen Consumption

Isoprenaline infusion resulted in a significant dose-dependent increase in hindlimb \(\dot{V}O_2\) during the contraction protocol (Figure 3.20). Mean \(\dot{V}O_2\) prior to isoprenaline infusion was 0.39± 0.08\(\mu\)mol·min\(^{-1}\)·gww\(^{-1}\) (n = 6) which was maximally increased by 0.59± 0.13\(\mu\)mol·min\(^{-1}\)·gww\(^{-1}\) after 100µM isoprenaline infusion.

Histamine administration significantly increased mean hindlimb oxygen consumption during the contraction protocol in a dose-dependent manner (Figure 3.21). Mean pre-infusion \(\dot{V}O_2\) was 0.43 ± 0.06\(\mu\)mol·min\(^{-1}\)·gww\(^{-1}\) (n = 6) where 100µM histamine infusion resulted in a maximal increase in hindlimb \(\dot{V}O_2\).
3.3.2.3 Force of Contraction

There was no significant effect on either $+\frac{dT}{dt_{\text{max}}}$ or $-\frac{dT}{dt}$ after isoprenaline infusion (Figure 3.26). Mean pre-infusion $+\frac{dT}{dt_{\text{max}}}$ was $9.46 \pm 0.57 \text{N} \cdot \text{s}^{-1}$ and $-\frac{dT}{dt_{\text{max}}}$ was $-7.38 \pm 0.52 \text{N} \cdot \text{s}^{-1}$ ($n = 6$).

Figure 3.26: The effect of Isoprenaline on maximal rate of contraction (■) and relaxation (□) during muscle contraction. The dashed line indicates pre-infusion maximal rate of contraction and relaxation. Significance from pre-infusion represented by * ($P < 0.05$). $n = 6$

Histamine infusion had no significant effect on the $+\frac{dT}{dt_{\text{max}}}$ but did significantly increase $-\frac{dT}{dt_{\text{max}}}$ for all doses except for 10nM (Figure 3.27). Mean pre-infusion $+\frac{dT}{dt_{\text{max}}}$ was $11.42 \pm 0.67 \text{N} \cdot \text{s}^{-1}$ whilst $-\frac{dT}{dt_{\text{max}}}$ was $-9.75 \pm 0.69 \text{N} \cdot \text{s}^{-1}$ ($n = 6$). 10nM histamine maximally increased $+\frac{dT}{dt_{\text{max}}}$ to $14.52 \pm 1.70 \text{N} \cdot \text{s}^{-1}$ and decreased $-\frac{dT}{dt_{\text{max}}}$ to $-15.32 \pm 1.31 \text{N} \cdot \text{s}^{-1}$ ($n = 6, P = 0.056$). After 100nM infusion, $-\frac{dT}{dt_{\text{max}}}$ was significantly decreased to $-14.99 \pm 2.32 \text{N} \cdot \text{s}^{-1}$. 
Figure 3.27: The effect of Histamine infusion on maximal rate of contraction (■) and relaxation (□) during muscle contraction. The dashed line indicates pre-infusion maximal rate of contraction and relaxation. Significance from pre-infusion represented by * (P < 0.05). n = 6

Isoprenaline and histamine significantly decreased mean hindlimb perfusion pressure during both basal and contraction protocols (Figure 3.28). Both vasodilators increased mean hindlimb oxygen consumption during both protocols however the greatest effects were observed during the contraction protocol (Figure 3.29).

Table 3.4: Summary of the greatest effect of Isoprenaline and Histamine on mean hindlimb perfusion pressure and oxygen consumption during basal and contraction protocols.

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Protocol</th>
<th>n</th>
<th>Perfusion Pressure (mmHg)</th>
<th>( \dot{V}O_2 ) (( \mu mol \cdot min^{-1} \cdot gww^{-1} ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoprenaline</td>
<td>Basal</td>
<td>6</td>
<td>*↓ 31 ± 2</td>
<td>*↑ 0.19 ± 0.06</td>
</tr>
<tr>
<td>Histamine</td>
<td>Basal</td>
<td>6</td>
<td>*↓ 22 ± 2</td>
<td>*↑ 0.19 ± 0.09</td>
</tr>
<tr>
<td>Isoprenaline</td>
<td>Contraction</td>
<td>6</td>
<td>*↓ 42 ± 7</td>
<td>*↑ 0.59 ± 0.13</td>
</tr>
<tr>
<td>Histamine</td>
<td>Contraction</td>
<td>6</td>
<td>*↓ 18 ± 5</td>
<td>*↑ 0.35 ± 0.13</td>
</tr>
</tbody>
</table>

Mean ± SEM. ↑ ↓ denotes trend, Significance from pre-infusion represented by *↑ *↓ (P < 0.05).
Figure 3.28: Summary of the maximal effect of Isoprenaline and Histamine on mean hindlimb perfusion pressure during basal (filled bars) and contraction (clear bars) protocols. Significance from pre-infusion hindlimb perfusion pressure represented by * ($P < 0.05$).

Figure 3.29: Summary of the maximal effect of Isoprenaline and Histamine on oxygen consumption during basal (filled bars) and contraction (clear bars) protocol. Significance from pre-infusion oxygen consumption represented by * ($P < 0.05$).
CHAPTER FOUR

Discussion and Conclusions
4.1 DISCUSSION

Summary

The aim of this thesis was to investigate the existence of dual vascular pathways within the skeletal muscle of a physiologically relevant model. This hypothesis has been primarily studied in the isolated perfused rat hindlimb during exogenous vasoconstrictor administration. The results from this thesis support the Clark classification of vasoconstrictors (Clark et al. 1995) in resting muscle in regards to Type B vasoconstrictors only. However, the effect of the Type B vasoconstrictor on oxygen consumption is diminished during times of increased metabolic demand (such as exercise). This could be due to local metabolite-mediated vasodilation. No significant effect on oxygen consumption was observed with adequate oxygen delivery to match basal metabolic rate after increased oxygen availability by increased nutritive flow after histamine, isoprenaline or noradrenaline infusion. During muscle contraction, vasodilator infusion significantly increased oxygen consumption possibly because of the direct effect of the agonist on the muscle or increased nutritive flow resulting in increased oxygen availability. The inability to identify an exogenously administered vasodilator that selectively vasodilates the non-nutritive bed suggests that the locale that a vasodilator is released from, rather than variations in receptor location, determines skeletal muscle blood flow redistribution.

Autoperfused Hindlimb

The autoperfused rat hindlimb model maintained physiological resting vascular tone, and hence the capacity to respond to either exogenous vasoconstrictors or vasodilators. Blood parameters were maintained at physiological levels throughout the experiments as discussed above (see blood profiles). This ability to provide high oxygen delivery ensured that the vasoconstrictor / vasodilator responses could be evaluated during the high demand conditions associated with repetitive muscle contraction. This was an improvement on previous models and provided conditions for physiological effects such as changes in shear stress, pH, CO₂ transport, haematocrit and haemoglobin saturation in which to occur.
Resting hindlimb perfusion pressure is very similar to systemic blood pressure \(~ 90\text{mmHg}\) at \(1\text{ml}\cdot\text{min}^{-1}\) flow rate in contrast to the perfusion pressure of \(~ 20\text{mmHg}\) observed at a flow rate of \(2\text{ml}\cdot\text{min}^{-1}\) in the isolated perfused hindlimb (Figure 4.1) (Colquhoun et al. 1988; Ye et al. 1990). Resting vascular tone, capable of increases or decreases, is present in the autoperfused hindlimb but not in the isolated perfused hindlimb. Hence, to obtain a perfusion pressure in the vicinity of \textit{in vivo}\ systemic blood pressure in the isolated perfused hindlimb, a flow rate in excess of \(20\text{ml}\cdot\text{min}^{-1}\) is required, which is much greater than that observed \textit{in vivo}. Janiak et al. (2002) reported that at rest, \textit{in vivo} iliac arterial blood flow is \(~ 1.5\text{ml}\cdot\text{min}^{-1}\). There was no effect on systemic blood pressure whilst hindlimb perfusion pressure increased to \(~ 160\text{mmHg}\) at \(2\text{ml}\cdot\text{min}^{-1}\) in the autoperfused hindlimb. The increase in flow rate during the contraction protocol was aimed to mimic the increase in cardiac output observed during exercise. However since there is no generalised increase in systemic sympathetic activity to act on the heart and vasoconstrict low metabolic demand tissue (such as the digestive organs during sciatic nerve stimulation) there was no change in systemic blood pressure, unlike in exercise.

