VASOCONSTRICTOR-MEDIATED CONTROL OF THERMOGENESIS

by

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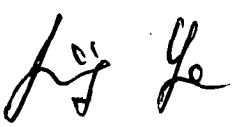
Thanks to:

Eric

Dedicated to:
my wife,
parents,
and son.

DECLARATION

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



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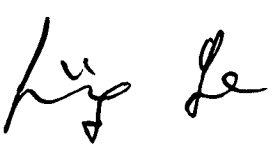

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ABSTRACT

Vasoconstrictors either stimulate or inhibit oxygen consumption ($\dot{V}O_2$) in perfused, but not in incubated or perifused, skeletal muscle. The mechanism of this phenomenon is not fully understood. This thesis attempted to investigate the implication of vasoconstrictor-mediated control of $\dot{V}O_2$ (thus thermogenesis) in perfused muscle as well as other tissue beds, and the mechanism involved.

Effects of vasoconstrictors on $\dot{V}O_2$ were examined in the perfused hindlimbs of rats, Tasmanian bettongs (*Bettongia gaimardi*) and toads (*Bufo marinus*) as well as in the perfused rat kidney, intestine and mesenteric artery. In line with *in vitro* experiments, the effects of adrenergic agonists on thermogenesis were further studied in conscious adult bettongs, an endothermic animal without detectable brown adipose tissue.

Norepinephrine (NE) and vasopressin (VP) stimulated, but serotonin (5-HT) inhibited $\dot{V}O_2$ in both constant-flow and constant-pressure perfused hindlimbs when causing vasoconstriction. In the constant-flow (once-through) perfusion, these changes were blocked by vasodilators (ie. nitroprusside) but not by tubocurarine. Vasoconstrictor-mediated changes in muscle metabolism were observed in the hindlimbs of all the three species. α-Adrenoceptors were shown to be responsible for NE-stimulated increase in $\dot{V}O_2$. Increasing the perfusion flow by resetting at different rates also stimulated changes in $\dot{V}O_2$ in the constant-flow perfused rat hindlimb, which could be augmented by NE but inhibited by nitroprusside.

Similarly, a vasoconstriction-associated increase or decrease in $\dot{V}O_2$ was observed in the constant-flow perfused rat kidney and intestine although the effect of a given vasoconstrictor to stimulate or inhibit $\dot{V}O_2$ was not necessarily the same as that in the perfused hindlimb. In the perfused rat mesenteric artery all vasoconstrictors stimulated increases in $\dot{V}O_2$ in parallel with the increased perfusion pressure.

By contrast, in the constant-pressure (recirculated) perfused rat hindlimb, β-adrenergic agonists, isoproterenol and BRL35135A were also found to stimulate $\dot{V}O_2$ in addition to α-adrenergic agonists and VP.

During the induced vasoconstriction or vasodilation, creatine phosphate (CrP), AMP, ADP, ATP and lactate remained normal in all the perfused mammalian tissues. In skeletal muscle, the energy status, as indicated by energy charge, was not significantly different among the normal (both *in vivo* and control perfusion), perfused

(high flow plus NE) and ischemic (for 60 min) muscles. In contrast, CrP and Cr differed in the three oxygenation states.

Consistent with the observations in the perfused hindlimbs *in vitro*, NE and isoproterenol elicited an increase in heat production in the intact bettong. α-Adrenergic agonists, phenylephrine and naphazoline were also found to stimulate heat production which was not blocked by propranolol.

These results taken together suggest that skeletal muscle is a potentially thermogenic tissue under the control of vascular functioning. Vasoconstrictor-mediated $\dot{V}O_2$ seems to be widespread across species and tissues. This control may play an important role in the regulation of non-shivering thermogenesis.

The results support the notion that working blood vessels may partially account for vasoconstrictor-stimulated changes in $\dot{V}O_2$. However, the data do not exclude the hypothesis that vasoconstrictors act on site-specific receptors on arterioles to redistribute flow to areas or cells that are metabolically more active. The results of this thesis can not definitively distinguish between these two hypotheses. They may not be mutually exclusive.

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ABBREVIATIONS

AMP	Adenosine-5'-monophosphate
ADP	Adenosine-5'-diphosphate
ATP	Adenosine-5'-triphosphate
AT II	Angiotensin II
BSA	Bovine serum albumin
CA	Cold adaptation
Cr	Creatine
CrP	Creatine phosphate
E	Epinephrine
HP	Heat production
HPLC	High performance liquid chromatography
5-HT	5-Hydroxytryptamine (serotonin)
Iso.	Isoproterenol
LDH	Lactate dehydrogenase
BMR	Basal metabolic rate
G-6-P	Glucose-6-phosphate
NAD	β-Nicotinamide-adenine dinucleotide
NADH	β-Nicotinamide-adenine dinucleotide (reduced)
NADP	β-Nicotinamide-adenine-dinucleotide phosphate
NADPH	β-Nicotinamide-adenine-dinucleotide phosphate (reduced)
NE	Norepinephrine
NP	Nitroprusside
P_aO_2	Arterial partial oxygen pressure
P_vO_2	Venous partial oxygen pressure
UCP	Uncoupling protein
$\dot{V}O_2$	Rate of oxygen consumption
VP	Vasopressin

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CHAPTER 1

Thesis introduction

1.1 General concepts and definitions

1.1.1 Thermogenesis

Endothermic animals are able to maintain a relatively constant body temperature in a wide range of environmental temperatures. Constant body temperature is achieved by balancing heat production and heat loss. In spite of voluntary adaptations in humans such as sheltering their bodies and adjusting the ambient temperature, maintenance of body temperature depends upon a high and controlled rate of heat production, or thermogenesis (Bligh & Johnson, 1973).

Under normal conditions heat is produced in the body as a by-product of metabolism involving the transformation of one kind of energy into another. However, for the endotherm or warm-blooded animal, extra heat is produced when the ambient temperature falls below its thermoneutral zone (Jessen *et al.*, 1980; Rothwell & Stock, 1980; Trayhurn, 1994).

When exposed to cold, an endotherm can adopt two strategies to raise heat production: shivering thermogenesis and nonshivering thermogenesis (NST). The former refers to heat production derived from skeletal muscle contraction whereas the latter means heat production from sources other than skeletal muscle contraction (Bligh & Johnson, 1973). If an endotherm is continuously exposed to cold, shivering thermogenesis gradually disappears and is replaced by NST. During the process of this acclimation, a physiological change occurs within the body which reduces the strain caused by stressful changes in the cold (Bligh & Johnson, 1973).

Heat production is also induced by dietary changes. During this process, extra heat (facultative thermogenesis) is generated besides the obligatory energy expenditure for the digestion (Rothwell & Stock, 1980; Webster, 1983; Astrup *et al.*, 1989). It is believed that diet-induced facultative thermogenesis (DIT) shares similar mechanisms with cold-induced NST (Rothwell & Stock, 1980).

It is this extra heat (NST or DIT) that this thesis will focus on. The term of thermogenesis in the thesis refers to NST as commonly used in the literature (Trayhurn, 1994).

1.1.2 Thermogenesis and O_2 metabolism

A study of thermogenesis requires the measurement of heat emission. Direct calorimetry measures the transfer rate of heat between a tissue, an organ, or an animal and its environment (Bligh & Johnson, 1973). However, the most common method of estimating heat production is to use indirect calorimetry which is based on calculations relating heat production to the measurement of oxygen consumption ($\dot{V}O_2$, the volume of oxygen consumed per unit time) and/or CO_2 elimination (Weir *et al.*, 1949; Bligh & Johnson, 1973; Cassel & Casselman, 1990). Thus $\dot{V}O_2$ in a tissue, an organ, or a whole animal is regarded as a synonymous parameter to heat production. In fact, $\dot{V}O_2$ is usually the most applicable indicator to assess the thermogenesis of a tissue.

1.2 Implications of vascular control of O_2 metabolism

1.2.1 Evidence to support this concept

The vascular control of blood supply to a tissue as a whole and at the onset of ischemia is relatively well understood. However, the role of vascular control of O_2 metabolism in the context of thermogenesis has only been put forward in the last several years (Colquhoun & Clark 1991; Clark *et al.*, 1994). This concept arose largely from comparing the effects of vasoconstrictors on $\dot{V}O_2$ (as well as other metabolites such as lactate efflux) in perfused muscle preparations and in non-perfused (incubated, perifused, superfused or homogenized) muscle preparations (Colquhoun *et al.*, 1988; Dubois-Ferrière & Chinet, 1981; Hettiarachchi *et al.*, 1992; Dora *et al.*, 1994). The major difference between perfused and non-perfused muscle preparation is the involvement of the vasculature. A significant difference in $\dot{V}O_2$ exists between these two preparations in response to vasoconstrictor exposure.

When infused via a vascular route (ie. in the perfused muscular bed), norepinephrine (NE) stimulates $\dot{V}O_2$ muscle thermogenesis by 60-100% (Grubb & Folk., 1977; Dubois-Ferrière & Chinet, 1981; Côté *et al.*, 1985; Colquhoun *et al.*, 1988). In contrast, NE has little effect on muscle $\dot{V}O_2$ if applied directly to a perifused or incubated preparation (Dubois-Ferrière & Chinet *et al.*, 1981; Hettiarachchi *et al.*, 1992). Other vasoconstrictors such as angiotensin II (AT II) and serotonin (5-HT) also affect muscle O_2 metabolism or muscle performance in perfused rat hindlimb, but do not show a direct effect on muscle themselves (Hettiarachchi *et al.*, 1992; Dora *et*

al., 1994). These findings seem to imply a key role for vascular functioning in muscle thermogenesis.

With a similar extrapolation, one would expect that vasculature may exert control of O_2 metabolism in other tissues as well. Thus, if these mechanisms apply to $\dot{V}O_2$ *in vivo*, it would suggest an important role for vascular function in whole body thermogenesis.

1.2.2 Metabolism and vascular system

Clinical observations have indicated links between metabolic disorders and vascular diseases. For example, obesity is a typical metabolic disorder related to a decrease in thermogenesis (Ravussin *et al.*, 1988). Yet, it is also a risk factor for cardiovascular diseases (e.g. hypertension) and diabetes mellitus (MacMahon *et al.*, 1987; Daly & Landeberg, 1991). Population studies have shown correlations between body weight and blood pressure in humans (Hall, 1993). Weight loss seems to attenuate the blood pressure of obese patients with hypertension (Reisin *et al.*, 1978).

The concept of vascular control of metabolism could reveal the hitherto unrecognized importance of the vascular effects of some hormones. For instance, insulin is known to mediate glucose uptake in skeletal muscle, but it has recently been shown to dilate blood vessels in the human leg (Baron, 1993). Likewise, glucagon was shown to induce NST in the bird (Barré *et al.*, 1987a; Duchamp *et al.*, 1993), yet it is also a strong vasodilator (Kazmers *et al.*, 1981). Similarly again, thyroid hormones are reported to reduce peripheral vascular resistance and raise cardiac stroke output (Klein, 1990).

Skeletal muscle is the largest tissue in the body (Elia, 1992). If its metabolism is largely controlled by vasculature, the impact of vascular functions on whole body metabolism would be expected to be significant.

1.3 Methodology for the study of thermogenesis

Theoretically, a direct measurement of increased temperature or heat efflux should be an ideal indicator of thermogenesis. In whole body studies, the measurement of body temperature is often conducted for this purpose. As temperatures differ at different parts of a body, the core temperature (often measured in the rectum) is perhaps most representative. The most accurate measurement of

thermogenesis would be the heat efflux. Unfortunately, this has proven to be difficult to measure in many cases, particularly at tissue, cellular or subcellular levels (Ducharme 1990).

For most endotherms, heat production (therefore thermogenesis) is coupled with $\dot{V}O_2$ since aerobic metabolism is the predominant pathway to generate heat. As mentioned earlier (Section 1.1.2.), $\dot{V}O_2$ is probably the most commonly employed parameter used to judge thermogenesis (Cassel & Casselman, 1990). This measurement can be readily undertaken at each level: whole body (Bligh & Johnson, 1973), isolated organs and tissues such as skeletal muscle (Bonen *et al.*, 1994) and subcelluar mitochondria (Cassel & Casselman, 1990). During the process of the conversion of substrates to products in aerobic metabolism, many intermediate metabolites (such as lactate, pyruvate, free fatty acid and glycerol, etc.) are involved. Thus the measurement of these substrates, intermediate metabolites and end products can provide important information for evaluation of metabolism and the mechanisms involved (Bonen *et al.*, 1994).

At the subcelluar level, the mitochondrion is the subcellular apparatus where a proton concentration gradient across the inner mitochondrial membrane that carries both potential and chemical energy is transferred to O_2. In this biochemical process, O_2 is consumed with the energy transferred into a chemical bond ("high energy phosphate bonds") of adenine nucleotide triphosphate (ATP). Thus the oxidation of metabolic substrates is coupled with phosphorylation of ADP to ATP. Since oxidative phosphorylation hinges on the oxidative capacity of mitochondria, properties of mitochondria, such as mitochondrial volume in the cell, mitochondrial respiration rate, and mitochondrial cytochrome oxidase are considered to be crucial to allow an increase in $\dot{V}O_2$ in a tissue (Jansky, 1973).

1.4. Facultative thermogenesis and effector tissues

For a particular biochemical system to contribute to facultative thermogenesis in the operational sense, Trayhurn (1994) proposed four criteria be fulfilled:

1. there should be the capacity for generating a considerable amount of heat;
2. the mechanism should be rapidly turned on or off;
3. there should be long-term adaptive changes consistent with variations in the requirement for thermogenesis;

4. the mechanism should be located in a tissue which physiological studies clearly indicate as being directly involved in facultative heat production.

Furthermore blood flow to this tissue is critical as a high blood flow enables heat to be rapidly distributed to the body core, and also ensure that the substrates (including O_2) required to fuel thermogenesis are provided at rates appropriate to the exceptional demands of the tissue.

1.4.1 Brown adipose tissue (BAT)

BAT has been found to be present in most small rodents and newborn mammals including humans. So far, BAT has been generally recognized as a specialized heater tissue which seems to fill all the criteria suggested by Trayhurn (1994). Histological study reveals that BAT is mainly located around the aorta, the heart and kidney vessels and within the interscapular, subscapular and axillary regions as well as at the nape of the neck (Himms-Hagen, 1990). Unlike white adipose tissue, which stores large amounts of triglyceride but contains very little mitochondria, BAT is rich in mitochondria as well as triglyceride stores..

In the rat, the estimated mass of BAT is 1-3% of the body mass (Foster & Frydman, 1978; Astrup, 1986; Himms-Hagens, 1990). The measured basal $\dot{V}O_2$ in the perfused BAT was reported to be 50-64 $\mu mol \cdot g^{-1} \cdot h^{-1}$ (Foster *et al.*, 1980; Matthias *et al.*, 1994) and increased to 2121 $\mu mol \cdot g^{-1} \cdot h^{-1}$ in response to various stimulations including NE (Foster *et al.*, 1980). In small rodents, BAT is estimated to account for approximately 60% of cold-induced thermogenesis (Foster *et al.*, 1980; Astrup *et al.*, 1986). Measurement of blood flow to BAT with radioactively-labelled microspheres reveals cold exposure can increase blood distribution to BAT by more than 30 fold (Foster and Frydman 1979).

A recent study, by detecting the expression of uncoupling protein (UCP) mRNA, indicates that some of the rat white adipose tissues, such as periovarian adipose tissue, express UCP following exposure to 4°C for three days (Cousin *et al.*, 1992). This change is associated with an increase in the density of mitochondrial cristae. Cold exposure or hyperphagia can stimulate the hypertrophy of BAT (Webster, 1983; Astrup, 1986; Himms-Hagen, 1990).

However, in larger animals the quantitative contribution of BAT to thermogenesis is yet to be fully elucidated. BAT is thought to be absent in pigs (Trayhurn *et al.*, 1989) and birds (Trayhurn, 1994) and has become functionally unimportant in the adult human even though NST still exists (Jamieson *et al.*, 1984;

Astrup, 1986; Duchamp *et al.*, 1993). Clearly, other tissues must contribute significantly to NST in these animals (Trayhurn 1994).

1.4.2 Skeletal muscle

Skeletal muscle might at first sight look unlikely to be a thermogenic effector because of its low resting metabolic rate (Ruderman *et al.*, 1971) and little increase in $\dot{V}O_2$ when exposed to NE in an incubated or perifused muscle preparation (Hannon *et al.*, 1963; Eaton, 1964; Dubois-Ferriére & Chinet *et al.*, 1981; Hettiarachchi *et al.*, 1992). In spite of its low energy cost at rest, resting skeletal muscle as a whole is thought to contribute to 20-40% of the whole body metabolic rate because of its large proportion in the whole body mass [approximately 40% (Astrup, 1986; Zurlo *et al.*, 1990; Elia, 1992)].

A number of research groups (Grubb & Folk., 1976, 1977; Richter *et al.*, 1982a, 1982b; Côté *et al.*, 1985; Chinet *et al.*, 1989; Colquhoun *et al.*, 1988, 1990) have clearly demonstrated that NE is able to elicit $\dot{V}O_2$ 60-100% above the basal level when administered via a vascular route to the tissue, such as in the constant-flow perfused hindlimb. Other thermogenic agents such as ephedrine, also stimulate $\dot{V}O_2$ in the constant-flow perfused skeletal muscle (Hettiarachchi *et al.*, 1991). In contrast to BAT, a coordination of vasculature function appears essential for skeletal muscle to be thermogenically functioning.

Perhaps, more direct evidence *in vivo* for muscle thermogenesis comes from the human muscle studies (Lundholm & Svedymr, 1965; Astrup *et al.*, 1985 & 1989). Lundholm & Svedymr (1965) found that administration of epinephrine (E) stimulates forearm $\dot{V}O_2$ by 50% at the peak. A similar result was observed in human legs when ephedrine is infused intravenously (Astrup *et al.*, 1985). More recently, Astrup *et al.* (1989) demonstrated that a carbohydrate diet significantly stimulates O_2, glucose and lactate uptakes by the forearm, which correlated well with the increase in whole body metabolism and with increases in plasma NE and E.

An increased blood flow distribution to skeletal muscle has been observed in various species during cold exposure. For example, when the ambient temperature was decreased from 25° C to 6 °C, the blood flow to non-respiratory skeletal muscle increased by 50% in rats. (Foster and Frydman, 1979). The blood flow to skeletal muscle doubled when exposed to cold in ducklings (Duchamp *et al.*, 1993) and a marsupial, red kangaroo (Needham & Dawson, 1984).

Skeletal muscle is histologically and biochemically heterogenous. Vertebrate skeletal muscle fibres are classified into three types as illustrated in Table 1-1 (Armstrong *et al.*, 1984). They show heterogeneity in metabolite contents as well as $\dot{V}O_2$. Slow-twitch oxidative muscle fibre (type I) has lower high energy phosphate (HEP) content but higher mitochondrial oxidative capacity. Conversely, fast-twitch glycolytic fibre has higher HEP but lower mitochondrial oxidative capacity (Pagliassotti & Donovan, 1990b; Philippi & Sillau, 1993). Zurlo *et al.* (1994) have found that there is a positive correlation between total fast-twitch muscle fibres and the forearm $\dot{V}O_2$.

Table 1-1 Classification of muscle fibre types

Muscle type	Colour	Physiological feature	Oxidative enzymes	Glycolytic enzymes	HEP enzymes
Type I	Red	SO	High	Low	Low
Type IIa	White	FOG	High	High	High
Type IIb	Intermediate	FG	Low	High	High

Modified from Armstrong *et al*, 1984; Zurlo *et al.*, 1994. SO: slow-twitch oxidative; FOG: fast-twitch oxidative glycolytic; FG: fast-twitch glycolytic. HEP: high-energy phosphate.

In the longer term, cold exposure has been shown to increase capillary density in skeletal muscle (Heroux & Pierre, 1957; Sillau *et al.*, 1980) and NE turnover rate (Dulloo *et al.*, 1988). Following cold acclimation at 4°C for 4 weeks, the activity of cytochrome oxidase increased by 34% per 100 g in the muscovy duckling, an animal devoid of BAT (Barré *et al.*, 1987b). Therefore it is likely that these changes can have an impact on whole body metabolic rate. However, the involvement of adrenoceptors in catecholamine-mediated muscle thermogenesis is still controversial. Most studies in human forearms show the stimulatory effect of only β-adrenergic receptors (Astrup *et al.*, 1985 & 1989; Blaak *et al.*, 1993). In contrast, results from the constant-flow perfused hindlimb seem to suggest that α-adrenoceptors are mainly responsible. In a constant-pressure perfused rat hindlimb, Hardeveld *et al* (1980) observed inhibitory effect of NE on $\dot{V}O_2$. There seems to be a need to further clarify the effect of α-adrenoceptors on muscle thermogenesis.

1.4.3 Working microvasculature

Smooth muscle is the contracting apparatus of blood vessels. The attachment of myosin with actin is regulated by the phosphorylation of the myosin light chain MLC. ATP is consumed during the phosphorylation of MLC (Hai & Murphy, 1988, 1989). Myosin light chain kinase (MLCK) which catalyses MLC phosphorylation is activated by Ca^{2+}-calmodulin complex. Compared with skeletal muscle, the work production of vascular smooth muscle is much less efficient when it contracts. However, during the maintenance of contraction, "latch-bridges" (attached dephosphorylated cross bridge) form and less ATP is required in the swine carotid artery (Hai & Murphy, 1988 &1989). The notion for working vasculature to be a thermogenic tissue resides in a fundamental principle: vasoconstriction costs energy.