\textbf{Figure 4.1:} The effect of flow rate on oxygen consumption and perfusion pressure in the isolated perfused hindlimb (adapted from Ye et al. (1990) (●) and the autoperfused hindlimb (data from this study) (○).
The use of the animal’s own blood and lungs facilitates the delivery of physiological oxygen supply (O\textsubscript{2} bound to Hb not saturated buffer) to both resting and contracting muscle. Inability to provide physiological delivery of oxygen to the muscle is a major limitation of both in vitro and in situ studies (Bon
den et al. 1994). Oxygen consumption at rest in the autoperfused hindlimb is 4-fold greater than in the isolated buffer-perfused hindlimb. Only 3% of oxygen transported within the blood in vivo is dissolved, with the remainder carried in the erythrocyte. Even with an arterial Po\textsubscript{2} greater than 400mmHg (compared with in vivo arterial Po\textsubscript{2} ˜ 100mmHg), metabolic demand is not matched by availability unless delivered at very high flow rates. Further, because cell-free perfusate oxygen content is not sufficient to match metabolic rate the muscle cells will be hypoxic even at rest at in vivo flow rates. Oxygen consumption in the autoperfused rat hindlimb at rest is within the normal in vivo range of 0.3 to 0.4 µmol·min\textsuperscript{-1}·gww\textsuperscript{-1} (Côté et al. 1985).

Hindlimb oxygen consumption decreased when flow rate was increased to 2 ml·min\textsuperscript{-1} with the muscle at rest then increased to above low flow resting values during repetitive muscle contraction. It has been clearly shown in the pulmonary system that a significant increase in cardiac output (increased flow rate) decreases erythrocyte transit time therefore affecting erythrocyte loading and unloading of oxygen and oxygen diffusion into the cell (Powers and Williams 1987). In this study, when the hindlimb flow rate was increased, it is assumed that erythrocyte transit time would be decreased and it is likely that unloading of oxygen would be limited in the capillary bed of the muscle. Furthermore, blood flow is distributed unevenly throughout the capillary bed (flow heterogeneity) at rest (Vetterlein et al. 1977; McDonagh et al. 1982; Lindbom and Arfors 1985; Pliper et al. 1985; Duling and Damon 1987). One-third to one-half of all capillaries are perfused at any time, with a homogeneous spatial distribution. The average erythrocyte velocity is less than 20% of the average maximal velocity obtained during maximal exercise (Lindbom 1983). Capillary recruitment during the transition from rest to exercise occurs within the first 15 seconds beginning in areas where capillary perfusion density is lowest. Mean capillary perfusion increases by 1.5- to 3-fold and flow increases 7-fold, which leads to a drop off in erythrocyte transit time by one-third (Honig et al. 1980). Further, at rest there are no other stimuli, such as changes in muscle temperature, pH, CO\textsubscript{2} or demand of O\textsubscript{2} to enhance this unloading, which is known as the Bohr effect (Rowell and Shepard 1996). There are also vascular limitations to oxygen exchange within the skeletal muscle. These include oxygen consumption to blood flow inequity;
limitation of diffusive transport of oxygen from the muscle microcirculation to the mitochondria; shunting of blood flow through the non-nutritive route; and diminished total flow (Wagner 2003).

Another possible influence on oxygen consumption at high flow rates would be blood flow redistribution into the low resistance non-nutritive vessels, which have lower metabolic demand therefore resulting in reduced oxygen consumption. It is likely that there is a combination of both reduced erythrocyte transit time and increase in blood flow into the low resistant non-nutritive pathway, which would explain the decrease in oxygen consumption with an increased flow rate. Therefore an increase in oxygen consumption with increase in flow rate at rest (as seen in buffer perfused isolated hindlimbs) most likely reflects increased delivery to meet previously unmet oxygen demand.

The effect of the infused saline vehicle upon both hindlimb perfusion pressure and oxygen consumption could be a result of a short-term change in viscosity and/or temperature of the perfusate. Saline was held at room temperature before infusion. To accommodate this saline effect, all measures of drug effects on perfusion pressure and oxygen consumption were made after the effects of the saline vehicle had subsided.

The stimulated twitch protocol was used to increase metabolic demand of the perfused hindlimb. This particular protocol was selected to reduce the chance of rapid fatigue. As a result the twitch tension produced was lower than many other studies. Mean twitch tension in this thesis was \(0.235 \pm 0.020\)N \((24 \pm 2\text{g}, n = 8)\) whilst Shiota and Sugano (1986) reported a mean twitch tension of \(0.422 \pm 0.049\)N \((43 \pm 5\text{g}, n = 4)\) in the isolated perfused rat hindlimb both at 5Hz. Shiota and Sugano (1986) used rats with a weight range of 180-230g compared with 450-785g used in this study. Therefore it can be speculated that maximal muscle contraction or tetanic stimuli would further increase hindlimb oxygen consumption with matched oxygen delivery above those reported here.

**Effects of Serotonin on Oxygen Consumption and Perfusion Pressure**

Serotonin significantly decreased hindlimb oxygen consumption at rest during vasoconstriction of the hindlimb vasculature, confirming the selective vasoconstriction observed by Clark *et al.* (1995). Changes in perfusion pressure were closely associated with changes in oxygen consumption at rest and during muscle contraction (Figure 3.13). The magnitude of the serotonin decrease in oxygen
consumption was however diminished during the high oxygen demand conditions of repetitive contraction which may be due to the local release of vasodilator metabolites to match oxygen delivery to oxygen demand.

Serotonin decreases oxygen uptake, free fatty acid efflux and lactate production which can be blocked by 5-HT₂-antagonist infusion (Dora et al. 1991; Clerk et al. 2003). Insulin-mediated glucose uptake is also inhibited especially in muscles rich in fast-twitch glycolytic fibres (Rattigan et al. 1993; Baron and Clark 1997). This decrease in hindlimb metabolism by Type B vasoconstrictors is thought to occur through selective vasoconstriction of the nutritive blood flow redirecting flow into the non-nutritive bed (Figure 4.2). It is proposed that this occurs at the terminal arteriole, proximal to the muscle tissue and distal to the connective tissue, increasing perfusion of the associated connective tissue capillary beds and decreasing oxygen uptake. However, further studies to identify the precise location of 5-HT₂ receptors within the microvasculature are necessary to link the vasculature and metabolic changes occurring during serotonin infusion.
Figure 4.2: A simplified schematic of the structure of the vasculature of skeletal muscle adapted from Borgström et al. (1988). TE represents Terminal Arteriole; TR: Transverse Arteriole; CAP: Capillary Bed.

At higher doses, the serotonin-mediated increases in perfusion pressure were diminished. This phenomenon has been reported in previous studies (Haddy et al. 1959; McCubbin et al. 1962; Emerson et al. 1973; McLennan and Taylor 1984; Blackshear et al. 1985). The diminished increase could be due to simultaneous vasodilation and vasoconstriction at differing sections of the vasculature by different subtypes of the 5-HT receptors. Serotonin has a direct vasodilator effect when the initial resistance is high, which can be unmasked during 5-HT₂ blockade (McLennan and Taylor 1984). Serotonin-mediated vasodilation has also been reported to involve the inhibition of release noradrenaline during sympathetic stimulation (Haddy et al. 1959; McCubbin et al. 1962; Emerson et al. 1973; Blackshear et al. 1985). However in this study, the serotonin-mediated increase in basal perfusion pressure was diminished whilst resting tone was maintained, therefore the possibility of two serotonin receptors in the microvasculature is most likely to explain this phenomenon; one that vasoconstricts (5-HT₂) and another that vasodilates (5-HT₁). The vasodilator phenomenon is unmasked when the vasoconstrictor receptors are saturated or blocked (Haddy et al. 1957; McLennan and Taylor 1984).
Dora et al. (1994) reported that muscle aerobic contractility is inhibited during serotonin infusion due to diminished nutrient delivery through redirection of blood flow by selective vasoconstriction. The vasoconstrictor action of serotonin was not overcome by local vasoactive metabolites released during skeletal muscle contraction. In contrast, serotonin had no significant effect on the maximal rate of muscle contraction or relaxation in this study. The effect of serotonin on hindlimb oxygen consumption was diminished during the contraction protocol although if nutritive flow was reduced it would be expected that the twitch characteristic would differ due to reduced oxygen availability. Despite this, the reduced nutritive flow might still provide sufficient oxygen for the twitch contractions in the presence of high oxygen content. The differing results could lie with the fact that Dora et al. used a tetanic protocol whilst this study used a twitch protocol.

**Noradrenaline and the α-Adrenoceptor**

Noradrenaline significantly increased hindlimb perfusion pressure in the autoperfused hindlimb, which supports previous findings (Powell and Skinner 1968; Shiota and Masumi 1988; Ye et al. 1990). However, the metabolic effects of noradrenaline administration in the autoperfused hindlimb do not equate with those reported by the Clark group in which noradrenaline infusion increased both perfusion pressure and hindlimb oxygen consumption at physiological doses (< 1µM) (Clark et al. 1995). Higher dose noradrenaline (> 1µM) decreases hindlimb oxygen consumption and inhibits insulin-mediated glucose uptake via α-adrenoceptor activation in the perfused hindlimb, but not in isolated muscle preparations. It is thought that high dose noradrenaline-induced vasoconstriction occurs in large arterioles downstream from branching points for shunting of blood into the venous circulation (Rattigan et al. 1995). The difference may lie in the experimental conditions.

Noradrenaline-induced metabolic changes are thought to occur by selective vasoconstriction of the non-nutritive route to redistribute skeletal muscle blood flow. This occurs at the transverse arteriole, distal to the muscle tissue and proximal to the connective tissue, therefore increasing perfusion of the muscle capillary beds via α-adrenoceptor activity to increase oxygen uptake (Figure 4.2) (Côté et al. 1985; Newman et al. 1997). This increase in oxygen consumption has been reported by a variety of groups (Powell and Skinner 1968; Grubb and Folk 1977; Clark et al. 1994;
Ye et al. 1995) and it can be totally blocked by $\alpha$-adrenoceptor blockade, but not by $\beta$-adrenoceptor blockade (Grubb and Folk 1977).

The inability to observe noradrenaline-induced increases in oxygen consumption during vasoconstriction in the autoperfused hindlimb has been reported previously (Gronert et al. 1980; van-Hardeveld et al. 1980; Shiota and Masumi 1988; Gainer et al. 1993). van-Hardeveld et al. (1980) reported that the oxygen consumption was significantly higher and the increase in perfusion pressure following noradrenaline infusion was significantly blunted in cold acclimated rats compared to control animals. Similarly, in a study carried out in cold acclimated animals by Shiota and Masumi (1988) found that hindlimb oxygen consumption was significantly increased with no change in lactate release, however they found that the increase in perfusion pressure was identical to the control animals. These studies support the evidence provided in this thesis and the theory that changes in oxygen consumption by noradrenaline can occur independently of the changes to vascular resistance.

Skeletal muscle arterioles appear to have both $\alpha$- and $\beta$-adrenoceptors, and activation of them influences erythrocyte velocity through the capillary ($\alpha$ decreases, $\beta$ increases) (Yu and Tyml 1997). It is thought that the occupation of $\alpha_2$-adrenoceptors does not stimulate the effector system but inhibits $\alpha_1$-adrenoceptor activity in a competitive manner (Kobinger and Pichler 1981). The $\alpha_1$-adrenoceptor is the most prolific $\alpha$-adrenoceptor in the peripheral vasculature although many studies have shown that the $\alpha_2$-adrenoceptor is found not only on the presynaptic membrane of adrenergic nerves but also on the postsynaptic membrane to mediate the constriction of vascular smooth muscle (Docherty et al. 1979; Timmermans et al. 1979; Timmermans and van-Zwieten 1981; Alabaster and Davey 1984; Ruffolo 1984; Chotani et al. 2004). Large arteriole smooth muscle cells and venules contain both $\alpha_1$- and $\alpha_2$-adrenoceptors. Whilst early reports suggested that precapillary arterioles such as the transverse and terminal arterioles contain predominantly $\alpha_1$-adrenoceptors (Faber 1988), more recent studies have concluded that precapillary vessels predominantly contain $\alpha_2$-adrenoceptors (Yu and Tyml 1997; Xu et al. 1998). The distribution of $\alpha_2$-adrenoceptors in the small, precapillary arterioles could be involved in prominent or unique regulatory features evident at this level of the microcirculation such as vasomotion. Differences in excitation-contraction coupling of vascular smooth muscle cells could also be due to the dominant subtype of $\alpha$-adrenoceptors at the various levels of the vasculature (Faber 1988).
Nitric oxide is thought to play a key role in functional sympatholysis, where systemic sympathetic vasoconstriction is negated by local metabolic vasodilation (physiological antagonism) (Segal and Kurjiaka 1995; Hansen et al. 1996). It is thought that this could be a mechanism to tightly regulate blood flow and oxygen delivery to skeletal muscle with the reflex control of the precapillary resistance arterioles (Anderson and Faber 1991; Buckwalter et al. 2001). In the human forearm, α-adrenoceptor-mediated vasoconstriction remains intact during infusion of nitroprusside (Rosenmeier et al. 2003). This is in contrast to animal studies where α₂ but not α₁-adrenoceptor sensitivity is diminished during nitric oxide infusion (Thomas and Victor 1997). During exercise, α-adrenoceptor-mediated vasoconstriction is diminished at various levels of the vasculature (Buckwalter et al. 2001) whilst glucose production is markedly increased during intense exercise via α-adrenoceptor stimulation (Sigal et al. 1994). Vasoactive metabolite-mediated vasodilation overcomes the α₂-mediated vasoconstriction of precapillary arterioles with no effect seen on the large arterioles during light exercise. During intense exercise, α₁-mediated vasoconstriction of the large arterioles can be overcome by the release of vasoactive metabolites (Anderson and Faber 1991; Buckwalter and Clifford 1999; Buckwalter et al. 2001). Therefore the effect of exogenous activation of skeletal muscle α-adrenoceptors during exercise would be dependent upon the intensity of exercise. The diminished sensitivity of the α-adrenoceptors during exercise suggest that the α-adrenoceptors may not selectively vasoconstrict the non-nutritive pathway, although it may play a role during basal conditions.

Selective vasoconstriction within skeletal muscle may play a crucial role in nutrient and hormone delivery and nutrient retention during basal conditions. The differing location of α-adrenoceptor subtypes within the microvasculature could play a crucial role in blood flow regulation. However, the use of the non-selective α-adrenoceptor agonist noradrenaline did not reveal the differing effects of the α-adrenoceptor subtypes upon basal blood distribution and skeletal muscle metabolism. Therefore an α₂-adrenoceptor agonist may be used to selectively vasoconstrict the precapillary arterioles of skeletal muscle to redistribute blood flow if the α₂-adrenoceptor is the most dominant subtype within the transverse and terminal arterioles. Nevertheless, endogenous control via the adrenergic nervous system must be mediated by the concentration of endogenous transmitters noradrenaline.
and adrenaline, the location of their release and the location of the receptor subtypes.

**Mechanisms for Communication of Metabolic Demand**

If the effect of serotonin on oxygen consumption is achieved by selective vasoconstriction of the nutritive pathway associated with the muscle fibres, the reduced effect on oxygen consumption during the contraction protocol may be due to the local effects of the twitch (such as the release of vasoactive metabolites) to increase local oxygen supply, hence overriding the exogenous vasoconstriction of the nutritive pathway (physiological antagonism). In a review of vasoactive metabolites released from skeletal muscle cells Altura (1971) reported that they all vasodilate arterioles (Table 1.1). It is known that systemic sympathetic vasoconstriction is negated by local metabolic vasodilation (functional sympatholysis) (Anderson and Faber 1991; Buckwalter and Clifford 1999; Buckwalter et al. 2001). The communication of metabolic demand from the tissue to the sites of regulation, the transverse and terminal arterioles, has been intensively investigated. The site for regulating blood flow at metabolically active muscle must be upstream from the active cells and the main mechanism for increased blood flow appears to be by vasodilatory agents.