Evidence for working vascular blood vessels was initially derived from observations of perfused skeletal muscle bed *in vitro*. In the constant-flow perfused rat hindlimb, vasoconstrictors such as NE, α-adrenergic agonists and AT II stimulate $\dot{V}O_2$. The vasoconstrictor-induced $\dot{V}O_2$ is tightly associated with an increase in perfusion pressure (Colquhoun *et al.*, 1988 & 1990). Both the increased pressure and $\dot{V}O_2$ are blocked by vasodilators, such as nitroprusside or isoproterenol. In addition, vasoconstrictor-mediated $\dot{V}O_2$ is additive to that induced by skeletal muscle contraction during sciatic-nerve stimulation (Colquhoun *et al.*, 1990). However, literature data for vascular $\dot{V}O_2$ vary many folds from 3 to approximately 300 $\mu mol \cdot g^{-1} \cdot h^{-1}$, probably due to the vascular tissues used (Paul, 1980; Sussman *et al.*, 1988).

1.4.4 Liver

Liver is the largest internal organ and has a high metabolic rate. In the rat, liver is approximately 2.5-5% of the body mass. The $\dot{V}O_2$ in isolated rat liver cells is measured at 145 $\mu mol \cdot g^{-1} \cdot h^{-1}$ under basal conditions, and this value can increase by more than 100% when palmitate is added (Berry *et al.*, 1989). After exposure to 4°C for 4 weeks, oxidative capacity, measured as mitochondrial cytochrome oxidase, increases by 122% and 54% per 100 g body weight in rats and muscovy ducklings, respectively (Barré *et al.*, 1987b). Measurement of blood flow with radio-labelled microspheres in muscovy ducklings showed a significant increase in blood flow to liver after cold adaptation (Duchamp *et al.*, 1993). However, the study of cold-induced NST in man shows that the splanchnic thermogenesis remains uninfluenced even though the whole body $\dot{V}O_2$ and plasma NE level increased by 25% and 132%,

respectively (Jessen *et al.*, 1980). In adult humans, the liver is estimated to contribute 21% of the metabolic rate (Elia 1992)

Table 1-2 Tissues considered to be responsible for NST in rats

	BAT[a]	Muscle[b]	Liver[c]	Microvessels[d]
Basal	64 $\mu mol \cdot g^{-1} \cdot h^{-1}$	25 $\mu mol \cdot g^{-1} \cdot h^{-1}$	145 $\mu mol \cdot g^{-1} \cdot h^{-1}$	288 $\mu mol \cdot g^{-1} \cdot h^{-1}$
Max.	2121 $\mu mol \cdot g^{-1} \cdot h^{-1}$	40 $\mu mol \cdot g^{-1} \cdot h^{-1}$	300 $\mu mol \cdot g^{-1} \cdot h^{-1}$	? $\mu mol \cdot g^{-1} \cdot h^{-1}$
% of body wt.	~ 1-3%?	~ 40%	2.5-5%	~ 3% ?
NE stimulation	$\dot{V}O_2\uparrow$	$\dot{V}O_2\uparrow$	$\dot{V}O_2$?	$\dot{V}O_2\uparrow$
After CA				
Blood flow	+++	++	++	
Cyto. oxidase	+++	++	++	?

CA: cold adaptation. Data were quoted for the rat except for microvasculature (bovine brain) from [a] Foster *et al.*, 1980; Matthias *et al.*, 1994; [b] Grubb & Folk, 1977; Foster *et al.*, 1979; Elia, 1992;[c] Berry *et al.*, 1989; Elia, 1992; [d] Sussman *et al.*, 1988.

1.5 Energy dissipating mechanism for NST

While extensive attempts have been made to elucidate the biochemical basis for NST (Chinet, 1989; Colquhoun & Clark, 1991; Clausen *et al.*, 1991; Block, 1994; Trayhurn, 1994), the energy dissipating process accounting for thermogenesis apart from BAT is poorly understood. Table 1-3 lists various mechanisms that have been suggested in the literature.

1.5.1 Oxidative phosphorylation uncoupling

Among these putative mechanisms, only uncoupled oxidative phosphorylation in BAT has been widely accepted as a specialized thermogenic mechanism (Trayhurn, 1994). The biochemical basis for this mechanism is the presence of a proton conductance pathway located in the mitochondrial inner membrane of BAT (Himms-Hagen, 1990).

Table 1-3 Putative mechanisms and sites for NST

Mechanism	Tissue	Reference
Sodium pump (Na^+, K^+-ATPase)	Skeletal muscle	Shiota *et al.*, 1988
Sarcoplasmic reticular Ca^{2+} cycle	Heater cells	Block, 1994
Microvasculature contraction	Working blood vessels	Colquhoun *et al.*, 1991
Uncoupling	BAT,	Himms-Hagen, 1990
	Skeletal muscle (?)	Barré *et al.*, 1986

Modified from Trayhurn, 1994.

Under normal circumstances, $\dot{V}O_2$ is coupled with the synthesis of ATP. This process is carried out by the respiratory chain in the inner membrane of mitochondria. When the electrons are transferred from NADH and $FADH_2$ to O_2, protons are pumped across the inner membrane to the intermitochondrial space, thus forming a proton gradient. ATP is formed when these protons flow back to the mitochondrial matrix though ATPase (Stryer 1988). UCP works as a proton conductance pathway to dissipate the proton gradient across mitochondrial inner membrane (Trayhurn 1989; Himms-Hagen, 1990). When protons permeate the mitochondrial membrane through UCP, energy is released as heat rather than generating ATP. During uncoupling, the respiratory chain still carries the electrons from NADH and $FADH_2$ to O_2. Thus O_2 is consumed to generate heat. This thermogenic process is controlled by sympathetic nervous system with free fatty acid involved (Astrup *et al.*, 1986, 1989; Himms-Hagen 1990).

UCP is a dimer of 3.2-3.3 kd. Up until now, it has been identified exclusively in BAT. Tissue specificity of UCP has led Trayhurn and his colleagues (1994) to identify whether BAT is present or absent in a species by detecting UCP with molecular techniques such as western blotting or northern blotting. Following an intensive search in a wide variety of animals, they concluded that UCP (thus functional BAT) is restricted to mammals and the pig is the only mammal with no detectable BAT (Trayhurn, 1994).

When an animal is exposed to cold or the sympathetic nervous system is stimulated, the released NE acts on the β-adrenergic receptor of the BAT and elicits the lipolysis of triglyceride via cAMP. Released free fatty acid then activates the function of UCP. When the proton conductance pathway is opened, the

electrochemical gradient across the inner membrane of the mitochondria is dissipated. Instead of coupled with the formation of ATP, heat is produced. The oxidative phosphorylation uncoupling is inhibited by purine metabolites such as ATP, ADP, GTP, and GDP (Himms-Hagen, 1990).

The finding of uncoupling as the biochemical basis for BAT thermogenesis has provoked the prediction that uncoupling of oxidative phosphorylation might also be a mechanism of heat production in other potentially thermogenic tissue(s) such as skeletal muscle. When a similar approach was applied to the billfish ophthalmic muscle, a specialized heat tissue to maintain brain temperature, immunological studies ruled out the expression of UCP (Block *et al.* 1987; 1994). Hence it can be predicted that the basis of uncoupling in skeletal muscle, if present, might be different from that of BAT.

1.5.2 Ion pumping

Ion pumping is another hypothesized mechanism for NST (Shiota *et al.*, 1988; Block *et al* ., 1994). The energy cost accounting for cation transport in the process of thermogenesis has recently been extensively reviewed by Clausen *et al.* (1991). The major cation transport systems suggested to account for thermogenesis are N^+-K^+ transport across a plasma membrane and Ca^{2+} transport across the sarcoplasmic reticulum (SR) membrane.

1.5.2.1 Na^+-K^+ transport

The strongest evidence in favour of Na^+-K^+ transport as a muscle thermogenesis mechanism, so far, arose from the report of the ouabain blockade of NE-mediated $\dot{V}O_2$ (Shiota *et al.* 1988). In constant-flow perfused hindlimbs at 32°C with a medium free of red blood cells (RBC) and BSA, they found that NE-stimulated $\dot{V}O_2$ was associated with a decrease in potassium efflux from cold-acclimated but not warm-adapted rats. The increased $\dot{V}O_2$ and decreased potassium efflux were blocked by 1 mM ouabain. These results led the authors to conclude that Na^+-K^+ transport was the biochemical basis for NE-induced thermogenesis in skeletal muscle (Shiota *et al.*, 1988).

However, their findings are in discord, in many aspects, with the data obtained from similar perfused muscle preparations by other groups who reported that NE is able to stimulate $\dot{V}O_2$ in the perfused muscle with RBC at 37°C (Grubb & Folk,

1977; Richter *et al.*, 1982a) or without RBC at 25°C (Côté *et al*, 1985; Colquhoun *et al.*, 1988, 1990) of warm-adapted animals.

Clausen and his colleagues (1991) estimated that the intact muscle utilizes only 4-10% of its total energy turnover for active Na^+-K^+ transport under basal resting or during contractile activity. In liver, Na^+-K^+ transport accounts for < 5% of its total $\dot{V}O_2$ under basal conditions (Clausen *et al.*, 1991). These data appear to suggest that Na^+-K^+ transport is not a major biochemical basis for NST.

1.5.2.2 SR Ca^{2+} cycling

Perhaps, the role of sarcoplasmic reticulum (SR) Ca^{2+} cycling is more attractive than Na^+-K^+ transport in terms of the role of cation transport in muscle thermogenesis. In normal muscle, intracellular Ca^{2+} is controlled by the T tubule and SR Ca^{2+} concentration. In the endothermic fish such as tuna and bill fish, thermogenesis is thought to be of muscle-origin (Block, 1994). The author hypothesized an excitation-thermogenic coupling model in the billfish heater cell with Ca^{2+} to play a key role. The specialized skeletal muscles have an exquisite mechanism for depolarisation-induced Ca^{2+} release. Such control is combined with stimulation of oxidative phosphorylation and substrate catabolism via Ca^{2+} release and reuptake at the SR in the heater cells.

The key element in this hypothesis would be the increased permeability of the SR membrane to Ca^{2+}. Immunological studies of the SR protein involved in Ca^{2+} cycling indicate that the heater cell has a unique expression pattern of the SR Ca^{2+} release channel with two isoforms: α and β while mammalian skeletal muscles express a single isoform of the SR Ca^{2+} release channel (Block, 1994). However, it could be argued that mechanisms for the ATP-dependent Ca^{2+} cycling in the SR membrane may only occur in the specialized skeletal cells such as heater cells in billfish. The specific inhibitor of Ca^{2+} release from SR through the ryanodine receptor/calcium channel, dantrolene, has no effect on $\dot{V}O_2$ in the perfused rat hindleg preparation (Chinet & Mejsnar, 1989). In the non-perfused soleus muscle, dantrolene only inhibited heat production by 8-9% with a concomitant 4% drop in $\dot{V}O_2$ (Chinet & Mejsnar, 1989).

Clausen *et al* (1991) concluded, based on an extensive review of literature data, that the energy cost of maintaining a steep Ca^{2+} gradient across the SR membrane is relatively small (5%) of the resting metabolic rate due to the low permeability to Ca^{2+} at rest and high thermodynamic efficiency of the Ca^{2+}-ATPase.

1.5.3 Vascular smooth muscle contraction

As vascular smooth muscle has mitochondria, contracting vasculature will consume O_2. $\dot{V}O_2$ by working microvasuclature has also been considered to be a possible candidate contributing to NST (see Section 1.4.3.). So far, it is not known quantitatively how much the working microvasculature can contribute to NST.

1.6 Endocrine control of thermogenesis

NST is under the control of the endocrine system. Either cold exposure or diet invokes a response of the endocrine system which acts on each effector to generate heat. The endocrine system controls thermogenesis in two different but also interwoven ways: (i) accelerate heat production directly from pre-existing thermogenic tissues; (ii) promote structural hypertrophy and other adaptive changes ie increasing vascularity and mitochondrial function.

1.6.1 Sympathetic system

Both cold exposure and diet mediate the release of catecholamines into plasma. In the human, a carbohydrate diet raised plasma NE and E by 35-45% and 130%, respectively (Welle & Campbell, 1983; Astrup *et al.*, 1989). In guinea-pigs, urinary NE increased by 360% following 4 weeks of cold challenge at 4°C (Kinnula *et al.*, 1983). The effect of NE on thermogenesis has been reviewed in previous sections (ie. Sections 1.4.1, 1.4.2 & 1.4.3). Most studies have demonstrated that β-adrenergic receptors are responsible for NE-mediated thermogenesis (Rothwell & Stock, 1980, Astrup *et al.*, 1989; Blaak *et al.*, 1993).

Several lines of evidence suggest that α-adrenergic receptors may also be involved in NE-mediated thermogenesis. Despite α-adrenergic stimulation of $\dot{V}O_2$ in the constant-flow perfused muscle vascular bed, the results from Foster (1984) indicate an auxiliary role of α_1-adrenergic receptors in rat BAT thermogenesis. The data from whole body metabolic rate studies in the rat (Rothwell, 1990), rabbit (Szreder, 1991) and dog (Liard, 1989) also indicate the involvement of α_1-adrenergic receptors in NE-mediated metabolism.

1.6.2 Thyroid hormones

The serum thyroxine (T_4) and tri-iodothyroxine (T_3) increased 85% and 87%, respectively, in guinea pigs following 4 weeks of cold challenge at 4°C (Kinnula *et al.*, 1983). Raised serum T_3 level has also been reported in rats exposed to cold (Jobin *et al.*, 1973) or overfeeding (Rothwell & Stock, 1980). Isolated hepatocytes from rats treated daily with T_3 have an elevated $\dot{V}O_2$.(Berry *et al.*, 1989). In BAT, T_3 exerts a permissive effect for NE and is thought to be essential for UCP expression (Himms-Hagen, 1990; Nedergaard, 1994). Additionally, thyroid hormones increase cardiac output and reduce peripheral vascular resistance (Klein, 1990). However, the extent and significance of T_3 and T_4 on thermogenesis is not clear.

1.6.3 Other hormones

Other hormones involved in thermogenesis may include glucagon as well as insulin and glucocorticoids (Himms-Hagen, 1990). In muscovy ducklings, thermogenesis can be induced by a direct injection of glucagon (Barré *et al.*, 1987a). In the same species, glucagon has also been shown to stimulate $\dot{V}O_2$ of leg muscle, and increase blood flow to muscle and liver (Duchamp *et al.*, 1993). In man and rat, acute cold exposure induces a rapid rise in plasma glucagon (Seitz *et al.*, 1981). In guinea pigs, there is a significant rise in serum cortisol (by 81%) after exposure to 4°C for 4 weeks (Kinnula *et al.*, 1983).

The effects of vasoconstrictive hormones on $\dot{V}O_2$ and other metabolites in the constant-flow perfused rat hindlimb have recently been reviewed by Clark and colleagues (1994). Based on their effect on $\dot{V}O_2$, vasoconstrictors can be classified as metabolically positive (type A) or negative (type B). Type A vasoconstrictors include NE at low concentrations ($\leq$ 1.0 μM), α-adrenergic agonists, AT II, vasopressin (VP), whereas type B vasoconstrictors include NE at high concentration ($\geq$ 2.5 μM), serotonin (5-HT, $\geq$ 0.1 μM). And yet, it has not been identified whether this classification applies to other perfused tissue preparations and ultimately to metabolism *in vivo*.

1.7 Signalling system of thermogenesis

Intracellular signals for thermogenesis are those coupled with specific receptors such as inositol tri-phosphate (IP_3) with α-adrenergic receptors and β-

adrenergic receptors with cAMP. In the perfused rat hindlimb, Dora (1993) has shown that NE-mediated $\dot{V}O_2$ is Ca^{2+} dependent. In BAT, cAMP is an important second messenger for β-adrenoceptor-mediated thermogenesis (Himms-Hagen, 1990). When NE elicits thermogenesis in BAT, it acts on β_3-adrenergic receptors to activate adenylate cyclase that catalyzes the formation of cAMP from ATP. The increased cAMP then elicits lipolysis of triglyceride to liberate free fatty acid.

Free fatty acid, resulting from lipolysis of triglyceride, is of particular importance in NST. On the one hand, it acts as a messenger to trigger the operation of UCP in BAT that dissipates the proton gradient across the mitochondrial inner membrane to generate heat. On the other hand, free fatty acid provides energy as a fuel for this thermogenic process (Himms-Hagen, 1990). Although a direct effect of FFA on muscle thermogenesis has not been described, it is considered to be involved in the SR membrane ATP-dependent Ca^{2+} cycling heater cell (Block, 1994). In the liver from normal rats, free fatty acid doubles $\dot{V}O_2$ (Berry *et al.*, 1989). The evidence to support the role of free fatty acid in NST also includes its stimulatory effect on hepatocytes $\dot{V}O_2$ (Berry *et al.*, 1989) and a rise in plasma free fatty acid in human when stimulated by NE (Kurpad *et al.*, 1994).

1.8 Vascular control of O_2 metabolism and thermogenesis

A comparison of the effects of vasoconstrictors on $\dot{V}O_2$ between the perfused and nonperfused muscle preparations has suggested a key role of vascular functioning in $\dot{V}O_2$. The following three issues will be addressed regarding the involvement of vascular system in thermogenesis. How does the vascular system control blood flow to increase $\dot{V}O_2$? Does the vasculature release thermogenesis-stimulating substance(s) to the supplied tissues? Can microvesels themselves consume enough O_2 to become thermogenic?

1.8.1 Structure of microcirculation

A typical structure of microcirculation involves small arteries, arterioles, precapillary sphincters, capillaries, venules and small veins (Greenberg *et al.*, 1983; Lundgren, 1984). The exchange between blood and the tissue occurs at the capillary level and largely relies on the access of blood flow to these areas. This is controlled by the upstream small arteries, arterioles and precapillary sphincters. The precapillary microvessels undergo regulation by the nervous system, blood hormones and intrinsic

mechanisms. The innervation of the microcirculation shown in Table 1-4 provides a structural basis for the control of microcirculation by the neuro-endocrine system.

Table 1-4 Adrenergic innervation in splanchnic and skeletal muscle microcirculation

	Evidence	
Microvessel	Electromicroscopic	Histochemical
Small muscular arteries	++++	++++
Arterioles	++++	+++
Terminal arterioles	++	++
Precapillary sphincters	±	–
Collecting venules	–	–
Muscular venules	±	±
Small veins	++	++

+, Degree of innervation; –, lack of innervation. Modified from Altura *et al.*, 1978a,b.

1.8.2 Autoregulation and intrinsic control mechanism

Autoregulation is defined as the tendency for blood flow to remain constant despite fluctuation in arterial perfusion pressure. This is a local property of the vasculature that exists in the absence of neural and humoral factors in most vascular beds. Three major mechanisms of the intrinsic vascular control include metabolic, myogenic and flow-dependent regulation (Meininger, 1991). Their interactions determine the blood perfusion in the microcirculation.

The function of precapillary arteries is controlled by two systems: a remote system and a local system (Folkow, 1991). The remote control system includes vasoconstrictive nerve fibres, vasodilator nerve fibres (in some circuits only) and blood-borne excitatory and inhibitory influences. On the other hand, the local control system involves myogenic activity, positive feedback (by the continuous and pulsative stretch of blood pressure), negative feedback (by vasodilator metabolites), and

endothelial activity (Folkow, 1991). The interaction between these two systems exerts important control on the parenchymal tissue metabolism.

In the resting state, some of the true capillaries are shut off by the upstream vasoconstriction of arteriole and precapillary sphincters predominantly controlled by remote vasoconstrictive nerves. The closure of the true capillaries leads to an accumulation of metabolites, such as H^+, CO_2, adenosine and an increase in osmolarity. As the concentration of these metabolites accumulates to a certain high level, their vasodilatory effect will override the vasoconstriction maintained by sympathetic nerves and dilates the closed blood vessels, thus opening the capillary bed for blood perfusion. When the blood flow washes away the accumulated local metabolites, the vasoconstrictive control becomes dominant again, thus closing the true capillaries (Folkow, 1991).

Recent studies have shown that the vasodilation induced by local metabolites can be conducted upstream to the larger arterioles and feed artery, leading to an increase in blood flow to skeletal muscle (Meininger *et al.*, 1991; Segal, 1992). The vasodilation is probably conducted along the arteriole wall via gap junctions between endothelial cells and /or smooth muscle cells (Segal, 1992).

1.8.3 Blood distribution in microcirculation

Heterogeneous perfusion of capillaries has been noted in the microcirculation of muscle (Lindbom & Arfors, 1985; Johnsson & Haraldsson 1988) and intestine vascular beds (Jacobson *et al.*, 1982; Lundgren 1984). Microscopic observations suggest that blood flow in individual capillaries is intermittent with constantly shifting perfusion of a variable fraction of the microvascular network. In the rat gracilis anticus muscle, a study with fluorescein dye showed a fraction of 61% open capillaries at rest (Connolly, 1976). The open-capillaries increase during tetanic muscle contraction with all of the capillaries opened at 12 tetani/min. Honig *et al.* (1980) counted red blood cell-containing capillaries in a cross section of dog gracilis muscle quick-frozen at rest and at various times during twitch stimulation. The results show that the open-capillaries are 655/mm^2 at rest and the recruited capillaries almost double after 15 min of contraction at 4 twitches/second. Hence, each true capillary bed maintains an interchanging state of opening-closing.