The mechanism for upstream communication has yet to be defined however there is no adrenergic innervation within capillaries of mammalian skeletal muscle (Dietrich and Tyml 1992b). It has been hypothesised that upstream communication via cell-to-cell coupling, erythrocytes as a sensor, or diffusion of a signal from the venule endothelium could be possible mechanisms for metabolites released by skeletal muscle cells to selectively influence blood flow to those cells. The following factors have been implicated in the communication of metabolic demand to increase supply.

*Endothelial-Derived Compounds*

The endothelium of capillaries and arterioles release a variety of compounds under different conditions that can regulate arteriolar resistance. The removal of vascular endothelium abolishes any flow-induced vasodilation *in situ* and *in vitro* (Pohl et al. 1986; Rubanyi et al. 1986). Arteriolar diameter is controlled by a variety of mechanism including endothelial-derived relaxing factors (EDRF), endothelial-derived hyperpolarising factors (EDHF) and endothelial-derived nitric oxide (EDNO). Rubanyi et al. (1986) demonstrated that both an increase in flow and the introduction
of pulsatile flow augments the production of prostacyclin and another EDRF’s in the
dog femoral artery. Bradykinin, prostacyclin, cyclic AMP, adenosine, 5’-AMP and
cyclic GMP have been ruled out as possible EDRF’s (Furchgott and Zawadzki 1980).

Arachidonic acid products, epoxygenase products, potassium or endogenous
cannabinoids have been suggested as possible EDHF’s (Triggle and Ding 2002).
EDHF activation of Ca\(^{2+}\)-dependent K\(^{+}\) channels at the local site is required to illicit a
conducted vasodilation in the hamster cremaster muscle, but nitric oxide and
prostaglandins play a less important role in initiating a conducted vasodilation (Hoepfl
et al. 2002). Potassium ions pass from the intra- to the extracellular space during
muscle activity (Kjellmer 1965), and low concentrations of exogenous potassium
dilate arteries and arterioles, whilst high concentrations constrict large arteries (>0.5mm) that lie external of the active muscle (Dawes 1941). Physiological
concentrations of exogenous potassium produces a vascular response similar to the
vascular responses observed during exercise (Kjellmer 1965).

Acetylcholine-induced vasodilation was initially thought to be mediated
through the release of an unknown EDRF (Furchgott and Zawadzki 1980; Moncada
et al. 1991) and EDNO (Hester et al. 1993). Acetylcholine activates calcium-
calmodulin-dependent nitric oxide synthase, which uses \(\text{L-arginine}\) as a substrate.
Inhibition of EDNO-release significantly decreases vasodilation of first- and second-
order arterioles during hyperaemia, but has no effect on third-order arterioles.
However, by using \(\text{L-arginine}\), the authors may have bypassed other receptor-linked
effects of acetylcholine and directly caused an increase in nitric oxide (Frame and
Sarelius 1995). The relationship between acetylcholine and nitric oxide is a good
example and exemplifies the complex nature of the regulation of skeletal muscle
blood flow.

Conducted Vasomotion

Conducted vasomotion describes the effect that the introduction of an agent
(be it pharmacological or metabolic) at the local site (capillary) has on the remote
site’s (arteriole) vasculature resistance. This could explain the vasculature changes
as a possible mechanism for upstream communication of metabolic demand. A
variety of agonists have been shown to induce a conducted vasomotion in a variety
of preparations (Table 4.1). During local administration of acetylcholine and
noradrenaline to skeletal muscle capillaries, propagation of the local vasomotor
response upstream to a remote site has been reported, where acetylcholine
increased, whilst noradrenaline decreased erythrocyte velocity. However, during post-propagation muscarinic, or $\alpha$-adrenoceptor blockade the effects of acetylcholine and noradrenaline were not blocked (Segal and Duling 1989; Kurjiaka and Segal 1995).

**Table 4.1:** Adapted from Gustafsson and Holstein-Rathlou (1999). Summary of local and conducted vasomotor responses in arterioles in a variety of models to various vasoactive agents applied extraluminally.

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Local Response</th>
<th>Conducted Response</th>
<th>Vasculature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetylcholine</td>
<td>VD</td>
<td>VD</td>
<td>Hamster Cheek Pouch</td>
</tr>
<tr>
<td></td>
<td>VD</td>
<td>VD</td>
<td>Hamster Striated Muscle</td>
</tr>
<tr>
<td>Methacholine</td>
<td>VD</td>
<td>VD</td>
<td>Hamster Cheek Pouch</td>
</tr>
<tr>
<td>Adenosine</td>
<td>VD</td>
<td>Variable</td>
<td>Hamster Cheek Pouch</td>
</tr>
<tr>
<td>Bradykinin</td>
<td>VD</td>
<td>Variable</td>
<td>Hamster Cheek Pouch</td>
</tr>
<tr>
<td>Nitroprusside</td>
<td>VD</td>
<td>NR</td>
<td>Hamster Cheek Pouch</td>
</tr>
<tr>
<td>Noradrenaline</td>
<td>VC</td>
<td>VC</td>
<td>Hamster Cheek Pouch</td>
</tr>
<tr>
<td>Adrenaline</td>
<td>VC</td>
<td>VC</td>
<td>Rat Mesentery</td>
</tr>
<tr>
<td>Phenylephrine</td>
<td>VC</td>
<td>VC</td>
<td>Hamster Cheek Pouch</td>
</tr>
<tr>
<td>KCl</td>
<td>VC</td>
<td>VC</td>
<td>Hamster Cheek Pouch</td>
</tr>
<tr>
<td>Vasopressin</td>
<td>VC</td>
<td>NR</td>
<td>Hamster Cheek Pouch</td>
</tr>
<tr>
<td>ATP</td>
<td>VC</td>
<td>VC</td>
<td>Hamster Striated Muscle</td>
</tr>
<tr>
<td>L-Arginine</td>
<td>NR</td>
<td>VD</td>
<td>Hamster Striated Muscle</td>
</tr>
</tbody>
</table>

VD: Vasodilation; VC: Vasoconstriction; NR: No response.

Blockade of $\alpha_2$-adrenoceptors abolished the noradrenaline-induced increase in erythrocyte velocity (Dietrich and Tyml 1992a). Noradrenaline application induces smooth muscle cell depolarisation, which is conducted between smooth muscle cells to induce contraction of adjacent cells and the depolarisation is conducted independently of endothelial cells. Acetylcholine acts on muscarinic receptors (Furchgott 1983) to induce a local and conducted hyperpolarisation along the arteriole between both smooth muscle and endothelial cell layers, which decay with distance up to 60\(\mu\)m (Figure 4.3) (Segal and Duling 1989; Song and Tyml 1993; Welsh and Segal 1998). Dual application of noradrenaline on two capillaries produced a greater decrease in erythrocyte velocity than a single application,
whereas dual application of noradrenaline and acetylcholine attenuated the acetylcholine response (Song and Tyml 1993). L-Cystine application in the microcirculation also initiates remote vasoconstriction whilst L-arginine induces a remote vasodilation $\geq 1000\mu m$ upstream similar to acetylcholine. Remote vasodilation by these agonists was found to have a rapid onset that cannot be explained by simple diffusion (Frame 1999).

Smooth muscle cells and endothelial cells are electrically coupled via gap junction proteins both homocellulary and heterocellulary in the microvasculature (Little et al. 1995; Xia et al. 1995). The molecule involved in signal transmission between vascular smooth muscle and the endothelium is unknown but it has been postulated that the increase in intracellular calcium generates a diffusion gradient that drives calcium through myoendothelial cell junctions and into the endothelial cells, which stimulate nitric oxide synthesis (Dora et al. 1997). The conducted vasomotion induced by L-arginine, noradrenaline and acetylcholine can be inhibited by non-specific gap junction inhibitors (Frame 1999).