Microscopic studies attempting to quantify microvascular flow patterns by measuring local RBC flow velocities were carried out by Johnson and Wayland (1967) who described five kinds of temporal flow variations in capillaries of cat mesentery. Pattern A is the thoroughfare channel where there is a high RBC velocity

with irregular and random variations. Pattern B is nonperiodic flow with large velocity variations. Pattern C is periodic flow. Pattern D is low RBC velocity with irregular and random variations. Pattern E is intermittent flow (on-off). The periodic and pulsatile flow patterns are attributed to the action of precapillary sphincters. In the cat sartorius muscle, Burton and Johnson (1972) described three RBC velocity patterns. Among them, 65% of capillaries maintain steady flow with small variations, 10% vary periodically in velocity and 25% fluctuate irregularly in velocity (going down to zero brief intervals).

A functional shunt of blood flow in muscle capillaries has been proposed based on clearance of substance (antipyrine, urea, sucrose) and ^{24}Na as well as inert gas studies (Renkin, 1971; Piiper, 1985). If there is such a physiological non-flow phenomenon which restricts O_2 delivery to certain capillaries, the improvement of the flow to these capillaries should enhance $\dot{V}O_2$ provided a biochemical basis for the consumption of this O_2 is present.

1.8.4 Effect of vasoconstrictors on $\dot{V}O_2$

As presented in Section 1.4.2, several studies in the constant-flow perfused muscle show that α-adrenergic receptors are responsible for NE-mediated $\dot{V}O_2$. In addition to NE, other vasoconstrictors such as VP and AT II also stimulate $\dot{V}O_2$ and are associated with a rise in perfusion pressure. However, not all vasoconstrictors increase $\dot{V}O_2$ in the perfused hindlimb. Dora *et al.* (1991) reported that 5-HT actually inhibits $\dot{V}O_2$ during vasoconstriction. Thus, vasoconstrictors may work as a switch to turn on or off skeletal muscle thermogenesis.

1.8.5 Relationship between perfusion flow and $\dot{V}O_2$ in skeletal muscle

In the perfused rat heart, $\dot{V}O_2$ increases when there is an increase in perfusion flow and therefore perfusion pressure (Sparks *et al.*, 1983; Kojima *et al.*, 1993). There is little doubt that increasing perfusion flow above the basal level augments $\dot{V}O_2$ in contracting skeletal muscle (Bockman, 1983; Mohrman *et al.*, 1988; Brechue *et al* ., 1993). Flow-induced $\dot{V}O_2$ is also found in canine intestine (Jacobson *et al.*, 1982; Gallavan *et al.*, 1983). In these cases, perfusion flow would appear to be a decisive factor to limit the metabolism and performance. It is also generally agreed that $\dot{V}O_2$ in resting skeletal muscle would be restricted when a perfusion flow falls below the critical level needed to meet basal aerobic metabolism.

Hence the concept of flow-induced $\dot{V}O_2$ in the resting skeletal muscle here is defined as the flow range beyond the need to maintain its basal metabolism. Such a conceptual flow-$\dot{V}O_2$ relationship has thermogenic implications. Since no energy is spent in skeletal muscle contraction under these conditions, the increased $\dot{V}O_2$ would become heat. This argument is strongly supported by a simultaneous measurement of flow-induced heat production and $\dot{V}O_2$ in the perfused rat gracilis muscle (Chinet & Majsnar, 1989). More importantly, a better understanding of flow-induced $\dot{V}O_2$ would provide insight into the role which the vascular system plays to control muscle thermogenesis.

In the literature, reports on the effect of perfusion flow on $\dot{V}O_2$ have been a matter of controversy. Listed in Table 1-5 are the results of many of these reports and the experimental conditions employed. In general, flow-induced $\dot{V}O_2$ is more readily observed in small animals such as rodents and cats, but is not always seen in dogs. The reason for these mixed reports are not clear and may lie with the animals or experimental conditions employed. For example, in isolated autoperfused dog muscle, Stainsby (1964) and Kohzuki (1991) reported that $\dot{V}O_2$ in the resting skeletal muscle remains at an almost constant value until blood flow reaches the critical O_2 delivery. In the dog, this level was reported to be 0.45 $ml \cdot min^{-1} \cdot 100g^{-1}$ (Kohzuki *et al.*, 1993). On the other hand, Pappenheimer (1941b) observed flow-induced $\dot{V}O_2$ in the auto-perfused dog hindlimb.

One explanation for flow-induced $\dot{V}O_2$ in resting isolated perfused skeletal muscle is that the increase in perfusion flow allows a perfusate or O_2 to reach more capillaries (Chinet *et al.* 1989). In the isolated gracilis muscle from cold-adapted rat, flow induced $\dot{V}O_2$ was parallel with heat production when perfused with RBC-free Krebs-Ringer buffer containing 5% BSA (Chinet *et al.* 1989). However, when perfused with the same medium with 20% washed RBC, the perfusion pressure was significantly higher at the same flow rate and $\dot{V}O_2$ had little dependence on flow rate. Their data reveal that flow-induced $\dot{V}O_2$ and heat production in the RBC-free perfusate still exist beyond the $\dot{V}O_2$ values where an increase in flow could no longer elicit $\dot{V}O_2$ with RBC-containing perfusate.

More recently, Wu *et al.* (1993) compared various perfusion temperatures and perfusate containing BSA from 2%-7% and 1.18 mM $CaCl_2$ without RBC in the perfused rat hindlimb and found flow-induced $\dot{V}O_2$ was present under all these conditions. However, when 20% RBC was added into the perfusate containing 2% BSA at the perfusion temperature of 37°C, $\dot{V}O_2$ became independent of flow rate (Wu *et al.*, 1993). The similar findings from these two groups would seem to suggest that RBC somehow inhibit flow-induced $\dot{V}O_2$.

Table 1-5 Literature reports on the effect of increasing perfusion flow rate on $\dot{V}O_2$ in resting skeletal muscle

Animal	Perfusion model	Perfusate	Metabolic change	Author
Rabbit	G-P-S group, gracilis, and soleus at 37°C	K-H buffer + RBC (2.5 mM $CaCl_2$)	$\dot{V}O_2$ ↑	Pagliassotti *et al.*, 1990a
Rat	Hindlimb at 37°C	K-H buffer + 4% BSA and RBC (2.5 mM $CaCl_2$)	$\dot{V}O_2$ ↑ in the limb; but not in the trunk	Gorski *et al.*, 1986
CA rat	Gracilis at 28°C	K-R buffer containing 5% BSA ± RBC (2.5 mM $CaCl_2$)	$\dot{V}O_2$ ↑	Chinet *et al.*, 1989
Rat	Hindlimb at 25°C, 37°C and 42°C	K-H buffer + 2, 4.7 or 7% BSA 1.18 mM $CaCl_2$	$\dot{V}O_2$ ↑	Wu *et al.*, 1993
	Hindlimb at 37°C	K-H buffer + 2% plus RBC	$\dot{V}O_2$ unchanged	
Cat	Autoperfused soleus and gracilis at 35-39°C	Blood	$\dot{V}O_2$ ↑	Whalen *et al.*, 1973
Dog	Autoperfused hindlimb at 37°C	Blood	$\dot{V}O_2$ ↑	Pappenheimer, 1941b
Dog	Autoperfused G-P-S group at 35.5-37.5°C	Blood	$\dot{V}O_2$ unchanged	Stainsby *et al.*, 1964
Dog	Autoperfused gracilis at 37°C	Blood	$\dot{V}O_2$ unchanged	Kohzuki *et al.*, 1991

CA rat: cold acclimated rat; G-P-S: gastrocnemius-plantaris-soleus; K-H: Krebs-Henseleite; K-R: Kreb-Ringer; BSA: bovine serum albumin; RBC: red blood cells.

However, flow-induced $\dot{V}O_2$ is also found in isolated autoperfused cat gracilis and soleus muscle (Whalen *et al.*, 1973). More interestingly, in the same study, where Chinet *at al* (1989) found flow-induced $\dot{V}O_2$ only in the rat gracilis perfused with RBC-free but not with RBC containing Krebs-Ringer buffer, heat production was clearly observed during a perfusion with heparinized whole blood. Therefore, flow-induced $\dot{V}O_2$ in resting skeletal muscle does not seem to be an artefact resulting from the lack of RBC in the perfusate.

In conclusion, the data from a variety of perfused muscle preparations (see Table 1-5) when taken together indicate that an increase in perfusion flow to muscle tissue can, under certain circumstances, stimulate thermogenesis in resting muscle. There appear to be some yet unknown mechanisms involved in the regulation of flow-induced $\dot{V}O_2$ in resting skeletal muscle. If, as proposed by Clark *et al.* (1994), an effective perfusion flow in the nutritive capillary bed is a major determinant for the ultimate $\dot{V}O_2$ and metabolism in the muscle, an increase in global perfusion flow may not necessarily elicit $\dot{V}O_2$ unless the increased flow reaches the nutritive capillaries. Over all, there must be a biochemical basis at cellular level to allow the increased flow to elicit $\dot{V}O_2$ above its physiological demand.

1.8.6 Coordination of vasculature and parenchyma in muscle thermogenesis

Despite the metabolic autoregulation of microcirculation (Section 1.8.2), Clark *et al.* (1994) postulate that vasoconstrictors may act on site-specific receptors to control nutritive and non-nutritive capillaries. Type A vasoconstrictors act on site-specific receptors of the arterioles or precapillary sphincters to close non-nutritive capillaries, thereby diverting flow to the nutritive capillaries. Conversely, type B vasoconstrictors shunt flow away from nutritive capillaries by closing the precapillary arterioles, thus leading to an inhibition of $\dot{V}O_2$. Yet, the hypothesized signal(s) for type A vasoconstrictors to stimulate $\dot{V}O_2$ is (are) not understood.

1.9 Aims for the present study

The role of the vascular control of $\dot{V}O_2$ in thermogenesis has recently emerged as a novel concept from the study of muscle metabolism in constant-flow perfused muscle preparations. There is obviously a need to clarify whether vasoconstrictors affect $\dot{V}O_2$ in other vascular beds. The classification of type A and type B vasoconstrictors from the results of constant-flow perfused hindlimb of rats

requires testing in other perfused tissues to assess their metabolic properties. In addition, a better understanding of changes in energy metabolites in skeletal muscle under different oxygenation states would appear informative in evaluating regulation of muscle thermogenesis. Overall, the ultimate effect of a vasoconstrictor on thermogenesis would need to be examined *in vivo*.

Thus, the aims of this thesis were to answer the following questions:

1. Is vascular control of $\dot{V}O_2$ in skeletal muscle a general phenomenon across different species?
2. Do vasoconstrictors mediate control of $\dot{V}O_2$ in other tissues?
3. Can flow-induced $\dot{V}O_2$ be manipulated by vasoconstrictors and vasodilators in muscle?
4. Can vasoconstrictors exert a thermogenic effect in a pressure-controlled perfused muscle preparation?
5. What are the characteristics of muscle metabolites in hypoxia and hyperoxemia?
6. What are the implications of vascular control of thermogenesis in whole body metabolic rate?

CHAPTER 2

Materials and methods

2.1 Animals

Male hooded Wistar rats housed at 20°C with a 12/12 h day/night cycle were used for the experiments. The animals were fed with a commercial diet containing 21.4% protein, 4.6% lipid, 69% carbohydrate, 6% crude fibre with added vitamins and minerals (Gibson, Hobart) and allowed to access to water at libitum.

Tasmanian bettongs (*Bettongia Gaimardi*) of either sex were obtained from the University's breeding colony (Dr. R.W. Rose, Department of Zoology, The University of Tasmania). The animals were housed in room-sized cages (approx. 6 m × 2 m) in an animal compound with free access to food and water. Diet was a choice of apples, bread, and Pal® dog pellets (Uncle Ben's of Australia), containing 15% crude protein and 10% fat. During the period of experiment, the seasonal temperature ranged between 18-28°C during the day time and 10-20°C at night.

Cane toads (*Bufo marinus*) of both sexes weighing 129.4 ± 16.6 g from Queensland (Australia) were used for the experiments. The animals were kept at 20°C with a 12/12 h day/night cycle and fed with beetles for one or two months before use.

The use of rats, bettongs and cane toads was both on the basis of phylogenetical classification and on the choice of the available species. Rats were used to represent endothermic animals with BAT and bettongs to represent marsupials which are thought not to have BAT. Cane toads were used to represent ectothermic animals. All experiments were approved by the Ethics Committee of the University of Tasmania under the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (1990).

2.2 Tissue perfusions for the study of O_2 metabolism

The perfused hindlimb has proven to be a reliable preparation for the study of muscle metabolism and peripheral vascular functions (Bonen *et al.*, 1994). The constant-flow perfused rat hindlimb at 25°C with cell-free medium at a flow rate *in vivo* [0.27 $ml \cdot g^{-1} \cdot min^{-1}$ (Armstrong & Laughlin, 1985)] was first described by Côté *et*

al. (1985) and confirmed by other researchers (Colquhoun *et al.*, 1988; 1990; Wu *et al.*, 1993; Dora *et al.*, 1994; Rattigan *et al.*, 1994) to be a reliable model for the study of the effects of vasoconstrictors on muscle $\dot{V}O_2$. Comparisons with the perfusion by the medium containing erythrocytes at 37°C have demonstrated that the perfusion with cell-free medium at 25°C yielded similar results, given that Q_{10}=2.5 (Hettiarachchi., Ph.D Thesis 1992; Wu *et al.*, 1993; Bonen *et al.*, 1994). The characteristics of the perfused hindlimb, including perfused rat hindlimb at 25°C, for the study of skeletal muscle metabolism have recently been reviewed by Bonen *et al.* (1994). Therefore, perfusions with cell-free medium were used for the present study in either constant-flow or constant-pressure model. The constant-flow perfusions were performed at 25°C whereas the constant-pressure perfusions were carried out at 37°C.

2.2.1 Constant-flow perfused hindlimbs

2.2.1.1 Perfused rat hindlimb

Sodium pentobarbital was injected intraperitoneally (i.p.) at a dose of 50 mg/kg body weight to anaesthetise rats (180-200g). The surgical procedures were previously described by Ruderman *et al.* (1971) and Colquhoun *et al.* (1988). After anaesthesia, the tail and the tarsus were ligated and epigastric vessels, iliolumber vessels were all tied off. An incision was made along mid-line of the abdomen. Following ligatures placed around the duodenum and the rectum, the gut was removed. About 200 I.U. of heparin was injected into the vena cava followed by the cannulation of the abdominal aorta and vena cava. The right iliac artery and vein were ligated so that only left hindlimb was perfused. In order to avoid a possible spillover of the perfusate during changes in pressure, a ligature was paced around the abdomen at the level of L_3-L_4. The rat was then killed with a further injection of sodium pentobarbital into the heart and placed onto a pad within the cabinet to start perfusion. Skeletal muscle in this preparation accounts for 60-70% of the whole perfused hindlimb (Ruderman *et al.*, 1971; Gorski *et al.*, 1986).

2.2.1.2 Perfused bettong hindlimb

As a result of successful breeding program, a limited number of bettongs which are under the protection of law, became available for experiment. Thus the experiments on the perfused bettong hindlimb were designed to make full use of these valuable animals. The animals (1.2 ± 0.11 kg) were anaesthetized with sodium

pentobarbital (i.p., 60 mg/kg). Additional injections were given to maintain the anaesthesia when required.

An incision was made distal to the femoral artery and vein to expose these vessels. Heparin (1000 I.U.) was injected into the femoral vein at the level of the inguinal ligament, and an arterial cannula and a venous cannula were inserted into the respective vessels just below the branch point for the superficial iliac circumflex artery and vein. A tight ligature was placed around the thigh approximately 1 cm above the cannula, and another tight ligature around the tarsus, so that only the muscle bed distal to the inguinal ligament of the limb was perfused. The mass of muscle perfused was determined for the first three animals by excising and weighing dye-containing muscle from the contralateral limb that had been infused with the perfusion medium containing Evans blue (1% w/v) following the same surgical procedures and heparinization of the animal, but before the perfusion commenced on the remaining hindlimb. A regression formula was then calculated out as: the muscle mass perfused (g)= 31.87 × body weight (kg) - 9.05 (r= 0.9993, $P<0.05$, n=3) and applied to the following experimental animals assuming that both hindlimbs were identical in muscle mass. The animals were killed with overdose sodium pentobarbital immediately after the commencement of perfusion. The measurement by dissection showed that skeletal mass accounted for 67% of the whole perfused hindlimb.

For the *in vivo* muscle metabolite measurement, gastrocnemius-plantaris-soleus group was freeze-clamped from the contralateral hindlimb before the animals were killed. After the sampling, the femoral artery and vein were ligated together with a circumferential ligature about the thigh to prevent any haemorrhage.

2.2.1.3 Perfused toad hindlimbs

Anaesthesia was introduced by sodium pentobarbital (i.p., 10 mg/100g), and additional injections were given when needed. In the amphibian, the venous system draining the hindlimbs consists of three main veins: an inferior abdominal vein running underneath the anterior abdominal wall and two renal portal veins. Therefore the surgical operations differed from those for the perfused rat or bettong hindlimb.

Two incisions were first made in parallel with the abdominal middle line leaving a strip of the anterior abdominal wall with the vein intact. A ligature was placed around the strip in the upper part before it was cut free above the ligature to expose the abdominal cavity. After the removal of the abdominal contents, each visible vessel running to or from the cut edges was tied off to prevent leaks during perfusion. To avoid spillover of the perfusate when the perfusion pressure rises, a

suture ligation was performed around the middle of the ilium and pubis bones. After the toad was heparinized by an intravenous injection of 200 IU heparin into a renal portal vein, both renal portal veins were tied off. An arterial cannula and a venous cannula were inserted into the dorsal artery and anterior abdominal vein, respectively, for the perfusion of both hindlimbs. The perfused muscle mass was measured by weighing the Evan's blue-dyed tissue at the end of the perfusion.

2.2.2 Constant-flow perfused other tissues of the rat

For the perfusion of kidney, intestine and mesenteric artery, 180-200 g male rats were used and anaesthetized as before (2.2.1.1).

2.2.2.1 Kidney

The abdominal aorta and vena cava were cannulated upward to the left renal vessels. The vessels to the adrenal gland and the surrounding fat tissue were tied off. A ligature was placed around the aorta and vena cava above the left renal vessels so that only the left kidney was perfused. The right kidney was weighed out to calculate the $\dot{V}O_2$ with the assumption that both kidneys were equal in weight.

2.2.2.2 Intestine

The inferior mesenteric artery and vein were cannulated for the perfusion. A ligature was placed between the stomach and duodenum and the between the small intestine and the large intestine. The perfused intestine mass was measured by weighing Evans-blue dyed tissue at the end of perfusion.

2.2.2.3 Mesenteric artery

The initial operation was as same as perfused intestine. Following the cannulation, the intestine was cut off along the edge. The left mesenteric artery was then hung within a 10 ml syringe submerged with 2.0 ml perfusate. The perfused flow rate was 2.0 ml/min. The perfused tissue was measured as mentioned above (2.2.2.2).

2.2.3 Perfusion system and medium

The perfusion system (shown in Fig. 2-1) consisted of a perfusion pump, an artificial lung, a thermostatic bath, a glass heat exchange coil, a Clark-type O_2

electrode, a pressure transducer, a magnetic stirrer, a chart recorder, and polyethylene tubing. The whole of the perfusion apparatus was set up in a thermostatic cabinet at 25°C unless indicated elsewhere.

The perfusion medium for the perfused mammalian tissues was modified Krebs-Ringer solution (Colquhoun *et al.*, 1988) composed of (in mmol/L): NaCl 118, KCl 4.7, $CaCl_2$ 1.27, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25.0 and Glucose 8.3 and 2% bovine serum albumin (BSA) unless indicated elsewhere. The perfusion buffer was filtered with a 0.45 μm pore filter under pressure after it was made to ensure that it was particle free. The perfusate was saturated with a mixture of 95% O_2 and 5% CO_2 by passing the artificial lung and the pH kept at 7.4. BSA (Sigma, Fraction V) was dialysed five times against distilled water before use.

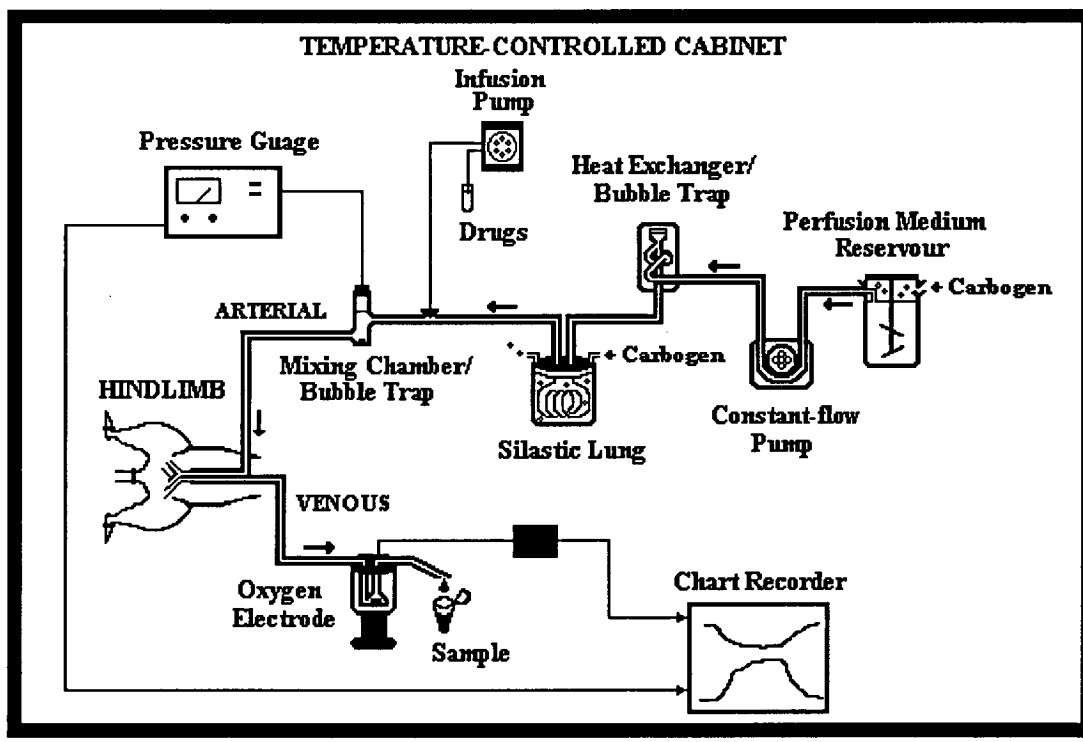

Fig. 2-1 Perfusion system for the constant-flow perfusion at 25°C. The same perfusion system was applied to the constant-flow perfused hindlimbs of rat, bettongs and toads as well as the rat kidney, intestine and mesenteric artery.

The toad hindlimb perfusion was conducted at 25°C with O_2 saturated modified amphibian Hepes-buffer solution (Pelster *et al.*, 1993) composed of (in mmol/L): NaCl 115, KCl 3.2, $MgSO_4$ 1.4, Hepes 10.0, $CaCl_2$ 1.3, glucose 5.0 and 1% BSA at pH 7.4. Whilst amphibian Ringer's buffer is used for many perfused amphibian tissues, Hepes buffer was employed in the present study for the perfused toad hindlimbs because it contains Mg^{2+}. As Hepes buffer does not contain HCO_3^-, it was saturated by O_2 to maintain a pH at 7.4 as reported by Pelster *et al.* (1993). The perfusion flow was set at a perfusion pressure of (15 ± 1.0 mm Hg) close to the physiological level *in vivo* (Altman & Dittmer, 1974; Withers *et al.*, 1988; Pelster *et al.*, 1993).

2.2.4 Measurement of $\dot{V}O_2$

The O_2 electrode was calibrated before and after each experiment with oxygen, air and nitrogen for each perfusion. Arterial PO_2 (PaO_2) was obtained from the perfusate without passing through a perfused tissue but with the same length of polyethylene tubing so that any loss to the atmosphere was constant. The mean value of PaO_2 was 697 ± 1.0 mmHg (Mean ± SE, n=20). Venous PO_2 (PvO_2) varied with the change in $\dot{V}O_2$ by perfused tissues but was never allowed to fall below 200 mmHg. $\dot{V}O_2$ by a perfused tissue was calculated from the arteriovenous difference of partial oxygen pressure and the flow rate according to Fick's principle as follows.

$$\underset{(\mu mol \cdot g^{-1} \cdot h^{-1})}{VO_2} = \frac{1.508 \times (PaO_2 - PvO_2)\ mmHg \times flow\ rate\ (ml/min) \times 60\ (min/h)}{1000\ ml \times tissue\ (g)}$$

where, 1.508 ($\mu mol \cdot L^{-1} \cdot mmHg^{-1}$) represents the oxygen solubility calculated from Bunsen coefficient at 25°C; at 37°C it is 1.256 (Christoforides *et al.*, 1969); 1000: 1L=1,000 ml.

2.2.5 Constant-pressure perfused hindlimbs

2.2.5.1 Surgical procedures

The constant-pressure perfusion of rat hindlimbs was performed at a physiological pressure of 80 mm Hg. Initial experiments showed that a high flow rate was required and 6% BSA was needed in order to prevent edema. Hence, smaller rats

(100 ± 5 g) were used for the study to reduce basal flow rate, thereby saving the cost of higher BSA expenditure.

The general surgical procedures were similar to those described above for the constant-flow perfused rat hindlimb with the following modifications. After the evisceration of the abdominal cavity, the iliac artery supplying the right hindlimb was cannulated with a heparin-filled 16 G cannula (I.D. 0.6 mm) and was then connected with a blood pressure transducer linked to a chart recorder. The cannula tip was positioned as close to the abdominal aorta as possible without disturbing flow to the perfused hindlimb. All other major vessels to and from this hindlimb were ligated. Both abdominal aorta and vena cava were cannulated, respectively, as described in Section 2.2.1.1 for the perfusion of the left hindlimb.

2.2.5.2 Perfusion system

The perfusion system was similar to that for the constant-flow perfusion with the following modifications. A three way tap was placed just before the arterial cannula as a by-pass flow (illustrated in Fig 2-2) to lead the excess flow through a T junction back to the reservoir. One end of the T junction was exposed to the atmosphere so that the pressure was controlled at 80 ± 0.5 mm Hg via the height of the T junction. During the perfusion, the perfusate was pumped at an 20-30% excess rate over the actual flow entering the hindlimb. Perfusion flow rate was noted intermittently by collecting venous effluent with a measuring cylinder. Perfusion pressure was monitored from the contralateral iliac artery with modifications of the surgery as described in 2.2.1, similar to the method used by Johnsson *et al.* (1991) who measured pressure from the tail artery.

In addition to the perfusion channel in the perfusion pump, a second channel was set up in a reverse direction to drain the venous perfusate, which was collected in a 100 ml beaker, back to the reservoir to form a recirculation. All metal needles and connections were replaced by polyethylene to eliminate a possible effect of trace metal elements on vasoactivity (Lew *et al.*, 1987). The reservoir was accurately calibrated and volumes of the tubing in the whole system were measured accurately. These volumes were taken into account when adding drugs into the reservoir to achieve a known concentration. As the total volume of the perfusate varied at times because of the perfusate samples taken, the calibrated reservoir allowed estimation of the actual volume at any given time.

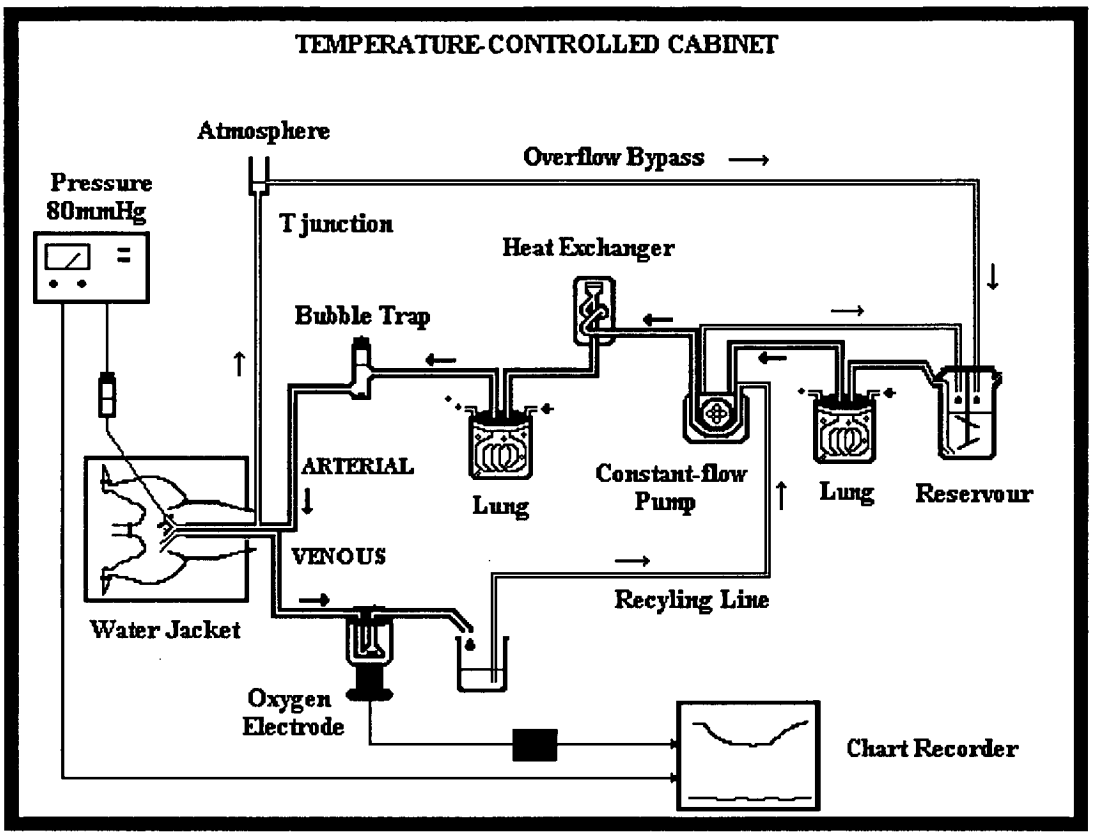

Fig. 2-2 Diagram for the constant-pressure perfusion system. The perfusions were conducted at 37°C in a recirculation model.

Two artificial lungs were used in series to ensure full saturation of the perfusate with 95% O_2 and 5% CO_2. Despite the lung normally used for constant-flow perfusion sitting (see Fig. 2-1), the other lung was before the pump and kept in ice. For each perfusion, 400 ml buffer was used and the first 50 ml venous effluent discarded. PaO_2 was obtained by measuring the perfusate before and after each perfusion at different flow rates. PvO_2 was continuously monitored by an in-line Clark-type O_2 electrode. $\dot{V}O_2$ was calculated from steady-state arteriovenous differences and the flow rate measured at the same time. Samples of arterial and venous perfusate were periodically taken and measured with an O_2 analyzer (TasCon, Department of Physiology, University of Tasmania) to check the results of from the in-line Clark-type O_2 electrode at different flow rates. Arterial perfusate pH was checked before and after recycling perfusate and no statistically significant difference was found.

The experiments were conducted at 37°C within a cabinet. As the ambient temperature, usually lower than 30°C, could still more or less affected the temperature within the cabinet, the animal was placed in another 37°C thermostatic water jacket during the perfusion. The composition of the perfusate was the same to that used for constant-flow perfused rat and bettong hindlimbs as mentioned in 2.2.1.3 except for 6% BSA and 2.5 mM $CaCl_2$.

2.3 Metabolite assays

For the assay of lactate, pyruvate and glycerol release from the perfused tissue, 2.0 ml of venous effluent was collected either at each new steady state, or at nominated times for the time-response curves. The collected perfusate was then centrifuged for 5 min at 3,000 rpm to remove any washed-out erythrocytes and frozen (-20°C) until assay. The arterial perfusate was used as the blank in the assays.

As preliminary experiments had shown that sampling procedures were extremely crucial for obtaining the closest bioenergetic data *in vivo*, special attention was paid to this process. Usually, the normal tissues (unless indicated elsewhere) were freeze-clamped by a pair of aluminium tongs *in situ* before being removed, leaving no ischemic time to elapse.

For skeletal muscle, gastrocnemius-plantaris-soleus muscle group was sampled for the assay. Studies by Armstrong & Phelps (1984) in the rat have shown that soleus muscle contains approximately 90% slow-twitch oxidative fibres (Type I) whilst plantaris muscle consists of 50% fast-twitch oxidative-glycolytic fibres (Type IIa), 40% fast-twitch oxidative fibres (Type IIb) and 10% slow-twitch oxidative fibres (Type I). Gastrocnemius muscle is composed of the mixture of the above three fibre types. Thus, gastrocnemius-plantaris-soleus muscle group represents all three muscle types. The skin covering the calf muscle was surgically peeled off to expose the muscle group. The muscle group was then gently separated from other muscle groups without disturbing blood or perfusate to the tissue. Then, the muscle group was lifted above the others at the distal end and freeze-clamped *in situ* with liquid N_2 precooled tongs before being cut free from the leg. The sampled gastrocnemius-plantaris-soleus muscle group consisted of (by mass) 7.0% soleus, 14.9% plantaris, 23.9% white gastrocnemius and 54.3% red gastrocnemius muscles. For the rat of 180-200 g, this muscle group was 1.25 ± 0.03 g (means ± SE, n=10), accounting for 0.62-0.69% of the body weight.

Other tissues except blood vessels were sampled in a similar way by freeze-clamping *in situ* with liquid N_2 precooled tongs before removal.

2.3.1 Sample preparation

The tissue samples were pulverised in liquid N_2 with a mortar and pestle. Tissues beyond the clamp edges and distinguishable tendon and connective fibres were discarded during grinding. The tissue powder was thoroughly mixed and stored at -80°C before analysis.

About 50 mg tissue powder was weighed out, dried in an oven at 100°C overnight and reweighed until of constant weight to determine the ratio of wet weight to dry weight.

Approximately 200 mg frozen tissue powder (except for the aorta) was weighed out and homogenized immediately in 2.0 ml of ice-cold 0.42 M $HClO_4$ (perchloric acid, PCA) with an electrically driven homogenizer (Ultra-Turrax Homogenizer) at 3/4 full speed for 30 seconds. 1.0 ml of the supernatant was transferred to another tube following centrifugation at 3,000 rpm for 5 min. The supernatant was titrated to pH 6.0-6.5 with K_2HPO_4 (1.0 M) whilst constantly vortexing. The neutralized $HClO_4$ extract was placed in ice for 5 min before it was centrifuged again in the same conditions as mentioned above. The supernatant was recovered and kept in ice for assays of creatine (Cr), creatine phosphate (CrP), adenine nucleotides, glucose-6-phosphate (G-6-P), and pyruvate within 2 hours as well as of lactate and glycerol within 24 hours.

For the assay of muscle glycogen, approximately 200 mg muscle powder was weighed out and incubated in 0.3 ml of 30% KOH in a glass tube with screw cap in boiled water for 30 min. After it cooled down, 0.1 ml of 2% Na_2SO_4 was added followed by 0.93 ml 100% ethanol to give a final concentration by 70%. Thin glass rods were used for thorough mixing. After standing overnight at 0°C, the glycogen was removed by centrifugation for 15 min at 2,000 rpm. The supernatant was then decanted and the tubes left inverted to drain on filter paper. The tubes were placed in ice for 1 hour before the wall of the tubes and the precipitate was washed with 0.5 ml 65% ethanol. After leaving the precipitate in ice for 15 min the tubes were centrifuged again (10 min at 2,000 rpm) to separate the glycogen. The supernatant was decanted and tubes left to drain as previously on to filter paper. The last traces of ethanol were expelled by evaporation in a water bath at 70°C and by lightly blowing carbogen into the tubes. The pellet was dissolved in 1.2 ml distilled water. 0.5 ml of the dissolved

sample was pippeted into a test tube containing 0.5 ml H_2O and 1 ml enzyme solution (4 mg amyloglucosidase/ml sodium acetate/acetic acid buffer, pH 4.5). The test tube was centrifuged (2,000 rpm, 5 min) following an incubation at 45°C for 2 hours. 0.025 ml supernatant was used for the assay of glucose

2.3.2 Enzymatic assays

Lactate, pyruvate, glycerol, glycogen, G-6-P and CrP were assayed with the method in "Methods of Enzymatic Analysis" edited by Bergmeyer (1974). Lactate, pyruvate, G-6-P and CrP were calculated as the follows:

$$\mu\text{mol/g dry wt} = \frac{\Delta E \times \text{cell (ml)} \times \text{total neutralized vol.(ml)} \times \text{wet wt}}{6.21 \times \text{tissue (g)} \times \text{sample (ml)} \times \text{recovery (\%)} \times \text{dry wt}}$$

where: 6.21 ($ml \cdot \mu mol^{-1} \cdot cm^{-1}$) represents the extinction coefficient for NADH or NADPH at 340 nm (Bergmeyer, 1974)

For glycogen, a standard curve for glucose was prepared using 25, 50 and 100 µl aliquot of 0.505 mM glucose. Calculation:

$$\mu\text{mol/g dry wt} = \frac{\mu\text{mol glucose (from Std)} \times 2.0 \times 1.2 \times \text{wet wt.}}{0.025 \times 0.5 \times \text{muscle powder (g)} \times \text{dry wt.}}$$

where 2.0 (ml): the total volume in the incubated test tube; 1.2 (ml): the volume of H_2O dissolving the pellet; 0.5 (ml): the volume of dissolved sample added into the incubated test tube; 0.025 (ml): the volume of supernatant used for the assay of glucose.

Glycerol was enzymically measured, essentially as described by Wieland (1974), using the change in fluorescence of NADH measured with an Aminco-Bowman spectrophotofluorometer set at 340 nm (excitation) and 460 nm (emission). The change in fluorescence intensity (ΔI) was used for the calculation of sample concentration from the standard solutions.

$$(\mu\text{mol/g dry wt}) = \frac{\mu\text{mol/ml (from Std)} \times \text{total neutralized vol.(ml)} \times \text{wet wt}}{\text{tissue (g)} \times \text{dry wt} \times \text{recovery (\%)}}$$

2.3.3 HPLC determination of high energy phosphate compounds

2.3.3.1 Anion exchange method

For the first part of this thesis (Chapter 3), adenine nucleotides were determined in Waters high performance liquid chromatography (HPLC) using an anion exchange Pharmacia Mono Q column as described by Colquhoun *et al.* (1990). The mobile phases included solution A and solution B. Solution A was 20 mM Tris-HCl buffer pH 8.0 and solution B was made up by adding 150 mM $(NH_4)_2SO_4$ into solution A. The buffers were filtered and degassed under vacuum before use.

HPLC settings: absorbance unit of full scale (AUFS) 0.5, chart feed 0.5 cm/min, flow rate 1.0 ml/min; wavelength 254 nm. A linear gradient elution started from 4.5 to 150 mM ammonium sulphate in 20 mM solution A. After all peaks were eluted (normally within 15 min), 5 min was given to regenerate the solid phase.

2.3.3.2 *Ion-pared reversed phase method*

In order to improve analytical efficiency, simultaneous determination of creatine compounds and adenine nucleotides (Shellevold *et al.*, 1986) were employed for the rest of the thesis after the instrument and column became available. A radial compression cartridge column (Radial-Pak, C18, 4 μm, 10 cm × 8 mm, Waters, USA) was connected with a HPLC (Waters or LKB). Both channels were used with the one at 210 nm for creatine compounds and adenine nucleotides and another at 254 nm as a reference for adenine nucleotides. AUFS was adjusted according to peak heights of creatine compound and adenine nucleotides. Isocratic elution was performed at a flow rate 1.5 ml/min and recorded at chart feeds of 20 or 40 cm/hour. All of the assayed compounds were washed out within 12 min. The mobile phase consisted of K_2HPO_4/KH_2PO_4 buffer (220 mM, pH 6.25), 3 mM tetrabutylammonium hydrogen sulfate and 5% acetonitrile. Usually, 10-20 μl of the neutralized $HClO_4$ extract was injected to HPLC. The contents of high energy phosphate compounds were. calculated according to following formula:

$$\mu\text{mol/g dry wt} = \frac{\text{peak height (or area) of sample} \times \text{Standard concentration}}{\text{Standard peak height (or area)} \times \text{inject sample(ml)}} \times \frac{\text{total neutralized volume (ml)} \times \text{wet wt}}{\text{muscle powder (g)} \times \text{recovery (\%)} \times \text{dry wt}}$$

Since preliminary tests showed that standard CrP converted to Cr by approximately 10% within 1 hour in the PCA solution, the recovery was used for the calculation. A batch of mixed standards of Cr, CrP, inosine, AMP, ADP and ATP were made in PCA solution and underwent exactly the same procedures as for the samples to work out the recovery for each compound.

Adenine nucleotide energy charge which reflects the energy state of a tissue was calculated as follows (Atkinson, 1968):

$$\text{Energy charge (EC)} = \frac{[\text{AMP}] + 0.5\,[\text{ADP}]}{[\text{AMP}] + [\text{ADP}] + [\text{ATP}]}$$

2.4 Metabolic rate studies on intact conscious animals

2.4.1 Calorimetric measurement on conscious animals

Bettongs of either sex (1.67 ± 0.10 kg) were used. The experiments were carried out between 10 am and 6 pm, which is in the bettong's normal sleep and quiescent phase. The animals were placed in a well-ventilated coarse-woven jute bag, to reduce ambient light influences to a minimum, and then moved into the respiratory chamber maintained at 25°C which is a thermoneutral zone for marsupials (Nicol, 1976, Wallis *et al.*, 1992) and the rat (Foster &Frydman, 1979, Maxwell et al., 1987). The resting metabolic rate was taken as the minimum 5 min average value when the animal stayed quiet. For the administration of drugs to the bettong, a venous indwelling cannula (θ 0.8/25 mm) was inserted into the tail vein with 0.9% NaCl solution continuously infused by a pump (LKB, Bromma) at a flow rate of 0.02 $ml \cdot kg^{-1} \cdot min^{-1}$. The drugs were freshly made in sterile physiological NaCl before use. Normally, animals had settled down within 15 min, but 30 min was allowed to elapse before collecting data. For comparison, metabolic rates were also determined on conscious rats (250 ± 10 g) in the same system but a smaller animal chamber.

2.4.2 Indirect calorimetry system

Heat production was determined by measuring $\dot{V}O_2$ of the whole animal in an indirect calorimetry apparatus. The system was composed of a sealed respiratory chamber sitting in a water bath, a coil of copper piping placed in the water bath to act as a heat exchanger for the inlet air, a vacuum pump for controlling air flow through

the chamber, a sampling pump, and a modified O_2/CO_2 gas analyzer (Datex, Labtech, Finland).