![Figure 4.3: An example of the relationship between the local and remote site during micropipette application of the agonist at the local site of the capillary adapted from Frame (2000).](image)

After intraluminal application of adenosine triphosphate (ATP) into skeletal muscle venules, an 8% increase in arteriolar diameter was recorded up to 450$\mu m$ upstream, which resulted in a 63% increase in secondary arteriolar and 50% increase in terminal arteriolar erythrocyte flux (Collins et al. 1998). Intra-arteriole infusion of ATP caused a similar increase in arteriolar diameter that, according to Poiseuille’s law, would lead to a 17% increase in flow (McCullough et al. 1997). Systemic administration of L-NAME (nitric oxide synthase inhibitor) prior to venous
ATP application eliminated any conducted responses and this effect was reversed by L-arginine systemic application, which suggests that nitric oxide plays a role in the conducted response of ATP (Collins et al. 1998). Adenosine is able to generate remote upstream arteriolar vasodilation which can not be explained by direct arteriolar stimulation, capillary stimulation along the same flow path, diffusion to the remote arteriole, increases in blood flow or veno-arteriolar transfer (Rivers and Frame 1999). It has therefore been speculated that changes in membrane potential could be a means for capillaries to communicate upstream to arterioles to optimise blood flow and oxygen delivery to downstream tissue (McGahren et al. 1998).

Muscle contraction not only causes local arteriolar dilation but also initiates conducted vasodilation upstream. The conducted vasodilation signal differs from the capillary level signal and from pharmacologically induced signal via acetylcholine directly at the arteriolar level (Murrant and Sarelius 2000b). It does not involve perivascular nerves or gap junctions, and it has been proposed by Murrant and Sarelius (2000b) that local paracrine signalling would account for transmission of the conducted vasodilation along the vessel wall. Although remote responses to some agonists are induced by primary signals to locally dilate, additionally, network changes in flow can stimulate extensive remote changes in diameter via separate or secondary mechanisms (Frame 2000). Skeletal muscle fibre capillaries can sense muscle contraction and initiate $K_{\text{ATP}}$ channel, adenosine, and nitric oxide dependent remote arteriolar vasodilation (Cohen and Sarelius 2002). Local vasodilation is $K_{\text{ATP}}$ channel, adenosine, and nitric oxide dependent whilst upstream dilation is $K_{\text{ATP}}$ channel, and adenosine, but not nitric oxide dependent whereas the conducted signal is independent of these components (Murrant and Sarelius 2002). The evidence reported supports the contention that the conduction of electrical signals upstream via gap junctions to the regulatory sites of vascular control is one possible mechanism to tightly regulate muscle metabolism to blood flow.

**Erythrocyte**

One group of researchers have focused on the erythrocyte as an oxygen sensor (Figure 4.4). ATP is released from the erythrocyte in response to hypoxia, hypercapnia, and low pH; conditions associated with decreased oxygen supply relative to demand (Ellsworth et al. 1995; McCullough et al. 1997). Extraluminal application of ATP induces a dose-dependent conducted vasoconstriction whilst, as reported above, intraluminal application induces a conducted vasodilation (McCullough et al. 1997). ATP is known to bind to the purine $P_{2y}$ receptors that are
located on the luminal surface of peripheral vascular endothelium. The binding of ATP to these receptors induce the production of nitric oxide and prostacyclin $\text{PGI}_2$ both of which are potent vasodilators of the peripheral vasculature (Ellsworth et al. 1995). The erythrocyte can also release nitric oxide as oxygen saturation is decreased during increased metabolic demand. The nitric oxide is bound to form S-nitrosohaemoglobin and is released, via allosteric transition, to induce vasodilation (Secomb and Pries 2002). Haemoglobin can also contract the vasculature by decreasing the steady state levels of free nitric oxide, resulting in increased blood pressure (Jia et al. 1996). The erythrocyte can act by two mechanisms to regulate arteriolar resistance. Therefore, it has been proposed that if the erythrocyte does act as an oxygen sensor via the release of ATP, it would eliminate the need for a diverse network of sensing sites throughout the microvasculature, and provide an efficient means for precisely matching supply with demand (Ellsworth 2000).

**Figure 4.4:** Taken from Ellsworth (2000) showing the hypothesis for erythrocyte derived ATP regulation of skeletal muscle blood flow. NO represents Nitric Oxide; EDHF represents Endothelial Derived Hyperpolarising Factor; SMC represents Smooth Muscle Cells; Endo represents Endothelial Cells; $P_2y$ and $P_2u$ represent subtypes of purine receptors

**Venule Endothelium**

A role for venules as a site of arteriolar diameter regulation has also been proposed since small arterioles and venules are paired within many branching levels of the microvasculature. It is thought that vasoactive metabolites released during times of increased metabolic demand could diffuse from the venule to the paired arteriole (Holtz et al. 1983; Hester and Choi 2002). Shear stressing of venular endothelium during rest releases nitric oxide, resulting in vasodilation of the
associated arteriole but this mechanism does not play a part during muscle contraction (Boegehold 1996). Venular endothelium is able to release multiple endothelium-derived relaxing factors in response to acetylcholine application onto the venular wall thus dilating the adjacent arteriole (Falcone and Bohlen 1990).

Venular endothelium can release PGI$_2$ which is a cyclooxygenase product that can diffuse from the venule to the paired arteriole. When the venule is treated with a cyclooxygenase inhibitor the paired arteriole does not dilate, unlike the unpaired arterioles (Hester and Choi 2002). The mechanism by which metabolic communication occurs in unpaired vasculature is unknown. The uptake of vasoactive metabolites (such as adenosine) by the venules can lead to vasodilation of the paired arteriole through diffusion of the metabolite from venule to arteriole (Hester 1990, 1993). When the venular endothelium is removed, arteriolar vasodilation is diminished in response to muscle contraction but the underlying mechanism is still unclear (Saito et al. 1994). Figure 4.5 combines the erythrocyte and venule endothelium as a possible mechanism that could regulate metabolic demand at a local level within the microvasculature.

**Figure 4.5:** Taken from Hester and Choi (2002). The proposed regulation of blood flow during exercise or rest. The change in the venous blood such as hypoxia, hypercapnea, and acidosis stimulate erythrocytes (RBC) to release adenosine triphosphate (ATP). ATP stimulates prostanoid release from the venular endothelium to vasodilate the adjacent terminal and/or transverse arteriole.
The evidence presented by this study and others (Dora et al. 1991; Dora et al. 1992; Dora et al. 1994; Clark et al. 1995; Newman et al. 1996) show that the metabolic effects of serotonin in resting and contracting muscle appear to be mediated via vascular redistribution of total flow into the non-nutritive pathway. However in contracting muscle, local metabolic vasodilation acts as a physiological antagonist to overcome the selective vasoconstriction of the nutritive bed, not only by serotonin but also sympathetic activation (functional sympatholysis). The ability of serotonin to significantly reduce nutritive blood flow may be crucial to retain nutrients during haemorrhage and/or platelet aggregation.

With the rationale that local metabolic-mediated changes in blood distribution are achieved via vasodilation, the identification of selective vasodilation in a model with resting tone could supply further evidence of a dual vascular pathway. Studies using the isolated perfused hindlimb were unable to study vasodilators without prior infusion of a vasoconstrictor to establish resting vascular tone. Selective vasodilation would arise according to the location of the receptors within the microvasculature, as is the case with the vasoconstrictors (Figure 4.2).

*Basal Nutritive Blood Flow and Oxygen Supply*

It would be expected that if selective vasoconstriction, or non-selective vasodilation increases the number of perfused capillaries to increase nutritive blood flow, this would result in an increase in basal skeletal muscle oxygen consumption, however this was not the case. At rest there was no significant effect of isoprenaline, histamine or low dose noradrenaline on oxygen consumption. With an increase of oxygen availability via vascular redistribution no change in basal metabolic rates was observed in the presence of high oxygen delivery in this study. Significant changes in perfusion pressure were observed at rest. This raises the question: does oxygen delivery dictate uptake?

Isoprenaline was chosen for further study as a vasodilator that decreases hindlimb perfusion pressure whilst increasing oxygen consumption. Histamine was chosen to study further because during two of the three screening trials an initial decrease in hindlimb oxygen consumption was observed followed by an increase. However this initial decrease was only observed in two of the six animals studied at rest. It was not observed during the contraction protocol.
Infusion of isoprenaline significantly decreased perfusion pressure during the basal protocol (1 ml·min⁻¹) as expected (Powell and Skinner 1968; Altura 1971; Bolme and Gagnon 1972; Nott and Head 1978; Gronert et al. 1980; Wickler and Horwitz 1984; Kuznetsova et al. 1998; Rohrer et al. 1999). In the β₁/β₂ knockout mouse this decrease in blood pressure is diminished (Rohrer et al. 1999), whilst β-adrenoceptor activation has a positive chronotropic effect which significantly increases cardiac output (Wickler and Horwitz 1984). Yu and Tyml (1997) reported a significant concentration-dependent increase in erythrocyte velocity through upstream arteriole vasodilation during local skeletal muscle capillary application of isoprenaline, which is diminished during β-adrenoceptor blockade. Belfrage (1978) reported that skeletal muscle vasculature dilatation is β₂-adrenoceptor-mediated, whereas in adipose tissue it is thought to be primarily β₁-adrenoceptor. However, more recently it was concluded that there are both β₁- and β₂-adrenoceptors in skeletal muscle capillaries that can modulate capillary module blood flow (Yu and Tyml 1997). It has been suggested that neurogenic β-adrenoceptor (G-protein-coupled) dilatation exists in the vasculature of skeletal muscle and appears to be limited to the resistance and precapillary arterioles (Lundvall and Jarhult 1974; Hillman and Lundvall 1981).

No significant effect upon basal oxygen consumption has been reported in previous studies for isoprenaline (Gronert et al. 1980; van-Hardeveld et al. 1980) or histamine (Bolme and Gagnon 1972). Bolme and Gagnon (1972) concluded that the infusion of vasodilating drugs into a constant pressure system does not affect the size of the capillary bed or change the oxygen consumption in resting or exercising skeletal muscle once steady-state is reached. However, the initial effect of these vasodilators significantly increased blood flow and oxygen consumption. Contrary to this Vetterlein and Schmidt (1974) reported that during infusion of vasodilatory agents, total blood flow increases in association with reduced perfusion of certain areas of the musculature. The infusion of vasodilators in a constant flow model results in increased number of perfused capillaries modules with diminished erythrocyte velocity (Bolme and Gagnon 1972; Shiota and Sugano 1986; Shiota and Masumi 1988; Ye et al. 1990; Kuznetsova et al. 1998), however, in a constant pressure model vasodilation increases total blood flow and the number of capillary modules perfused (Ye et al. 1995). This increase in total flow has a diminished laser Doppler signal which is thought to be a result of decreased erythrocyte velocity or erythrocyte numbers or a combination (Kuznetsova et al. 1998). Vasodilation results in a significant decrease in the number of perfused capillaries (Vetterlein and
Schmidt 1975, 1980) and decreases erythrocyte velocity in isolated preparations (Takakura et al. 1995). These studies show that vasodilation increases the number of perfused capillary modules in vivo. This increased surface area would be expected to increase oxygen uptake by the skeletal muscle, however, this was not the case with high oxygen content perfusates with reduced erythrocyte velocity through the capillary. This result may differ in an autoperfused constant flow model where there is an increase in the number of perfused capillaries with an increase in erythrocyte transit time, which are conditions found in vivo.

The significant decrease in basal and contraction hindlimb perfusion pressure after histamine infusion supports previous studies (Haddy 1960; Hashimoto and Kumakura 1965; Kjellmer and Odelram 1965; Rang et al. 1995). Histamine-mediated vasodilation is via H<sub>1</sub>-receptor activation in humans and a combination of H<sub>1</sub>- and H<sub>2</sub>-receptors in some experimental animals (dogs and rats) (Rang et al. 1995) and has been suggested to induce both pre- and post-capillary vessel vasodilation (Kjellmer and Odelram 1965).

Previous studies support the lack of significant effect upon basal oxygen consumption by noradrenaline in a variety of models (Grubb and Folk 1977; Gronert et al. 1980; van-Hardeveld et al. 1980; Shiota and Masumi 1988; Gainer et al. 1993). Furthermore, neither low nor high dose noradrenaline had any significant effects on the hindlimb twitch characteristic. If selective vasoconstriction that results in an increased nutritive flow does occur, it would be expected that there would be significant change in the muscle twitch characteristics of the stimulated hindlimb due to greater oxygen availability unless oxygen delivery was already optimal. However, since there is local metabolite-mediated vasodilation of the nutritive bed, if selective vasoconstriction of the non-nutritive bed by noradrenaline was to occur then there may not be any overall significant increase in nutritive blood flow hence no resulting effect on twitch.

Therefore, when basal metabolism is matched with adequate delivery of oxygen to skeletal muscle cells (autoperfused hindlimb), the cells remain normoxic. With an increase in oxygen availability during redistribution of flow no further increase is possible due to adequately matched oxygen delivery and metabolism. The changes observed in basal oxygen consumption in other studies would arise from models where inadequate delivery of oxygen occurs, such as cell-free perfusate. Any increase in oxygen availability via redistribution would increase oxygen uptake to
reduce the basal oxygen debt. However, in a constant flow model, vasodilation results in decreased perfusion pressure and decreased erythrocyte velocity through the capillary. In a constant pressure model (as found in vivo) vasodilation increases total flow which would increase the number of perfused capillaries with maintain flow rate. The study of the effects of blood redistribution during adequate oxygen availability in autoperfused constant pressure model is required for further understanding of the relationship between oxygen delivery and uptake.

*Increased Oxygen Uptake During Repetitive Contraction.*

Isoprenaline and histamine infusion significantly increased oxygen consumption during repetitive muscle contraction, but not at rest. The ability to further increase oxygen consumption during muscle contraction could arise from either a) selective vasodilation of the nutritive pathway; b) non-selective vasodilation to further increase the number of perfused capillaries; c) unmatched oxygen delivery and metabolic demand; or d) a direct metabolic effect on the skeletal muscle cells.

Both vasodilators produced dose-dependent decreases in perfusion pressure whilst isoprenaline caused an associated dose-dependent increase in oxygen consumption. Histamine also increased oxygen consumption but this was not dose-related or closely associated with the change in perfusion pressure. Histamine did, however, significantly increase the maximal rate of relaxation with a negative dose-dependent relationship. Isoprenaline had no corresponding effect upon the twitch during changes in perfusion pressure and oxygen consumption. The metabolic effects of the exogenous vasodilator infusion may be brought about by differing mechanisms. Beta-adrenoceptor activation has a direct positive metabolic effect upon the skeletal muscle cell whereas histamine may have an indirect effect upon increased oxygen uptake via increased oxygen availability through vasodilation.

Isoprenaline-mediated vasodilation is associated with an increase in skeletal muscle oxygen consumption which may not be causal (Wickler and Horwitz 1984; Ye et al. 1990; Gainer et al. 1993; Ye et al. 1995). Isoprenaline has been shown to be a potent stimulator of non-shivering thermogenesis as measured by increases in oxygen consumption (Bolme and Gagnon 1972; Wickler and Horwitz 1984; Gainer et al. 1993). However, isoprenaline has varying effects on oxygen uptake dependent upon the duration of the application (Powell and Skinner 1968). Activation of β-adrenoceptors results in skeletal muscle glycogenolysis and lipolysis activation which can impede glucose and free fatty acid uptake during intense exercise (Richter et al.
1982; Sigal et al. 1994; Rohrer et al. 1999; Sigal et al. 1999). Beta-adrenoceptor agonists are responsible for increased oxidative metabolism resulting in increased lactate production, muscle glycogen breakdown, and oxygen consumption with no effect on ATP or creatine phosphate levels observed in skeletal muscle (Li and Jefferson 1977). Williams et al. (1984) concluded that β-adrenoceptor density correlated positively with oxidative capacity in rat skeletal muscle. The significance of this could be that β-adrenoceptor activity increases oxygen uptake resulting in increased oxidative metabolism in fast and slow oxidative muscle fibres. These studies suggest that there is a direct action by activation of β-adrenoceptors on skeletal muscle metabolism independent of vascular changes.