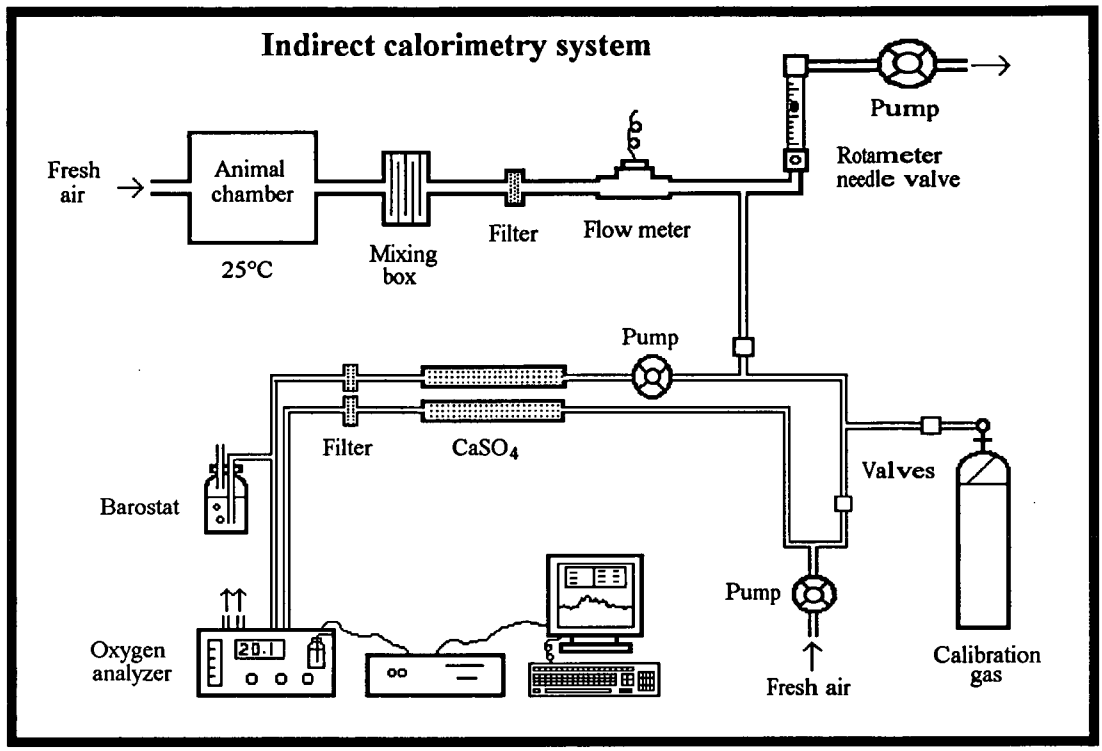

Fig. 2-3 Diagram of indirect calorimetry system for whole animal metabolic rate study.

The sealed chamber was temperature controlled (25°C) and at negative pressure with a water trap on the exit line. The air flow was controlled by a needle valve and measured by a rotameter on the exit line. The flow rate of air through the chamber was adjusted to a level to keep the expired CO_2 below 0.2%. A sample was drawn off by the sampling pump and passed through a T-junction containing a humidity and temperature probe to give correction to the flow figure from the rotameter. The sample then travelled through a drying column ($CaSO_4$) and into a constant pressure head device (Barostat) where it was sampled by the O_2/CO_2 analyzer. A peristaltic pump sampled the air and passed it through a drying column to the reference air inlet of the O_2/CO_2 analyzer. The main source of vacuum for the system was a large diaphragm pump that has sufficient power to extract air from a hood for 100 L/min.

Data from the electronic device were controlled via interface boxes by an A/D board on an IBM compatible computer, and processed by the program "Labtech Notebook" and stored in a file form compatible with "Lotus". The expired air was continuously monitored with the gas analyzer, and the data collected and stored in a computer for every 1 min interval during the period of experiment.

2.4.3 Calculation

Whole body heat production was calculated from $\dot{V}O_2$ measured under conscious conditions by an indirect calorimeter according to the method of Weir (33). The standard average energy yield from mixed food per liter of oxygen consumed was assumed to be 4.83 kcal (9). Thus, the formula was modified as:

$$\text{Heat production } (\text{kJ}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}) = \frac{0.2088 \times (O_I - O_E) \times V \times 60 \times 24}{\text{Body weight (kg)}}$$

where 0.2088 is a constant calculated from Weir's formula (33) using 4.83kcal/L $\dot{V}O_2$, V= flow (L/min) at STP (dry), $(O_I - O_E)$= the difference in oxygen content between incoming air and exiting air, 60= 60 min, 24= 24 hours.

For comparison between the bettong and other species, the metabolic body size of 0.75 power of body weight ($W^{0.75}$) was used. The proportional change in metabolic rates to $W^{0.75}$ of adult animals is recommended by the International Commission of IUPS for Thermal Physiology for comparison to be made between the metabolic levels of different animals (Bligh & Johnson, 1973).

The proportion of metabolism that skeletal muscles contribute to the whole body metabolic rate was estimated according to the equation (Flanigan *et al.*, 1991):

$$\% \text{ contribution} = \frac{\text{Muscle } \dot{V}O_2 \times \text{Entire muscle mass}}{\text{Whole body } \dot{V}O_2}$$

where the proportion of muscle mass relative to total body mass was obtained from literature reports for the rat (Grubb & Folk, 1976) and for the red kangaroo (Tribe & Peel, 1963).

2.5 Chemicals, instruments and statistics

2.5.1 Chemicals

Sodium pentobarbital (Nembutal) was obtained from Boehringer Ingelheim (Australia) and heparin sodium (porcine mucous) from David Bull Laboratories Pty. Ltd (Australia). [-]-Norepinephrine bitartrate, L-phenylephrine-HCl, naphazoline-HCl, [-]-isoproterenol-HCl, phentolamine-HCl, DL-propranolol-HCl, Arginine-vasopressin, 5-hydroxytryptamine creatinine sulfate, nifedipine-HCl and Evans-blue were obtained from Sigma (USA). Nitroprusside sodium was purchased from E. Merck (Germany). BRL 35135A was a gift from SmithKline Beecham Pharmaceuticals, UK. BSA (Fraction V) was obtained from Boehringer Mannheim Biochemicals (Indianapolis, USA).

AMP-Na_2, ADP-Na_2, ATP-Na_2, inosine, creatine phosphate-Na_2, NAD, NADH, NADP, LDH, α-glycerophosphate dehydrogenase, glycerol kinase, hexokinase, creatine kinase, G-6-P dehydrogenase, almyglucoase, and GOD-Perid agent were purchased from Boehringer, Mannheim (Germany). Creatine was provided by BDH (England). Tetrabutylammonium hydrogen sulfate was obtained from Aldrich Chemical Company, Inc. (USA). Other chemicals were analytical grade from Ajax Chemicals (Australia).

Gases including O_2, carbogen (95% O_2 and 5% CO_2), N_2 and the calibration gases for the O_2/CO_2 analyzer were purchased from Commonwealth Industrial Gases Limited, Australia.

2.5.2. Instruments

Perfusion apparatus included a perfusion pump (Masterflex, Chicago Ill, USA), infusion pumps (2132-Microperpex® LKB, Bromma), a thermostatic circulator (TE-8J, LKB), an artificial lung, a Clark-type O_2 electrode, a pressure transducer (Department of Biochemistry, University of Tasmania), and a chart recorder (Omniscribe, D5000, Houston Instrument, Austin, Texas, USA). The oxygen content analyzer (TasCon) was manufactured by Department of Physiology, University of Tasmania. The electrical stimulation system consisted of a square-wave stimulator (HSE, NERV-MUSKEL-REIZGRÄT, Germany) and an isometric transducer (Harvard, Bioscience).

Analytical instruments used in the study included an electrically driven homogenizer (Ultra-Turrax Homogenizer), centrifuges [DPR-6000, ICE (USA); Eppendorf], spectrophotometers [Cary-219, Varian (USA); DU-30, Beckman (USA); Spectronic 3000 Array, Milton Roy (USA)] and a spectrophotofluorometer (Aminco-Browman, American Instrument Company).

High performance chromatography (HPLC): a) Waters-HPLC (USA) consisted of four 6000A pumps, an U6K injector, a 440 absorbance detector, a 660 solvent programmer, and a SP 4100 computing integrator; b) LKB-HPLC included four 2150 pumps, a 2141 variable wavelength monitor, a 2152 LC controller and a 2210 recorder.

2.5.3 Statistical analysis

Data are presented as means ± standard error (SE). Student's *t*-tests were used for the statistics of the study. A paired *t*-test was used when indicated. $P<0.05$ was regarded as statistically significant, $P<0.01$ as extremely significant.

CHAPTER 3

Flow-induced increase in $\dot{V}O_2$ is inhibited by vasodilators and augmented by NE in the perfused rat hindlimb

3.1 Introduction

Thermogenesis in the resting skeletal muscle has been long considered as a significant contributor to whole body heat production (Jansky 1971; Mejsnar *et al.*, 1980; and Astrup *et al.*, 1986). Indeed data of Foster and Frydman (1979), which focus on the role of BAT, were also indicative of a substantial role for skeletal muscle. Their findings showed that for warm-adapted and normal rats NE administration resulted in a 30% increase in skeletal muscle blood flow and a 60% increase in $\dot{V}O_2$. Such data suggested that muscle could produce equal amount of heat to brown fat, even though $\dot{V}O_2$ was very much less per gram of tissue.

Attempts to address the issue more directly by using the perfused rat hindlimb have supported the above conclusion. The perfused rat hindlimb has been found to respond reproducibly to NE administration with a marked increase in $\dot{V}O_2$ (Mejsnar *et al.*, 1973; Grubb & Folk, 1976; Richter *et al.*, 1982b; Côté *et al.*, 1985; Colquhoun *et al.*, 1988). This response occurred at rest when the hindlimb was perfused with erythrocyte-containing medium at 37°C (Mejsnar *et al.*, 1973; Grubb & Folk, 1976; Richter *et al.*, 1982b) or medium without erythrocytes at 25°C (Côté *et al.*, 1985; Colquhoun *et al.*, 1988, 1990; Hettiarachchi *et al.*, 1992). A surprising finding has been that the $\dot{V}O_2$ was mediated by an α-adrenergic receptor mechanism that was invariably associated with a rise in perfusion pressure. Other vasoconstrictors such as VP and AT II also stimulated $\dot{V}O_2$ during vasoconstriction with both effects being blocked by nitroprusside, thereby supporting the possibility that perfusion pressure (possibly flow) controls $\dot{V}O_2$ of the skeletal muscle (Colquhoun *et al.*, 1988).

As reviewed in Chapter 1 (Section 1.8.5), many studies have shown flow-induced $\dot{V}O_2$ in resting skeletal muscle. Yet the mechanism underlying it remains unclear. In addition, it is not known whether flow-induced $\dot{V}O_2$ is affected by vasoconstriction or vasodilation. Thus in the present study, perfusion flow and pressure have been varied over a wide range and the effects of NE and vasodilators compared during varied flow.

3.2 Methods

3.2.1 Hindlimb perfusion

180-200 g male rats were used for the experiments. The method for the hindlimb preparation is described in Chapter 2 (Sections 2.2.1 & 2.2.3). The perfusions were conducted at 25.0 ± 0.2°C using Krebs-Ringer buffer containing 1.27 mM $CaCl_2$ and 4% BSA.

For perfusions where pressure was varied stepwise, the perfusate was allowed to flow under gravity from a reservoir kept at 4°C. The pressure was adjusted by relocating the perfusate reservoir to predetermined positions above the hindlimb. Flow was monitored by collection into a fraction collector set on time mode. $\dot{V}O_2$ was calculated from arteriovenous difference in PO_2 and flow rate after allowing a time delay in PvO_2 measurement due to electrode characteristics and tubing volume.

For perfusions where flow was varied, the hindlimb was initially equilibrated at 4 ml/min (per 15 g fresh weight of muscle) for 40 min. Flow was then adjusted to 2.0 ml/min using a calibrated pump and maintained at that rate for 30 min. The flow was then subsequently adjusted to 4, 6, 8, and 18.5 ml/min maintaining constant flow until steady-state pressure and PvO_2 were achieved. These set flow rates were noted by collecting the effluent in a measuring cylinder over a prescribed period of time, usually one minute.

3.2.2 Electrical Stimulation

An incision was made in the back of the thigh, and then the superficial gluteal muscles were carefully separated in parallel with the sciatic nerve to expose this nerve. The proximal portion of the nerve was divided and the distal end sucked into a saline-filled plastic syringe mounted with silver electrode probes. The gastrocnemius-plantaris-soleus muscle group was then stimulated to contract by electrically depolarizing the sciatic nerve. Twitch tension was recorded via a force transducer during electrical stimulation (200-ms trains of 100 Hz applied every 2 s; each impulse in the train being of a duration of 0.1 ms) and the voltage was adjusted (15-20 V) to attain full fibre recruitment as shown by maximal contraction. Muscles were stimulated for three periods of 2 min each with a rest period of 30 min between consecutive runs. PvO_2 was monitored continuously and maximum $\dot{V}O_2$ noted; this occurred 1-2 min after commencement of stimulation.

3.2.3 Metabolite assay

The production of lactate and pyruvate were determined from arteriovenous differences and the flow rate of the perfusate. The gastrocnemius-plantaris-soleus muscle group was removed and then freeze-clamped for the measurement of muscle metabolites. The detail of the mass and fibre types of the gastrocnemius-plantaris-soleus muscle group was outlined in Section 2.3 (p. 31-32). Lactate, pyruvate, glycogen, and CrP were enzymatically assayed as described in Section 2.3.2. AMP, ADP, and ATP contents were determined by anion-exchange column with HPLC (Waters Associates) according to the method described by Colquhoun *et al.* (1990) and Section 2.3.3.1.

3.3 Results

3.3.1 Pressure-induced changes in flow and $\dot{V}O_2$

These experiments were conducted to assess the effects of pressure on flow and $\dot{V}O_2$. The conditions chosen were considered likely to minimize autoregulation of flow and the related control of capillary density. Fig 3-1 shows the effect of periodically varying the perfusion pressure from high to low while allowing flow to self-regulate. Initially the rat hindlimb was perfused at a pressure of 44 mm Hg for 30 min to allow complete equlibration; a constant flow rate of 5.4 ml/min was attained and the $\dot{V}O_2$ was 7.0 $\mu mol \cdot g^{-1} \cdot h^{-1}$. The perfusate reservoir was then raised to a new level to give a pressure of 82 mm Hg. Perfusate flow immediately increased to 13 ml/min then, over the next 30 min, gradually decreased to 11 ml/min. $\dot{V}O_2$, calculated from the arteriovenous difference and flow rate, peaked at 19.5 $\mu mol \cdot g^{-1} \cdot h^{-1}$, then gradually decreased over the next 30 min to 8 $\mu mol \cdot g^{-1} \cdot h^{-1}$. When the pressure was decreased from 82 to 28 mm Hg by lowing the pressure reservoir, the flow decreased to 1.5 ml/min and then increased marginally to 2.0 ml/min. $\dot{V}O_2$ decreased immediately following the fall in pressure and flow to 0.5 $\mu mol \cdot g^{-1} \cdot h^{-1}$ but then increased over the next 30 min to 3 $\mu mol \cdot g^{-1} \cdot h^{-1}$. A similar relationship between pressure, flow, and $\dot{V}O_2$ as described above was again evident when the pressure was reset to 44 mm Hg.

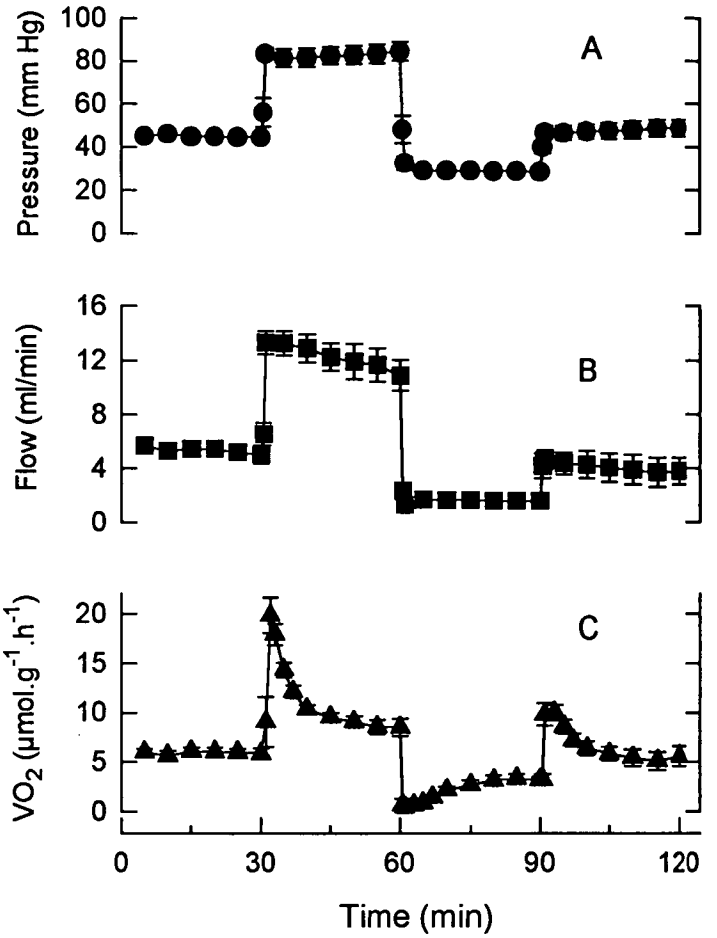

Fig. 3-1 Effect of perfusion pressure on flow and $\dot{V}O_2$ of the perfused rat hindlimb. Data are means ± SE, n=4.

3.3.2 Flow-induced changes in $\dot{V}O_2$ and pressure

The relationship between flow, steady-state $\dot{V}O_2$ and pressure development , as well as the effect of vasodilators and NE on these latter parameters is shown in Fig. 3-2. Perfusate flow was set at 2, 4, 6, 8, and 18.5 ml/min for at least 15 min and sometimes 40 min during which time pressure and $\dot{V}O_2$ were recorded. Fig. 3-2A shows that $\dot{V}O_2$ increased as the flow increased and this increase was further augmented by 50 nM NE. Conversely, nitroprusside (acting via cyclic GMP) inhibited the pressure and $\dot{V}O_2$ induced by flow. However, a combination of three vasodilators, nitroprusside, nifedipine (a Ca^{+2} channel blocker), and isoproterenol (acting via cyclic AMP) did not further inhibit pressure and $\dot{V}O_2$ compared with nitroprusside alone (Fig. 3-3).

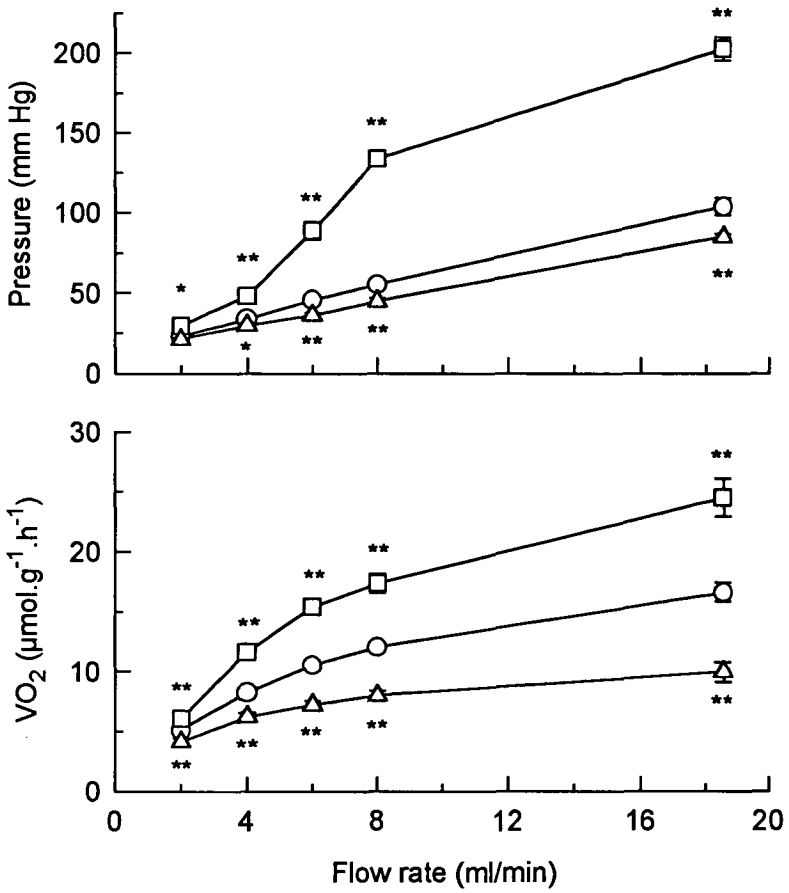

Fig. 3-2 Effect of perfusate flow, NE, and NP on pressure and $\dot{V}O_2$. Additions to the perfusate were none (O, n=6-7), 50 nM NE (□, n=6) and 0.5 mM NP (Δ, nitroprusside, n=6). * P<0.05, ** P<0.01, vs no addition. Means ± SE.

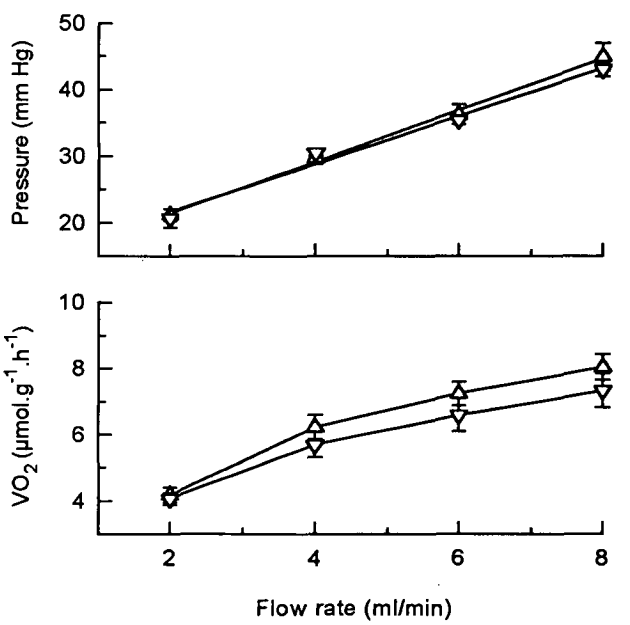

Fig. 3-3 Effect of NP alone and a combination of vasodilators on $\dot{V}O_2$ and pressure. Δ: 0.5 mM NP (nitroprusside, n=6); ∇ : a combination of 0.5 mM NP, 1.0 µM nifedipine and 1.0 µM Iso, (isoproterenol, n=5). Data are steady state values (means ± SE).

3.3.3 Vasodilators and working skeletal muscle

Since $\dot{V}O_2$ in the hindlimb at all flow rates was markedly inhibited by the vasodilators, it was considered relevant to assess the effects of these agents on working skeletal muscle $\dot{V}O_2$. Table 3-1 shows that neither nitroprusside alone nor the combination of nitroprusside, nifedipine, and isoproterenol had any significant effect on the increase in $\dot{V}O_2$ by the perfused contracting hindlimb at constant flow (4 ml/min). More importantly, the twitch tension force development was also unaffected by the vasodilators.

Table 3-1 Effect of vasodilators on twitch tension development and $\dot{V}O_2$ of the rat perfused hindlimb

Addition	n	Twitch tension (g)		$\Delta \dot{V}O_2$
		0 min	2 min	(μmol h^{-1} g^{-1})
None	3	603 ± 132	220 ± 49	3.90 ± 0.38
NP	3	641 ±75 ns	200 ± 64 ns	4.50 ± 0.34 ns
NNI	3	547 ± 51 ns	217 ± 47 ns	3.86 ± 0.42 ns

Note: NNI = NP (nitroprusside, 0.5 mM) + nifedipine (1 μM) + Iso (isoproterenol, 1 μM). ns, not significantly different from the "none". The perfusions were conducted at 4 ml/min. Data are means ± SE.

3.3.4 Muscle metabolites

The experiments recorded in Table 3-2 were conducted to assess whether NP-inhibition of hindlimb $\dot{V}O_2$ was associated with a decrease in high energy phosphates in muscle. Table 3-2 shows metabolite concentrations for the gastrocnemius-plantaris-soleus muscle group from the hindlimb freeze-clamped before surgery (*in vivo*) and following perfusion for 120 min with no additions, 0.5 mM nitroprusside, and a combination of nitroprusside (0.5 mM), nifedipine (1 μM), and isoproterenol (1 μM). None of the differences between muscle perfused with or without the vasodilators and muscle *in vivo* was significant. However, NE significantly reduced lactate content and lactate/pyruvate ratio in the muscle and tended to increase CrP.

Table 3-2 Metabolite contents in the gastrocnemius-plantaris-soleus muscle group of the rat hindlimb.

Muscle origin	n	Wet/dry wt	ATP	CrP	Lactate	Pyruvate	L/P
			μmol/g dry wt				
In vivo	3	4.4 ± 0.1	27.3 ± 1.1	71.9 ± 4.1	10.5 ± 1.6	0.7 ± 0.1	16.8 ± 5.3
Perfused 120 min							
No additions	5	4.5 ± 0.2	29.7 ± 0.9	72.5 ± 1.9	9.2 ± 1.0	1.0 ± 0.1	9.2 ± 1.1
NE	3	5.4 ± 0.2*	30.2 ± 1.2	80.6 ± 4.1	2.5 ± 0.5**	0.8 ± 0.1	3.1 ± 0.7**
NP	5	5.3 ± 0.3	27.2 ± 1.7	70.1 ± 2.3	9.7 ± 1.2	1.1 ± 0.1	8.9 ± 1.3
NNI	4	4.9 ± 0.2	26.0 ± 0.9	63.3 ± 0.9	9.0 ± 0.9	0.9 ± 0.2	10.3 ± 2.0

Note: Perfusions were conducted at 8.0 ml/min. NNI, combination of nitroprusside (0.5 mM), nifedipine (1 μM) and isoproterenol (1 μM). * $P<0.05$, ** $P<0.01$, vs no addition. The gastrocnemius-plantaris-soleus muscle group was removed and then quickly freeze-clamped. Neutralized $HClO_4$ extract was enzymatically assayed (see Sections 2.3.1 & 2.3.2) for CrP, lactate and pyruvate. ATP was measured by an anion exchange method in HPLC (Section 2.3.3)

3.3.5 Perfusate lactate/pyruvate ratio

The lactate/pyruvate ratio is considered a reliable indicator of muscle oxygenation (Bylund-Fellenius *et al.*, 1981; Shiota & Sugano, 1986) and was thus used in the present study.

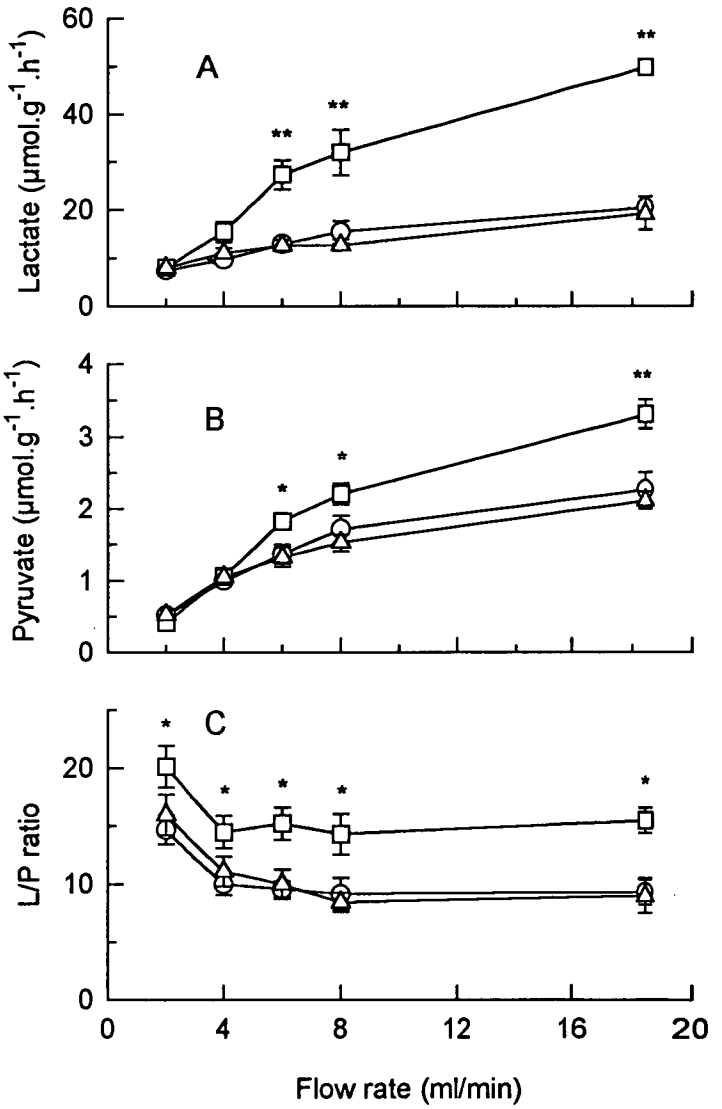

Fig. 3-4 Effect of vasoactive substances on flow-induced lactate and pyruvate release as well as the ratio of lactate/pyruvate. Additions to the perfusate were none (O, n=6-7), 50 nM NE (□, n=6) or NP (Δ, nitroprusside, n=6). Data are the steady values. * $P<0.05$, ** $P<0.01$, vs no addition.

Fig 3-4 shows lactate and pyruvate release at each flow rate with and without addition of NE or nitroprusside. Lactate release increased as the perfusate flow was changed from 2 to 18.5 ml/min (Fig. 3-4 A). Pyruvate release also increased over this range of flow rates (Fig. 3-4B). Addition of 50 nM NE significantly elevated lactate and pyruvate release. The increases became larger at higher flow rates. In contrast, the vasodilator, nitroprusside, had no significant effect on either lactate release or pyruvate release. Thus the net effect of changing flow rate from 2 to 18.5 ml/min was to lower the perfusate lactate/pyruvate ratio from approximately 15 at 2 ml/min to a constant value of approximately 9 at 4 ml/min and beyond. In addition, and as shown in Fig 3-4C, the ratio was shifted up in parallel by NE but not affected by the presence of nitroprusside. Overall these data suggest that inadequate O_2 supply to the resting perfused rat hindlimb at 25°C occurs only at flow rates less than 4 ml/min.

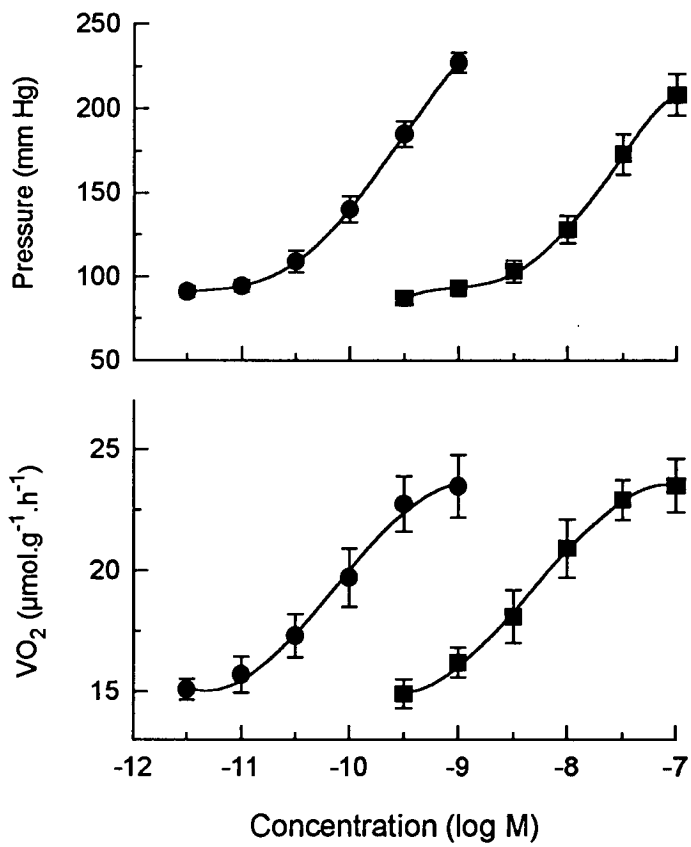

Fig. 3-5 Dose-response curves for the effect of VP and NE on perfusion pressure and $\dot{V}O_2$ at a high flow rate. The rat hindlimb was perfused at a constant-flow rate of 18.5 ml/min. ●: VP, ■: NE. Data are means ± SE, n=3.

3.3.6 Dose-response curves of $\dot{V}O_2$ and pressure for NE and VP at high flow rate

These experiments were carried out to compare results with those obtained at low flow rate under the same conditions in the same laboratory (Colquhoun *et al.*, 1988 & 1990). VP was employed here as another example of the effect of a vasoconstrictor on $\dot{V}O_2$ and perfusion pressure. As illustrated in Fig. 3-5, the dose-response curves of $\dot{V}O_2$ and perfusion pressure induced by VP and NE at 18.6 ml/min were essentially similar, in both the response dose ranges and proportion, to those at 4 ml/min with ED 50 for $\dot{V}O_2$ at 0.06-0.10 nM for VP and 10 nM for NE (Colquhoun *et al.*, 1988 & 1990). There were no significant differences in maximal increase in $\dot{V}O_2$ and perfusion pressure induced between NE and VP.

3.3.7 Effect of tubocurarine on $\dot{V}O_2$ mediated by NE and sciatic nerve stimulation

In order to assess the possibility that NE increases $\dot{V}O_2$ by increasing the skeletal muscle tension, tubocurarine, a drug which paralyzes skeletal muscle, was also infused during NE infusion. Fig. 3-6 demonstrates that 1 μM tubocurarine had no effect on either $\dot{V}O_2$ or pressure induced by NE. By contrary, the same dose of tubocurarine completely blocked both $\dot{V}O_2$ and skeletal muscle contraction produced by sciatic nerve stimulation.

3.4 Discussion

In the present study, the effects of flow, NE and vasodilators on $\dot{V}O_2$ has been assessed using a system where rapid autoregulation has been avoided (Fig. 3-1). A principal finding was that the $\dot{V}O_2$ by the perfused rat hindlimb increased as flow was increased to approach a plateau at 18.5 ml/min or 1.3 $ml.min^{-1}.g^{-1}$ (Fig 3-2). At flow rate less than 8 ml/min, the hindlimb data illustrate a widely noted property of perfused non-contracting skeletal muscle preparation where $\dot{V}O_2$ increases with an increase in perfusion pressure or flow. This relationship has been reported for rat gracilis muscle (Honig *et al.*, 1971), dog gastrocnemius-plantaris muscle group at low but not high blood flow levels (Stainsby & Otis, 1964), dog hindlimb both with NE (Coburn & Pendleton, 1979) or without NE (Pappenheimer, 1941b), isolated perfused cat soleus and gracilis muscles (Whalen *et al.*, 1973), resting and isolated gracilis muscle of dogs (Beer & Yonce, 1972), and hindlimbs of lemmings and rats (Grubb & Folk, 1978; Shiota & Sugano, 1986). The relationship appears to be readily observed

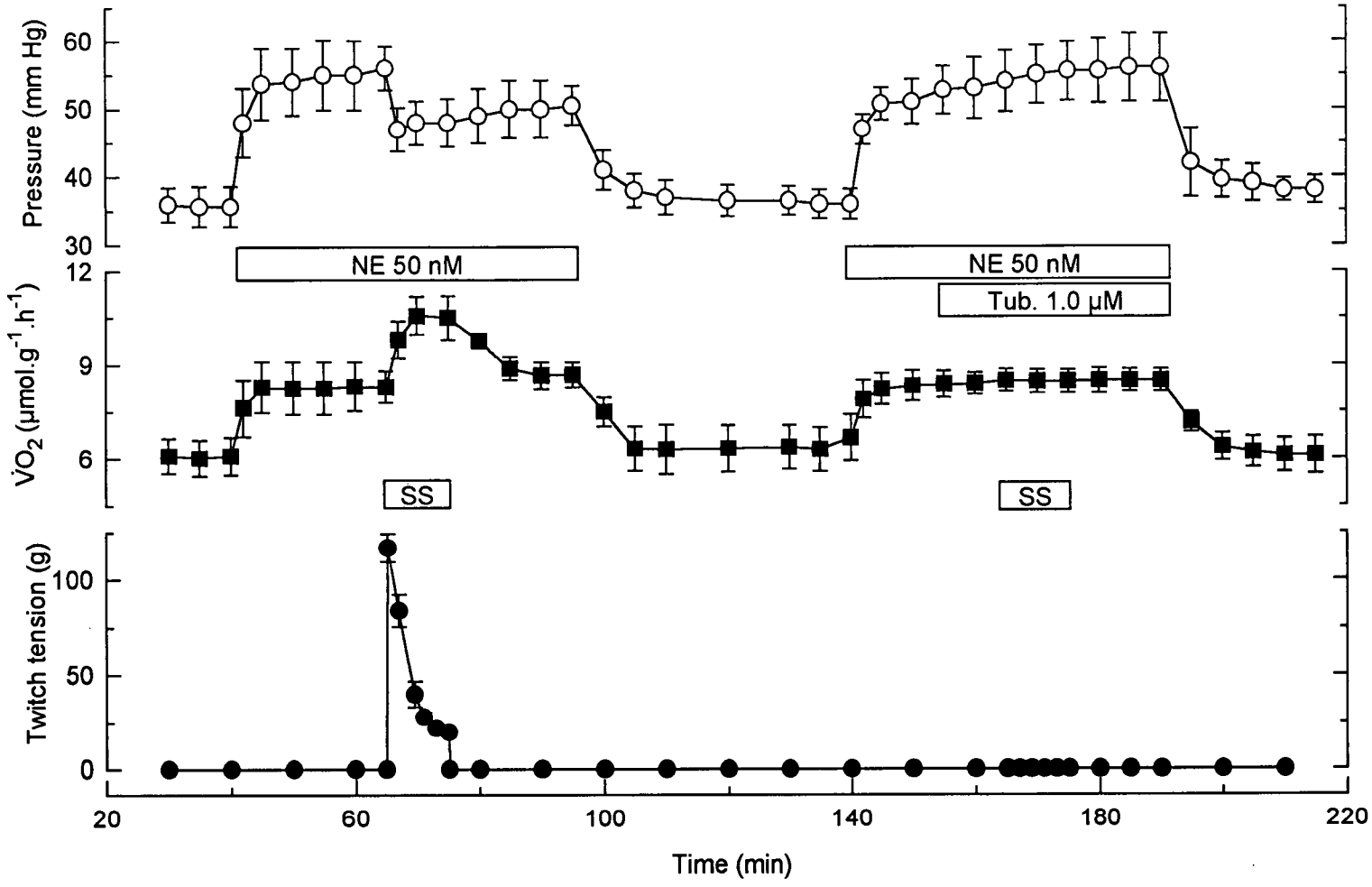

Fig. 3-6 Effect of tubocurarine on $\dot{V}O_2$ induced by NE and sciatic nerve stimulation. Hindlimbs were perfused at 4 ml/min for the experiment. The length of each bar represents duration of infusion or stimulation. SS: sciatic nerve stimulation; Tub.: tubocurarine. Data are means ± SE, n=3.

in rat hindlimb preparations without exception (Honig *et al.* 1971; Grubb & Folk, 1978; Shiota & Sugano, 1986) but, for as yet unknown reasons, is not always apparent in dogs (Durán & Renkin, 1974; Honig *et al.*, 1971; Chapler *et al.*, 1980).

To date, the dependence of $\dot{V}O_2$ on blood flow has been interpreted as a physiological limitation of $\dot{V}O_2$ in resting muscle by O_2 transport, owing to the inhomogenity of capillary functions (Lindbom & Arfors, 1985) and uneven distribution of O_2 within an organ (Honig *et al.*, 1971; Shiota & Sugano, 1986). To address this issue, a range extending from half to four times the normal hindlimb blood flow rate of the conscious rat (Armstrong & Laughlin, 1985) has been used in the present experiment. Under these conditions it was noted that nitroprusside inhibited $\dot{V}O_2$ and pressure at all flow rates, in particular at the highest flow where tissue perfusion could be expected to be approaching the maximum. Furthermore, the effect of NE increasing $\dot{V}O_2$ and pressure was greater at high flow rates than at lower flow rates. This reasoning is based on the notion that NE results in increased perfusate O_2 delivery to previously underperfused regions of skeletal muscle and that these regions are more extensive at low pressure.

The question of underperfusion was also addressed by assessing the biochemical indices of hypoxia. Poorly perfused muscle has a lower CrP concentration and increased tissue lactate concentration (Ruderman *et al.*, 1971; Challisss *et al.*, 1986). The sensitivity of CrP concentration measurement to evaluate ischemic muscle is further supported by the study on the bioenergetic profiles of skeletal muscle during ischemia (shown in Chapter 7), where CrP content is reduced by 18% of the original level following 5 min of ischemia. Thus, to address the possibility that nitroprusside-induced inhibition of $\dot{V}O_2$ resulting from tissue underperfusion, a representative sample of muscle (the gastrocnemius-plantaris-soleus group) was freeze-clamped and assayed for the content of adenine nucleotides, CrP, lactate and pyruvate. If the vasodilators decreased $\dot{V}O_2$ by decreasing the area of skeletal muscle actually perfused, it could be expected that CrP level would be lower following this treatment. Despite the fact that the vasodilators had resulted in a 40% inhibition of $\dot{V}O_2$ under the conditions chosen, there was no significant difference between vasodilator-treated and untreated muscle with respect to metabolite concentration (Table 3-2). On contrary to vasodilators, NE reduced lactate content in the muscle and tended to improve CrP concentration. The latter is consistent with the observations in the constant-pressure perfused rat hindlimb where CrP was significantly improved by NE (Chapter 6).

The lactate/pyruvate ratio is a sensitive index of hypoxia (Bylund-Felleneius *et al.*, 1981) and was also used in the present study to assess whether the increase in

$\dot{V}O_2$ by the hindlimb induced by the increase in flow rate was the result of elimination of previously underperfused muscle regions. Since the ratio was higher at 2 ml/min per hindlimb than at all other flow rates and was constant at rates equal to or greater than 4 ml/min per hindlimb, it appeared unlikely that hindlimb tissue was underperfused at flow rates other than 2 ml/min per hindlimb. It is also worthy of note that 0.5 mM nitroprusside which inhibited $\dot{V}O_2$ at 18.5 ml/min per hindlimb to a value similar to that of the untreated hindlimb perfused at 5 ml/min had no effect on the lactate/pyruvate ratio at high flow rate. Although NE increased the lactate/pyruvate ratio, the change also reached the steady state at 4 ml/min and remained unaffected at flow rates beyond. Lactate is transported across the cell membrane and its gradient between muscle and blood drives its release or uptake. Pyruvate is also thought to cross the cell membrane in the same way (McDermott & Bonen, 1992). These data taken together imply that flow- and agonist-induced $\dot{V}O_2$ results from processes other than simply supplying flow and O_2 to hypoxic regions of skeletal muscle fibres.

It appears that NE-mediated increase in lactate release in the effluent perfusate was associated with a lowering lactate content in the muscle. The higher lactate/pyruvate ratio in the presence of NE may be due to NE-induced high production in lactate from blood vessels (Hettiarachchi *et al.*, 1992). The similarities of the profiles of lactate and perfusion pressure induced by NE (Fig. 3-2A and 3-3A) appear to be supportive of this argument. Alternatively, if NE redirects the perfusate to more nutritive capillaries (Clark *et al.*, 1994) or increases the perfused capillary surface areas, a lower lactate in the tissue associated with a higher washout of lactate could be expected. However, the present data are not sufficient to identify the exact mechanism for the opposite effects of NE on lactate efflux and intracellular lactate concentration. Further studies are needed to address this observation.