In a variety of studies β-adrenoceptor activity has been linked to skeletal muscle tremor. The increased muscle activity during skeletal muscle tremor would result in an increase in oxygen uptake due to increased metabolic demand. This tremor can be increased by infusion of isoprenaline and adrenaline, and blocked by propranolol, but not by atenolol (β₁-antagonists). Therefore, the β₂-adrenoceptor mediates skeletal muscle tremor (Marlin and Turner 1975; Abila et al. 1985). It has been shown more recently that β₂-adrenoceptors are the predominant subtype found on the sarcolemma of mammalian skeletal muscle (Cairns and Dulhunty 1993). Hallberg and Almgren (1985) reported that β-adrenoceptor stimulation with hydrophilic agonists neither induced nor potentiated established centrally evoked mild tremor in conscious unrestrained rats. This is contrary to results found in human skeletal muscle, where increased metabolic rate during catecholamine release occurs via β₁-adrenoceptor activation (Lamont et al. 1997). It was concluded that β₂-adrenoceptors do not mediate the same functions in rat skeletal muscle as in humans. These studies suggest that the role of the sub-types of β-adrenoceptors varies within different species. Furthermore, increased muscle fibre recruitment via β-adrenoceptor-mediated muscle tremor may play a role in the increased oxygen consumption observed during the contraction protocol in this study.

There is no significant change in resting heart rate or mean blood pressure in the β₁/β₂ knockout mouse, however the effect of isoprenaline on blood pressure and heart rate is significantly diminished during basal conditions (Rohrer et al. 1999). Isoprenaline has no significant effect on resting oxygen consumption but does increase lactate output (Gronert et al. 1980; van Hardeveld et al. 1980). The β₁/β₂ knockout mouse has a significantly lower oxygen consumption, carbon dioxide production, and heart rate during 30 minutes of treadmill running. It can be concluded
that rodent β-adrenoceptors influence skeletal muscle metabolic activity during exercise. It was suggested that the decrease or attenuated increase of oxygen consumption could arise through β-agonist hyperpolarisation of the muscle membrane resulting in the decrease in oxygen demand (Gronert et al. 1980). These studies suggest that there is a heightened effect of β-adrenoceptor activity during exercise. This may explain the increase in oxygen consumption after isoprenaline infusion during the contraction protocol.

Isoprenaline had no significant effect on muscle twitch in the autoperfused hindlimb. Beta-adrenoceptor agonists have been reported to reduce the tension and fusion of incomplete tetanic contraction in slow-twitch skeletal muscle such as cat soleus (Bowman and Nott 1978). This has been contested more recently by Cairns and Dulhunty (1993) who reported that β-adrenoceptor agonists increase force production through increased cyclic AMP activity in rat soleus muscle. Beta-adrenoceptor activation alters the rate of relaxation of both twitch and tetanic contractions however this is dependent upon fibre type. In the isolated cat and guinea pig soleus muscle, isoprenaline significantly depresses the tension as well as the degree of fusion of incomplete tetanic contractions (Nott and Head 1978). However in a more recent study, β₂-adrenoceptor activation resulted in an increase in peak twitch and peak tetanic force in innervated or denervated isolated rat soleus muscle (Cairns and Dulhunty 1993). Twitch force is increased by activation of β-adrenoceptors, and since cyclic AMP application mimicked the effects of β-adrenoceptors, the activation of β-adrenoceptors is mediated by increases in the myoplasmic cyclic AMP concentration. This suggests that the increase in force development is mediated by cyclic AMP levels and therefore the increase in oxygen consumption may not result in increased cAMP levels.

Overall, this study and others suggest that the effects of isoprenaline on hindlimb oxygen consumption are most likely to be due to direct activation of skeletal muscle β-adrenoceptors which lead to increases in metabolic activity during muscle contraction rather than selective vasodilation. The increase in oxygen consumption during contraction is the result of increased sensitivity of the β-adrenoceptor leading to increased oxidative metabolism.

In the past, it has been suggested that histamine produces exercise-induced vasodilation in both skeletal and cardiac muscle and reactive hyperaemia (Berne 1964) although this has not been supported by subsequent experiments. However,
histamine and/or prostaglandin release has been suggested to play a role in post-exercise prolonged vasodilation (Morganroth et al. 1977). Unlike isoprenaline, there was no clear relationship between increased oxygen consumption and reduced perfusion pressure after histamine infusion during the muscle contraction.

Histamine infusion significantly increased oxygen consumption but only during muscle contraction. Even though there was no significant effect on the rate of force development, there was a significant increase in the rate of relaxation which could be due to an increase in nutritive flow through increased substrate availability, as relaxation is a highly energy dependent component of muscle contraction which is reliant on active removal of calcium from the contractile apparatus. Bolme and Gagnon (1972) reported that histamine initially increases oxygen consumption. However once steady state is restored one to five minutes post-infusion, there is no significant effect. Using the contracting perfused cat hindlimb Sonnenchein and Hirvonen (1961) assumed that maximal muscle force is an index of nutritive blood flow. Histamine decreased force production by either an increase in non-nutritive flow or a decrease in nutritive flow. However, Kjellmer and Odelram (1965) observed that histamine increased nutritive blood flow at rest in the perfused cat hindlimb due to increases in capillary filtration coefficient. Neither of these two studies measured oxygen consumption, so any changes in force production or capillary coefficient may not be causative through redirection of blood flow into or away from either vascular route. In this study, the relationship between perfusion pressure and the maximal rate of relaxation gives rise to the possibility that the increase in the number of perfused capillary modules increase metabolite efflux resulting in a greater rate of relaxation.

Selective vasodilation by histamine in this thesis cannot be concluded or dismissed. The increase in hindlimb oxygen consumption during muscle contraction and the increased rate of relaxation could arise through selective vasodilation of the nutritive route or through non-selective vasodilation of the transverse arteriole, which would increase perfusion of more capillaries hence increasing oxygen consumption and nutrient delivery as well as metabolite efflux. The direct effect of histamine on skeletal muscle cells has not been reported, but cannot be ruled out as an explanation of these phenomena.

The effects of isoprenaline and histamine infusion during muscle contraction on skeletal muscle metabolism are via different mechanisms. Isoprenaline has a direct effect upon the skeletal muscle cell by stimulating β-adrenoceptors resulting in
increased oxidative metabolism, whilst histamine has no direct effect. It is doubtful that histamine does not selectively increase nutritive blood flow, as there was no strong correlation between changes in perfusion pressure and oxygen consumption.

4.2 FUTURE DIRECTIONS

4.2.1 Application of the Findings

The results of these studies show that skeletal muscle metabolism can be influenced by redistribution of blood flow. This result could be explained by the existence of parallel vascular pathways within the skeletal muscle. Selective vasoconstriction by noradrenaline (Type A) to increase perfusion of muscle fibre associated capillaries does not increase oxygen consumption during adequate oxygen delivery. Increased availability of oxygen when basal metabolic rate is matched does not increase oxygen consumption. On the other hand, serotonin (Type B) decreased oxygen consumption which could be a result of selective vasoconstriction of the nutritive bed to increase perfusion of the connective tissue and adipose (Figure 4.2). Physiological control of skeletal muscle blood flow in vivo occurs via vasodilation. However, we were unable to identify a vasodilator that could selectively vasodilate the non-nutritive pathway resulting in a decrease in hindlimb oxygen consumption in a similar fashion to serotonin. Eight vasodilators with a variety of mechanisms such as nitric oxide donors, inflammatory mediators, G-protein coupled receptors, calcium channel blockers, and M₃-glandular receptors that release nitric oxide were screened. The inability to selectively vasodilate different parts of the microcirculation does not negate the existence of a dual circulation. However, it appears that the location from which the vasodilatory agents are released maybe the crucial factor to such selective vasodilation.

Increased capillary module perfusion via increase nutritive flow to increase oxygen availability above adequate supply does not increase resting oxygen consumption. Resting muscle can contribute to whole body thermogenesis, which is thought to be regulated by the vascular system and delivery of nutrients (Clark et al. 1994), however increasing supply above demand does not increase uptake. Therefore the contribution to whole body thermogenesis by skeletal muscle is regulated by a combination of central and peripheral mechanisms, such as neural and circulating hormones to stimulate metabolism resulting in thermogenesis.
Skeletal muscle metabolism is tightly regulated with blood flow. However, increased oxygen availability resulting from vasoconstriction, does not increase metabolic rate when there is no change in metabolic demand. Even so, when oxygen availability is reduced via selective vasoconstriction, metabolism is diminished.