Another finding in the study was that vasodilator nitroprusside did not inhibit $\dot{V}O_2$ and tension development by the perfused working skeletal muscle. Recently, Murrant *et al.*, (1994) found that in the superfused mouse soleus muscle, prolonged muscle contraction is improved by addition of 3 mM of nitroprusside. This contrasts with its marked effect to inhibit $\dot{V}O_2$ and pressure induced by vasoconstrictors (Colquhoun *et al.*, 1988) and pressure (Fig. 3-2) where dilation of thoroughfare channels might have occurred. However, the opening of thoroughfare channels would be expected to compromise O_2 availability to working skeletal muscle and thereby reduce working skeletal muscle $\dot{V}O_2$ and twitch-tension development. Clearly, this was not evident from the present experiment (Table 3-1). and thus further investigations are required.

A direct effect of NE on skeletal muscle to increase $\dot{V}O_2$ would be consistent with the observation made in the present study. However, it must be kept in mind that the effect of NE is mediated by α-adrenergic receptors (Grubb & Folk 1977; Richter *et al.*, 1982b; Chapter 4) and $\dot{V}O_2$ is also increased by VP and angiotensin II (Colquhoun, *et al.*, 1988; Fig. 3-5). There are relatively fewer α-adrenergic receptors on striated muscle cells (Rattigan *et al.*, 1986) and the majority of these receptors are located on blood vessels (Martin *et al.*, 1990). A biochemical mechanism or reaction of skeletal muscle to account for such a large increase in $\dot{V}O_2$ is yet to be defined.

Working muscle is capable of high rates of $\dot{V}O_2$ and rates of approximately 6 $\mu mol \cdot g^{-1} \cdot min^{-1}$ (Stainsby *et al.*, 1984) have been reported for contracting dog gastrocnemius muscle *in situ*. But the data from Fig 3-6 show that NE stimulates $\dot{V}O_2$ without affecting basal skeletal muscle tone. More importantly, the increased $\dot{V}O_2$ is entirely distinguishable from muscle contraction-induced increase in $\dot{V}O_2$ by tubocurarine that blocks only sciatic nerve stimulation-induced, but not NE-induced, $\dot{V}O_2$.

The increase in perfusion pressure (Fig. 3-1) or flow (Fig. 3-2) would have two hemodynamic forces on vascular walls: stretch or pressure, acting perpendicular to the surface, and shear stress, a tangential, dragging component resulting from friction with the endothelial surface (Davies 1989; Meininger *et al.*, 1991). These two effects on vascular vessels may be involved in the flow (pressure)-induced $\dot{V}O_2$ of the perfused hindlimb. The transient large increase in $\dot{V}O_2$ is probably due to a delay of the response of the O_2 electrode to a sudden increase in flow rate. In addition, blood vessels are known to consume more O_2 during the process of contracting (Murphy, *et al.*, 1983).

It is well established that the $\dot{V}O_2$ of vascular smooth muscle is proportional to its active tension (Paul *et al.*, 1975; Paul, 1980). The increased stretch pressure on vascular walls as a result of increase in perfusion flow (or pressure) could be expected to elevate vascular wall tension. The latter would enhance vascular myogenic responsiveness and work load, thus stimulating vascular $\dot{V}O_2$ ("hot pipes") (Colquhoun *et al.*, 1990; Colquhoun & Clark, 1991). In resting skeletal muscle, the myogenic mechanism acts as the more dominant form of local control of the resistance vasculature (Meininger *et al.*, 1991). This probably explains the inhibition of flow-induced perfusion pressure by vasodilators (Fig, 3-2). The inhibitory effect of nitroprusside on flow-induced $\dot{V}O_2$ may be attributed to its relaxation of blood vessels (Fig. 3-2) whereas NE enhances $\dot{V}O_2$ by further increasing vascular tension. If flow- or vasoconstrictor- induced $\dot{V}O_2$ results from the increased active vascular wall

tension, it would be expected that vasoconstrictor-stimulated $\dot{V}O_2$ is a general feature in muscle vascular bed and may occur in other perfused vascular beds as well.

Renkin *et al.* (1971) proposed that there are nutritive and nonnutritive capillaries in muscle. Some experiments suggested only a part of the nutritive capillaries is recruited under basal conditions (Burton & Johnson, 1972; Johnsson, 1988). Thus, another possibility for flow-induced $\dot{V}O_2$ may be due to an increased recruitment of nutritive capillaries as perfusion flow rate increases. The present perfusions show little autoregulation as indicted by a slow response of flow to a change in pressure (Fig. 3-1). The loss of autoregulation and presumably control of capillary distribution within the muscle may allow perfusate to reach more and more capillaries, thereby increasing $\dot{V}O_2$. In addition, a dye-washout study (Dora *et al.*, 1991; Newman, 1992) at 4 ml/min at 25°C suggested that NE increases perfused vascular volume. NE may redirect the perfusate to nutritive areas that consume more O_2. Based on this hypothesis, the vasodilator-inhibited $\dot{V}O_2$ could be interpreted as a result of redirecting perfusate to less nutritive muscle capillaries. This would imply that the increased flow of perfusate to nutritive capillaries somehow stimulates mitochondrial respiration to consume O_2 or other yet unknown thermogenic process.

CHAPTER 4

Vascular control of $\dot{V}O_2$ in the perfused hindlimbs of a marsupial (bettong) and an amphibian (cane toad).

4.1 Introduction

Marsupials are a diverse group of mammals with well-developed thermoregulation. The Tasmanian bettong (*Bettongia gaimardi*) is a rat-kangaroo closely related to the larger kangaroos and wallabies. At birth, the bettong is ectothermic. The young becomes progressively endothermic before it finally vacates the pouch (Rose, 1987). Intramuscular injection of NE elicited a significant increment in $\dot{V}O_2$ *in vivo* in the potoroo *Potorous tridactylus* (Kerr), a close relative of the bettong, and this increase was blocked by propranolol but not by phenoxybenzamine (Nicol 1978). Hayward and Lisson (1992) have presented strong anatomical and microscopical evidence that marsupials, including bettongs, do not possess BAT. The lack of BAT in bettongs is further supported by a recent molecular biology study using Northern blotting which failed to identify any UCP in this species (Trayhurn, personal communication, 1994). This characteristic makes the bettong a valuable animal for the study of non-shivering thermogenesis occurring in tissues other than BAT. To date, no study on $\dot{V}O_2$ in a perfused marsupial skeletal muscle has been found in the literature. Accordingly, the first aim of the present study attempted to examine whether the vasoconstrictive hormones, NE, VP and 5-HT, have any effect on $\dot{V}O_2$, and lactate and glycerol efflux from the perfused hindlimb of the bettong as an example of marsupial muscle metabolism.

By comparison, amphibians are typical ectothermic animals with a very low blood pressure of 15-30 mmHg (Withers *et al.*, 1988; Pelster *et al.*, 1993) and much lower $\dot{V}O_2$ which ranges between 0.4-1.2 $ml \cdot kg^{-1} \cdot min^{-1}$ (Altman & Dittmer, 1974; Degani & Meltzer, 1988). The acclimation of metabolic rate in amphibians is still controversial. The data from Feder *et al.* show an increase in metabolic rate of salamanders (*Desmognathus*) following cold exposure (reviewed by Lawrence *et al.*, 1992). Wood (1991) found no evidence for thermal acclimation of $\dot{V}O_2$ in the salamander (*Trarichа granulosa*). In a small frog (*Neobatrachus pelobatoides*), the measured muscle $\dot{V}O_2$ varied between 2.0-5.6 $\mu mol \cdot g^{-1} \cdot h^{-1}$, and contributed 60-77% of the whole body metabolic rate with a total mass of 35% of the body weight (Flanigan *et al.*, 1991). Yet little is known about the effect of vasoactive hormones on

muscle metabolism in amphibians. Hence, comparisons of vasoconstrictor-mediated alterations in skeletal muscle O_2 metabolism between the endotherms (rats and marsupials) and ectothermians (toads) will provide useful information for addressing the role of the vascular system in controlling or contributing to muscle thermogenesis. To this end, the cane toad (*Bufo marinus*) was used as a representative of ectothermic amphibians in the present study. Therefore the second aim in this study was to examine the effect of NE on $\dot{V}O_2$ in perfused toad hindlimbs to observe whether the relationship between vascular function and $\dot{V}O_2$ was universal regardless of whether an animal is endothermic or ectothermic.

4.2 Methods

The experiments for both species were performed in a constant-flow (once-though) model at 25°C. The bettong hindlimb was perfused with modified Krebs-Ringer buffer with 1.27 mM $CaCl_2$ and 2% BSA whilst the toad hindlimbs were perfused with modified amphibian Hepes buffer containing 1% BSA and 1.3 mM $CaCl_2$. The details for the perfusion were described in Chapter 2 (Section 2.2). The efflux of lactate and glycerol was measured enzymatically as previously described (Section 2.3). Muscle metabolites of bettongs were measured in freeze-clamped gastrocnemius-plantaris-soleus group. High energy phosphate compounds were determined by HPLC (Waters Associates, USA) with a Radial-Pak column (C18, 4 μ m) according to the method of Shellevold *et al.* (1986, see Section 2.3.3.2).

4.3 Results

4.3.1 Validation of perfusion for the perfused hindlimb

Table 4-1 summarize the mean values for body weight of these animals, the mass of perfused skeletal muscle, as well as the perfusion pressure, $\dot{V}O_2$, and the rate of release of lactate and glycerol. $\dot{V}O_2$ as well as lactate efflux was maintained stable in the perfused hindlimb(s) of both species throughout the experiment. At no stage did the PvO_2 in the perfused hindlimb(s) during the maximum O_2 extraction decline below 200 mm Hg for the bettong perfused hindlimb or below 400 mm Hg for the perfused toad hindlimbs. Both the basal $\dot{V}O_2$ and lactate efflux of the toad muscle were lower in comparison with those of the bettong.

Table 4-1 Basal measurements for the perfused bettong and toad hindlimb(s)

Species	n	Body wt. (g)	Perfused muscle (g)	Flow ($ml \cdot g^{-1} \cdot min^{-1}$)	Pressure (mm Hg)	$\dot{V}O_2$	Lactate ($\mu mol \cdot g^{-1} \cdot h^{-1}$)	Glycerol
Bettong *(B. gaimardi)*	7-8	1200 ± 110	25.0 ± 2.3	0.28 ± 0.02	32.0 ± 2.30	4.18 ± 0.35	4.41 ± 0.39	0.28 ± 0.03
Cane toad *(Bufo marinus)*	5	129.4 ± 16.6	18.4 ± 3.2	0.20 ± 0.02	15.8 ± 3.2	1.36 ± 0.10	0.70 ± 0.17	not done.

Table 4-2 Metabolites of bettong gastrocnemius-plantaris-soleus muscle group

	n	Lactate	Cr	CrP	AMP	ADP	ATP	ΣAN	Cr+CrP	EC	CrP/ATP
		($\mu mol/g$ dry weight)									
In vivo	5	14.02	318.4	99.78	0.16	2.63	23.09	25.87	418.2	0.943	4.33
		± 2.74	± 6.08	± 1.84	± 0.01	± 0.06	± 0.64	± 0.66	± 6.58	± 0.002	± 0.10
Perfused	5	6.55**	324.2	126.6**	0.16	2.58	25.28	28.03	450.8	0.948	5.03*
		± 0.59	± 12.50	± 4.45	± 0.02	± 0.09	± 0.79	± 0.80	± 14.03	± 0.002	± 0.21

ΣAN: total adenine nucleotides, Cr: creatine, CrP: creatine phosphate. * $P < 0.05$; ** $p < 0.01$, vs *in vivo*. Data are means ± SE.

Measurement of metabolites in the frozen bettong gastrocnemius-plantaris-soleus muscle group at the end of the perfusion period showed that there were no significant statistical differences of adenine nucleotides between the muscle samples obtained *in vivo* and after perfusion. In fact, the concentration of lactate decreased ($P<0.05$), and the CrP concentration and the CrP/ATP ratio were both improved ($P<0.01$) after the perfusion (Table 4-2). These results show an essentially normal metabolite profile of the perfused muscle without evidence of hypoxia.

4.3.2 Actions of vasoconstrictors

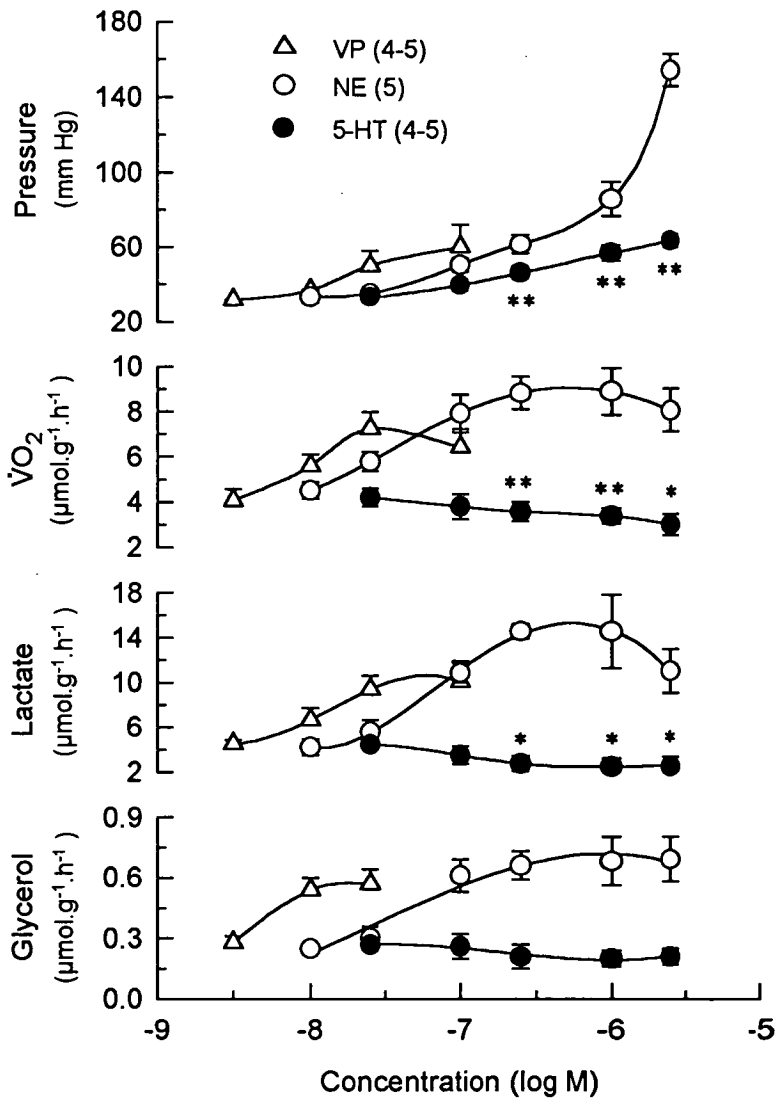

Fig. 4-1 Dose-response curves for vasoconstrictor-mediated changes in pressure, $\dot{V}O_2$, lactate and glycerol efflux in the constant-flow perfused bettong muscle. Significance for 5-HT data is indicated: * $P<0.05$, ** $P<0.01$. Data are means ± SE.

As shown in Fig. 4-1, NE, VP and 5-HT each exerted a qualitatively similar dose-dependent vasoconstrictive effect in the perfused bettong hindlimb. Their other effects on $\dot{V}O_2$, lactate and glycerol efflux aggregated into two different sets of responses. Thus qualitatively, NE and VP induced increases in $\dot{V}O_2$, lactate and glycerol efflux which appeared to be closely associated with the changes in perfusion pressure. NE-induced changes attained a maximal value at approximately 1.0 μM NE with increases greater than 100% over the basal in the three variables. Above this dose, values of $\dot{V}O_2$ tended to decline, although the perfusion pressure continued to rise.

Similarly, VP stimulated, but to a lesser degree, the $\dot{V}O_2$, and the efflux of lactate and glycerol with maximal values occurring at approximately 25 nM. Perfusion pressure continued to increase with dose. In contrast to NE and VP, 5-HT significantly inhibited $\dot{V}O_2$ ($P<0.05$) in the concentration range 0.1-2.5 μM during vasoconstriction. After termination of infusion of each of the vasoconstrictors, values for PvO_2 and perfusion pressure returned to basal values throughout the periods of perfusion which lasted at least 3-4 hours.

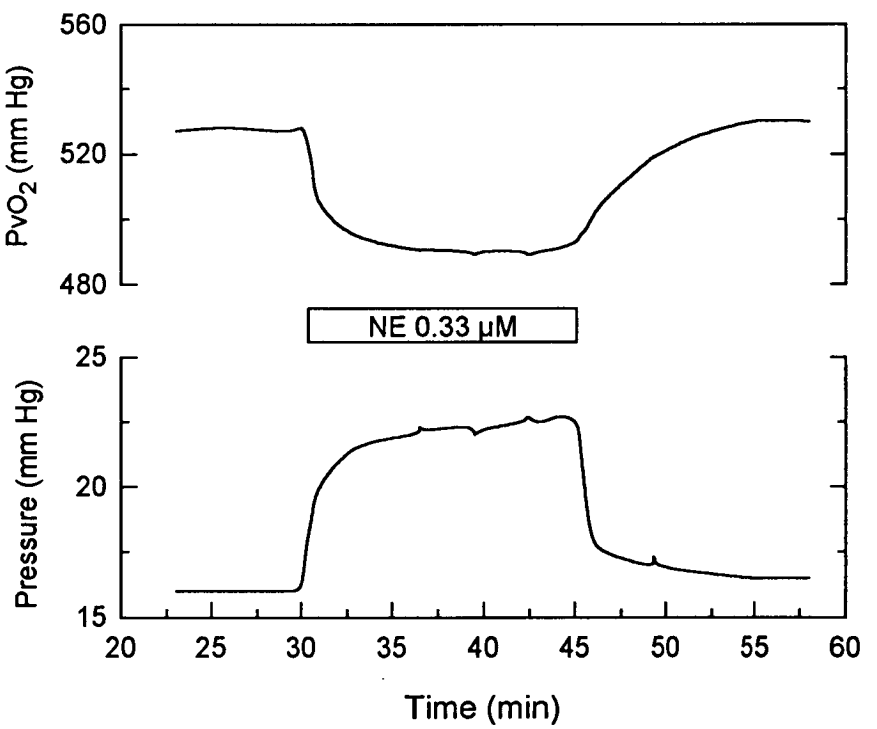

Fig. 4-2 Representative tracing of the effect of NE on PvO_2 and perfusion pressure in the constant-flow perfused toad hindlimbs.

In the perfused toad hindlimbs, the infusion of NE increased both the perfusion pressure and $\dot{V}O_2$. The changes in perfusion pressure and $\dot{V}O_2$ responded to the infusion of NE rapidly in the onset, stayed steady during the infusion period and returned to the basal state within 10 min after terminating the administration (Fig 4-2). These responses were readily repeatable during each perfusion. As shown in Fig. 4-3, NE started to have effect on $\dot{V}O_2$ at a concentration of 10 nM and attained the maximum effect on$\dot{V}O_2$ at 0.3 μM. The change in perfusion pressure was closely associated with the changes in $\dot{V}O_2$ but did not reach a plateau within the dose ranges applied. The maximal increase in $\dot{V}O_2$ reached approximately 28% above the basal value, and stayed around that level without an appreciable trend of inhibition up to NE concentrations of 30 μM. Lactate initially showed no significant change followed by a significant decline ($P<0.05$).

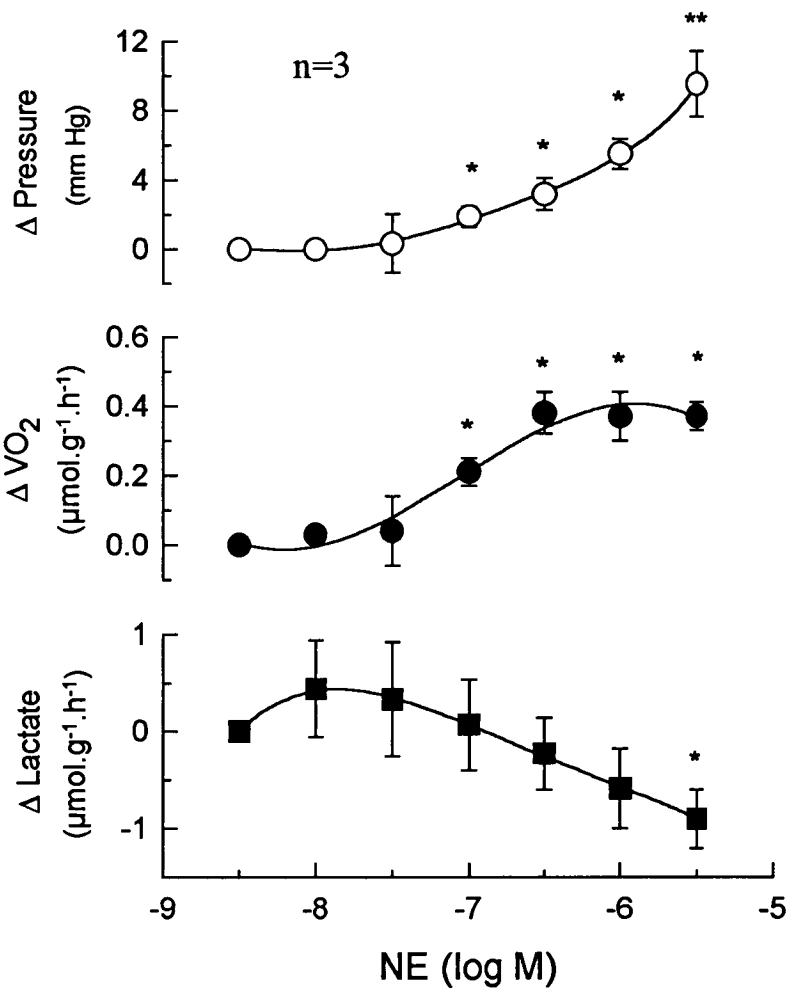

Fig. 4-3 Dose-response curves of NE on perfusion pressure, $\dot{V}O_2$ and lactate in the constant-flow perfused hindlimbs of toads. The basal values are shown in Table 4-1. * $P<0.05$, **$P<0.01$ vs the basal values (Paired *t* test).