The existence of a dual vascular arrangement in skeletal muscle is a distinct possibility. The clearance rate studies, pharmacological studies and direct observation studies all support the notion of a dual circulation, although definitive evidence in weight bearing muscles (unlike the tenuissimus, cremaster muscles) is required. The redistribution of blood flow by serotonin resulting in reduced oxygen consumption could play a part in nutrient retention when released from platelets during times of aggregation such as haemorrhage. During the early phase post-haemorrhage (30% haemorrhage), Borgström et al. (1990) observed that the reduction in total blood flow was diverted into the non-nutritive at the expense of nutritive flow to the muscle tissue.

Speculating on the significance of his and his colleague’s work, Clark stated that reduced physical activity and stress each contribute to poor nutritive flow in muscle, which leads to diminished ability to deliver nutrients, such as glucose and amino acids, and hormones, such as insulin, to the muscle fibres. This speculation leads to the conclusion that reduced physical activity may cause insulin resistance and reduced protein synthesis.
4.2.2 Further Investigations

The development of the autoperfused hindlimb as a physiological model used in pharmacological studies has possibilities for improvement. Application of pharmacological agents without the injection artefact, and slow infusion to increase the duration of the stimulus is one possibility. Add to this real time continuous recording of oxygen consumption and the ability to determine if the change in oxygen consumption during agonist infusion is associated with perfusion changes or the direct effects of the agonist on the muscle cells will be improved.

The study of a vasodilator that is released exclusively from adipose tissue could result in an increase in non-nutritive flow. This vasodilator could have receptors located exclusively in an area of the vasculature resulting in selective vasodilation to reduce oxygen consumption.

Increasing the duration of the contraction stimulation to greater than three minutes and with greater duration of agonist application will enhance the ability to observe any changes in twitch characteristics associated with prolonged redistribution of blood flow and hence nutrient delivery.

The use of radio-labelled vasoactive agents such as serotonin and noradrenaline or their antagonists could be used to localise their sites of action with a beta-imager. This could be used to determine whether there is a distribution difference between the respective receptors which gives rise to the differences observed in hindlimb oxygen consumption and metabolism.

A more in-depth study that includes antagonists in the autoperfused hindlimb could help to determine whether the changes in oxygen consumption and perfusion pressure induced by agonist infusion can be blocked. This was attempted, however, the combination of doses of agonist and antagonist as well as time to reach maximal saturation of receptors by the antagonist prior to agonist infusion made it difficult to achieve in time allocated to perform this study. The most effective application of the antagonist was also a source of difficulty. The choice of whether to infuse intra-arterially into the hindlimb, or intra-venously for maximal mixing within the blood or intra-peritoneal/intra-muscularly added to possible combinations which could not be determined within the time frame of this study.

The effect of diabetes and obesity on vasoactive agents could be studied to determine whether changes in the vasculature of the skeletal muscle are detrimental to nutrient delivery, metabolic rate and performance during exercise (twitch
characteristics). In these two populations or a normal population, the effects of exercise training (such as running or swimming) could be studied to determine whether there is a beneficial relationship between nutrient delivery and lifestyle. The effect of high fat or high n-3 fatty acid diet on nutrient delivery could further this study with the hypothesis that a sedentary lifestyle leads to an increased non-nutritive flow. It has been shown that a diet high in n-3 fatty acids has a positive effect upon cellular metabolic rate and have antiarrhythmic effects whilst lipid infusion prevents insulin-mediated capillary recruitment and muscle glucose uptake in skeletal muscle (McLennan et al. 1990; Charnock et al. 1992; Pepe and McLennan 1996; McLennan 2001; Clerk et al. 2002; Janiak et al. 2002). This increase in non-nutritive flow could result in a diminished ability of skeletal muscle to take up glucose and possibly results in vascular rearrangements within the musculature (Rattigan et al. 1997b; Sigal et al. 1999; Rattigan et al. 2001; Vincent et al. 2002). It has recently been shown that vascular remodelling occurs in syndrome X animal models, which may result in reduced perfusion capacity and peripheral microvascular disease (Stepp et al. 2004). Further studies might also focus on whether a diet high in n-3 fatty acids result in changes in blood flow or whether exercise in a diabetic or insulin resistant animal overcome the detrimental effects of a sedentary lifestyle on nutrient and hormone delivery and hence metabolism through changes in vascular structure.
4.3 **FINAL CONCLUSIONS**

The capability to retain vascular tone in the autoperfused hindlimb is of great importance when studying skeletal muscle metabolism, particularly during contraction, as metabolically driven increases in blood flow arise from upstream vasodilation. The retention of vascular tone also results in the ability to maintain blood flow within the range observed in vivo. Furthermore, the use of erythrocytes results in high oxygen content, which is important for high oxygen availability to sustain muscle contraction and basal metabolism with in vivo blood flow rates. Therefore, these physiologically relevant conditions are important for the study of in vivo skeletal muscle metabolism during pharmacological or physiological stimuli.

The effects of vasoconstrictors were largely overcome by increased muscle metabolic activity. This taken together with the inability to identify vasodilators with differential effects on oxygen consumption suggests that blood flow redistribution via vasodilation is determined by the locale of vasodilator release rather than receptor distribution. The exogenous regulation of dual circulation appears most relevant during resting conditions. Whilst during exercise, it is under local control via vasoactive metabolites.

The existence of dual circulation within skeletal muscle is a distinct possibility. The ability of serotonin to decrease basal and exercising skeletal muscle oxygen consumption during changes in perfusion pressure gives rise support to this notion. A non-nutritive pathway may act as a low metabolic route during times where nutrient retention is of great importance (such as a large haemorrhage). Many studies have suggested that the non-nutritive route is the dominant route at rest. Therefore, blood flow may be the redistributed from the non-nutritive route into the nutritive route during the early phases of increased metabolic demand, such as exercise. This would result in an increase in oxygen availability to reduce, but not negate, the severity of skeletal muscle oxygen debt, which is observed during the transition from rest to exercise.
In the autoperfused hindlimb at rest, selective increases in oxygen consumption by vasoconstrictors or vasodilators were not demonstrable. On the other hand, reduced oxygen consumption was demonstrated with serotonin or high noradrenaline dose. The ability to greatly increase hindlimb oxygen consumption by increasing metabolic demand through muscle contraction but not through increasing muscle blood flow suggests that \textit{in vitro}, muscle oxygen delivery is adequate and redistribution of perfusate to increase oxygen consumption is largely a phenomenon of the high flow, low oxygen delivery preparations. Considering the ability of muscle metabolism to override the exogenously produced flow distribution, a role for noradrenaline \textit{in vivo} might be in the expectant rise in sympathetic nervous activity that precedes exercise to provide an initial redistribution prior to the onset of muscle activity. While not producing any increase in oxygen consumption, it would provide increased delivery in preparation for (anticipation of) the impending muscle activity.


hindlimb; implications for non-shivering thermogenesis in muscle tissue."

**General Pharmacology** 21(1): 141-8.


Proceedings of the National Academy of Sciences of the United States of America 94(12): 6529-34.


APPENDIX
Appendix 1: The autoperfused hindlimb model during a basal experiment, wide shot.
Appendix 2: The autoperfused hindlimb model during a basal experiment, close shot.
**Appendix 3:** Oxygen Consumption Calculation Excel Spreadsheet and Formulae.

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<td>Arterial_HCO3 (mmol/L)</td>
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<td>(1.39*(U2<em>Y2)+(0.003</em>R2)</td>
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<p>| (K2<em>Y2+1000)</em>(U2<em>Y2) | (K2</em>Y2+1000)<em>(U2</em>Y2) |</p>
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<td>0.0307+ (0.00057*(37-P3))+0.00002*(37-P3)(37-P3)</td>
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<th>BL</th>
<th>Basal Venous O₂ conc.(mol/100ml WB)</th>
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<td>(1.39<em>BC3</em>BH)+(0.003*AZ3)</td>
<td>2</td>
<td>(S3*(BK3/1000))/(T3*Q3)</td>
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<td>BM</td>
<td>BN</td>
<td>BO</td>
<td>BP</td>
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<tr>
<td>Basal Venous O₂ conc. (umol/ml)</td>
<td>Basal (A-V) O₂ difference (ml/100ml)</td>
<td>Basal (A-V) O₂ difference (umol/ml)</td>
<td>Basal O₂ consumption (ml/100ml/min)</td>
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<td>(BL₃*1000000)/100</td>
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<td>AF₃-BM₃</td>
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<td>BR₃/O₃</td>
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