4.3.3 Effects of α- and β-adrenergic antagonists on NE-mediated effects on the perfused bettong hindlimb

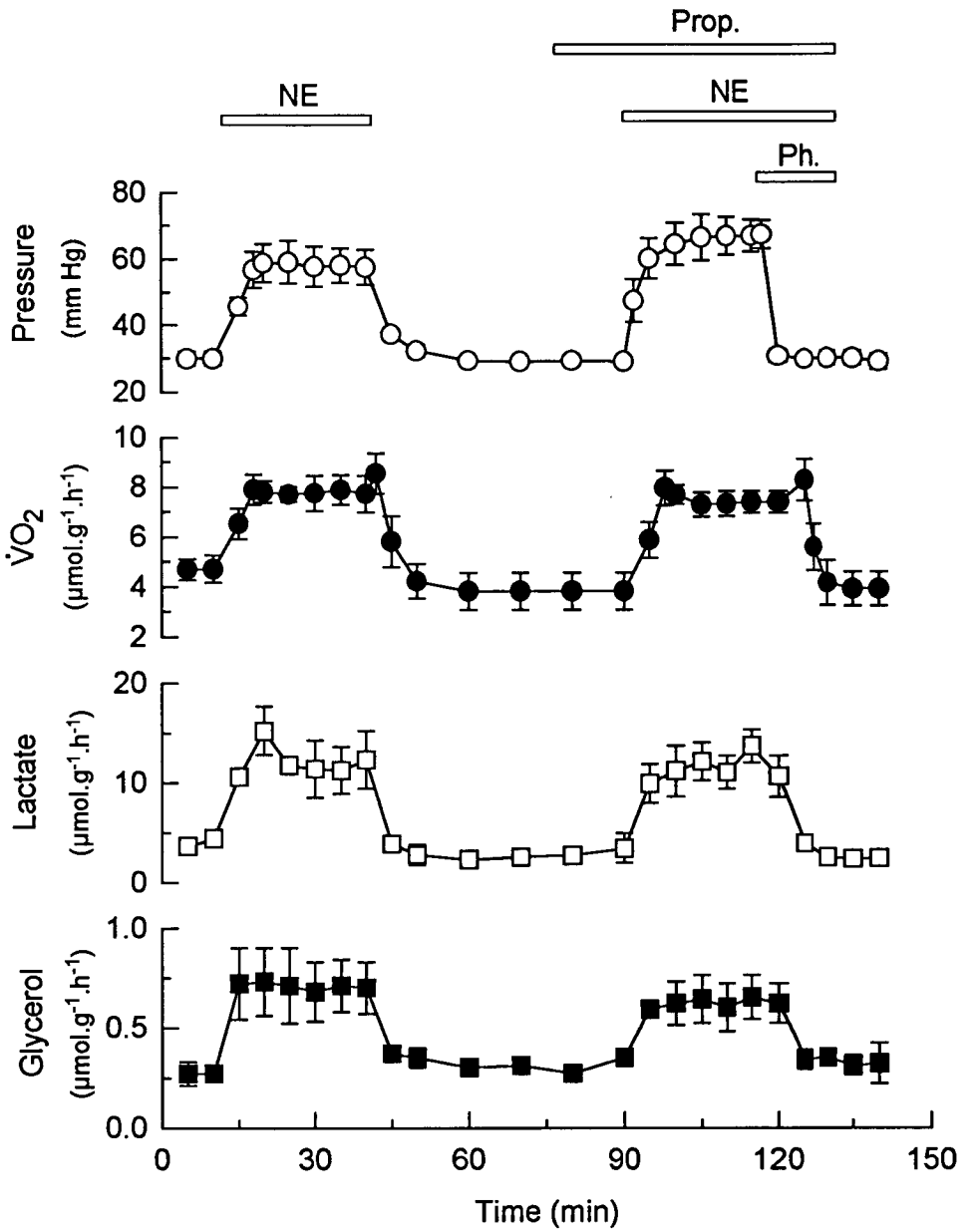

Fig. 4-4 Effects of α- and β-adrenergic antagonists on NE-mediated changes in perfusion pressure, $\dot{V}O_2$, lactate and glycerol efflux in the constant-flow perfused bettong hindlimb. Prop.: propranolol (10 µM, racemic), Ph.: phentolamine (10 µM), NE (0.1 µM). The length of each bar represents the duration of infusion of a given drug (mean ± SE, n=3).

Changes in perfusion pressure, $\dot{V}O_2$, lactate and glycerol release in response to NE were rapid in onset, sustained and rapidly reversible (Fig. 4-4). In the presence of the β-adrenergic antagonist propranolol (10 μM D,L-racemic), NE-induced perfusion pressure tended to be higher, but none of $\dot{V}O_2$, and lactate and glycerol release were statistically different when compared to NE alone. However, the additional infusion of the α-adrenergic antagonist, phentolamine (10 μM), almost completely blocked the rise in perfusion pressure as well as the increases in $\dot{V}O_2$, and lactate and glycerol release elicited by NE.

4.3.4 Effect of nitroprusside on the perfused bettong hindlimb

Fig. 4-5 shows a representative tracing of changes in perfusion pressure and P_VO_2 induced by VP, NE and 5-HT. These changes were rapid in onset and sustained. The infusion of nitroprusside (1.0 mM) completely blocked the rise in perfusion pressure and the changes in $\dot{V}O_2$ stimulated by NE, VP and 5-HT (Fig. 4-5).

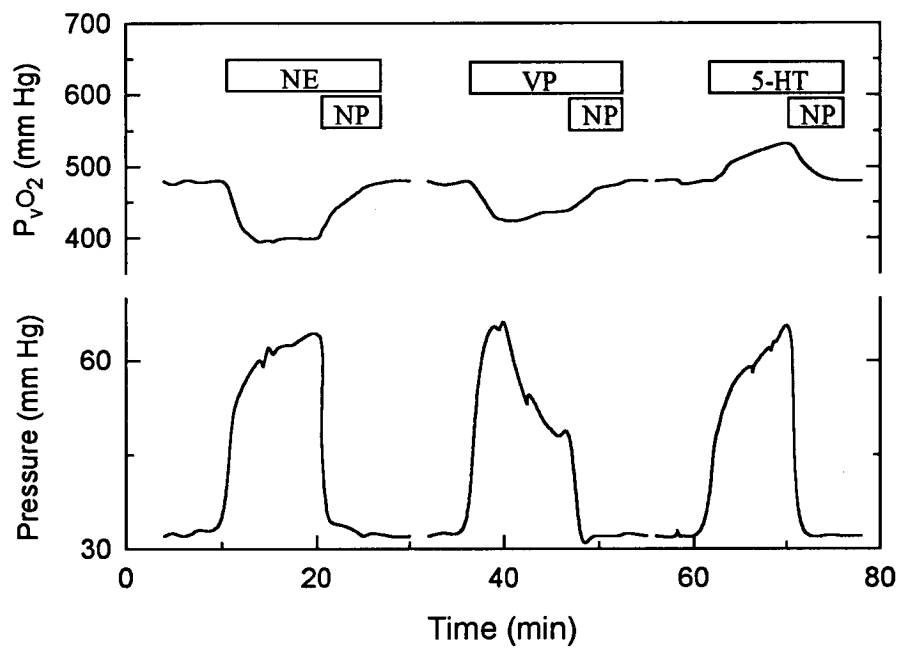

Fig. 4-5 Representative tracing of the inhibitory effect of nitroprusside on perfusion pressure and PvO_2 mediated by vasoconstrictors in the constant-flow perfused bettong hindlimb. Nitroprusside (NP), 1.0 mM, NE: 0.1 μM, VP: 25 nM and 5-HT: 2.5 μM. Two to three perfusions were performed.

4.4 Discussion

In the present study, bettong hindlimbs were perfused at 25°C at flow rates chosen to give a constant flow per unit mass of muscle of 0.28 $ml \cdot min^{-1} \cdot g^{-1}$ (Table 4-1). This constant flow rate was the same as that employed for rat hindlimb perfusions at the same temperature (Colquhoun *et al.*, 1988; 1990, Dora *et al.*, 1991, and Hettiarachchi *et al.*, 1992).

Whilst resting hindlimb perfusion pressures are at first sight low, arterial pressures will necessarily be lower than *in vivo* when employing a cell-free, low viscosity perfusate. In addition, there is neither resting sympathetic tone nor circulating vasoconstrictors in the perfused hindlimb. Thus the arterial tree is almost fully relaxed as shown by the fact that nitroprusside does not lower the perfusion pressure (Fig. 4-5). The *in vivo* blood flow rates to the skeletal muscle of the bettong's larger relative, the red kangaroo (*Macropus rufus*), at rest under different environmental temperatures ranged from 0.010 to 0.053 $ml \cdot min^{-1} \cdot g^{-1}$ (Needham & Dawson, 1984). Thus if similar flow rates apply to the bettong, the flow rate we employed is approximately 5 to 28 times greater than *in vivo*. Furthermore, because there is little diminution of pressure on progressive passage through the dilated arterial and arteriolar beds, the capillary exchange pressures should be similar to those occurring in vivo. Adequate perfusion and O_2 delivery to the hindlimb preparation at rest and under the maximal vasoconstriction or $\dot{V}O_2$ are indicated by the normal values for intracellular lactate, high energy phosphate compounds and the calculated energy charge in perfused muscle (Table 4-2). This is further supported by values for PvO_2 which at no stage fell below 200 mm Hg.

As for the perfused toad hindlimbs, the basal perfusion pressure of 15.8 ± 3.2 mm Hg was close to the blood pressure of 20-30 mm Hg *in vivo* (Withers *et al.*, 1988) of this species. Although the number of the toads was not available for a similar metabolite analysis for the perfused muscle, the lower requirement for O_2 as shown by the basal $\dot{V}O_2$ (Table 4-1) and higher PvO_2 (compared with the bettong perfused hindlimb) indicated an adequate O_2 supply to the hindlimbs had been achieved. Hepes-buffer containing 1.4 mM rather than amphibian Ringer buffer was employed in the present experiment and is the same as that used for the perfused bullfrog heart (Pelster *et al.*, 1993). 1% BSA was added to this perfusate to prevent edema. In the bullfrog tadpole, plasma protein concentration was 0.2 mmol/l (Just *et al.*, 1977). If this value applies to the cane toad as well, the concentration of albumin used in the perfusate would be similar to the concentration *in vivo*.

The basal $\dot{V}O_2$ of 4.18 ± 0.35 μmol·g^{-1}·h^{-1} for the perfused bettong hindlimb is approx. 65% that of perfused rat hindlimbs of 6.4 μmol.g^{-1}.h^{-1} (Colquhoun *et al.*, 1988; 1990; Hettiarachchi *at al.*, 1992). An even lower $\dot{V}O_2$ of the perfused toad hindlimbs (1.36 ± 0.10 μmol·g^{-1}·h^{-1}) was noted (Table 4-1). These relativities were expected when the whole body metabolic rates of these species were considered. The whole body $\dot{V}O_2$ (see Table 8-1, Chapter 8) for 250 g rats ranged 19.0 ± 1.3 ml·kg^{-1}·min^{-1} (51 ± 3.5 μmol·g^{-1}·h^{-1}) whereas the $\dot{V}O_2$ of the intact bettong (1.7 kg) attained 8.6 ± 0.7 ml·kg^{-1}·min^{-1} (23 ± 1.9 μmol·g^{-1}·h^{-1}). At the same temperature (25 °C), the whole body $\dot{V}O_2$ of toad (*Bufo marinus*, 88.7 g) was reported to be 1.2 ml·kg^{-1}·min^{-1} [3.2 μmol·g^{-1}·h^{-1}, (Altman & Dittmer, 1974)]. Thus the basal $\dot{V}O_2$ of the skeletal muscle paralleled body metabolic rates across the eutherian, marsupial and amphibian.

Skeletal muscle has been thought to be a thermogenic tissue in mammals. The lower muscle metabolic rate in the toad perfused hindlimb is consistent with the fact that the toad is a ectothermic species and there is no need for its skeletal muscle to be thermogenic. The differences in skeletal muscle mitochondria between mammals and reptiles reported in the literature (Else & Hulbert, 1985) seem to support this argument. In that study, the mitochondrial cell volume density of reptile skeletal muscle was 0.89% compared with the 8.16% reported in mammals and the mitochondrial surface area (m^2) per volume of mitochondria (cm^3) was also lower in reptiles (93.2 in reptiles and 110.0 m^2/cm^3 mitochondria in mammals). These mitochondrial characteristics in reptiles are consistent with the lower muscle metabolic rate seen in perfused toad hindlimbs in the present experiments.

Although there are a number of reports on the effect of infused NE on $\dot{V}O_2$ in the perfused muscle in eutherian mammals such as the rat (Grubb & Folk, 1977; Dubois-Ferrière & Chinet, 1981; Richter, 1982b, Côté *et al.*, 1985; Colquhoun *et al.*, 1988 & 1990), and the dog (Pappenheimer, 1941a), to the best of my knowledge, this is the first report on the effects of vasoconstrictors (ie. NE, VP and 5-HT) on $\dot{V}O_2$, and lactate and glycerol efflux in perfused marsupial hindlimbs and hence in a non-eutherian homoeothermic species.

In the perfused bettong hindlimb, increases in $\dot{V}O_2$, and in both lactate and glycerol effluxes were observed in response to NE and VP, whereas decreases in $\dot{V}O_2$, and lactate and glycerol efflux were noted with 5-HT (Figs. 4-1 and 4-5). The changes in $\dot{V}O_2$, and lactate in response to these vasoconstrictors were qualitatively similar to those observed previously in the perfused rat hindlimb (Colquhoun *et al.*, 1988; 1990; Dora *et al.*, 1992). A new observation for muscle perfusion was that changes in the glycerol efflux closely paralleled the changes in the hindlimb $\dot{V}O_2$ and

lactate (Figs. 4-1 & 4-4). The production of lactate and glycerol will have thermogenic potential *in vivo* if they are subsequently taken up by other tissues such as the liver and resynthesised to carbohydrate or lipid as these processes would require additional energy expenditure.

Analysis of the dose response data (Fig. 4-1) for the perfused bettong hindlimb shows that the thermogenic response to NE started at 25 nM which is almost 25 times higher than that required for the perfused rat hindlimb under the same conditions (Colquhoun *et al.*, 1988). The increase in $\dot{V}O_2$ stimulated by NE of approximately 100% above the basal value was greater than that of the perfused rat hindlimbs which increases by approximately 60-70% at the same flow rate (Colquhoun *et al.*, 1988; Hettiarachchi *et al.;* 1992). At present, no data on circulating NE are available for the bettong and in general, data are sparse for all marsupials. However a small marsupial, the sugar glider (Stoddart *et al.*, 1991), and the platypus (McDonald *et al.*, 1992) show resting and stressed values of NE approx 3-10 fold higher than those of the rat. The plasma levels of NE and E for the unstressed carnivorous marsupial [*Antechinus stewartii* (Bradley, personal communication, 1994)] is 4-5 times those of the rat (Eriksson & Persson, 1982). So that the present observations may be relevant to field conditions.

Vasoconstrictor-mediated changes in $\dot{V}O_2$ occurred not only in the perfused hindlimbs of endotherms as mentioned above but also in the toad perfused hindlimbs where NE stimulated $\dot{V}O_2$ during vasoconstriction (Fig. 4-2). NE started to elicit $\dot{V}O_2$ changes at 10 nM and achieved the maximum effect with 28% increase above the basal at 0.33 μM. In the constant-flow perfused rat hindlimb, NE stimulates $\dot{V}O_2$ at low concentrations (0.01-1 μM) and the increased $\dot{V}O_2$ began to fall at the concentration higher than 1 μM (Dora *et al.*, 1992). Fig. 4-1 shows a similar tendency of decline in $\dot{V}O_2$ at 2.5 μM of NE. In contrast, the NE-mediated dose-response curve for $\dot{V}O_2$ did not show a tendency to decline up to a dose of 33 μM (Fig. 4-3). Compared with the bettong (or rat), NE-mediated a marked lower increase in $\dot{V}O_2$ in the toad perfused hindlimbs. As argued above, the toad skeletal muscle is not as potentially thermogenic because of the significantly lower content of mitochondria and mitochondrial surface area (Else & Hulbert, 1985). A comparatively lower increase in NE-stimulated $\dot{V}O_2$ in the perfused toad hindlimbs may result from their structural bases of skeletal muscle mitochondria.

In amphibians, E is the sympathetic neurotransmitter whereas NE is the neurotransmitter in the higher vertebrates. Withers *et al.* (1988). reported plasma levels of 2.1 nM NE and 13.1 nM E in the toad (*Bufo marinus*) at rest. When the animal was continuously and repeatedly flipped onto its dorsum and then allowed to

right itself for 3 min,, the plasma levels for NE and E increased to 9.8 nM and 72 nM respectively, resulting in significant increases in the heart rate and blood pressure. Coinciding with the increased catecholamines was the maximal rate of $\dot{V}O_2$ in the animal (Hillman *et al.*, 1979; Walesberg *et al.*, 1986). As skeletal muscle is believed to be the greatest contributor to the whole body metabolism *in vivo* in amphibians (Flanigan *et al.*, 1991), the present findings indicate the catecholamine-induced whole body metabolic rate may largely originate from skeletal muscle under the control of the vascular system *in vivo*.

In the present study, lactate did not significantly increase during the infusion of NE and started to decline when the increased $\dot{V}O_2$ reached the maximum. The mechanism for the NE-induced dissociation of lactate release from $\dot{V}O_2$ in the perfused toad hindlimbs (Fig. 4-3) is not known. Since the toad muscle metabolic rate is significantly lower compared with that of the eutherian mammals (Table 4-1), it is possible that an increased aerobic metabolism would accelerate lactate oxidation faster than glycolysis.

Marsupials appear to have more skeletal muscle than eutherians. Red and grey kangaroos have 52% of body mass as skeletal muscle which is 6-8% more than in the wildebeest or Thomson's gazelle, and approx 20% more than in domestic eutherian species (Tribe & Peel, 1963). The blood flow to the marsupial skeletal muscle varies considerably in response to the environmental temperature. For example, the distribution of cardiac output to skeletal muscle increased from 4% at 37 °C to 23% at 7°C (Needham & Dawson, 1984). When exposed to the cold, the maximal heat production of small marsupials can match or exceed that of rodents of similar size (Dawson & Dawson, 1982). Thus the present study, when taken in conjunction with these observations, suggests that marsupial skeletal muscle may contribute a larger proportion to whole body thermogenesis than the skeletal muscle in eutherian species. As the bettong and other marsupials have been shown not to possess histologically apparent BAT (Hayward & Lisson, 1992), the bigger relative increase in NE (or other vasoconstrictor)-induced energy metabolism and the greater proportion of skeletal muscle may compensate for the lack of BAT in these species.

Unexpectedly, the NE-induced glycerol release was found to be mediated through α-adrenergic receptors as shown by its blockade by phentolamine plus propranolol but not by propranolol alone (Fig. 4-3). A recent study in the constant-flow perfused rat hindlimb also revealed an increased glycerol efflux mediated by α-adrenoceptors (Clark *et al.*, 1994). Activation of lipolysis by epinephrine has been reported in the perfused mouse hindlimb and β-adrenergic receptors were thought to be responsible (Chan *et al.*, 1983). On the other hand, inhibition of adipose tissue

lipolysis has been associated with activation of the α_2 -adrenergic receptor (Lafontan *et al.*, 1992). β-Adrenoceptors are known to be present in skeletal muscle (Martin *et al.*, 1990) and some appear to be atypical or putative β_3 subtype (Roberts *et al.*, 1993; Sillence *et al.*, 1993) whilst the α-receptors are much more sparse and are 30 times more prevalent in arterioles than in the surrounding skeletal muscle (Martin *et al.*, 1990). Nevertheless the NE-mediated increases in $\dot{V}O_2$, lactate and glycerol effluxes in the present experiment are α-receptor dependent rather than β-receptor dependent (Fig. 4-3), suggesting blood vessels are somehow involved in the increased metabolism.

The close parallel relationship between vasoconstrictor-mediated glycerol release and $\dot{V}O_2$ change implies that the vasoconstrictors may play a role in modulating lipid mobilisation either directly by receptor activation or indirectly by association with smooth muscle contraction to adjust the fuel sources for skeletal muscle thermogenesis. Chin *et al.* (1991) have shown that infused lactate can lead to increased glycerol production in the perfused rat hindlimb. Hence in the present study even though there is no recirculation of perfusate, another potential source of glycerol would be the conversion of a small proportion of lactate to glycerol via glycerol-3-phosphate and triacylglycerol.

The lack of a β-adrenergic stimulation of $\dot{V}O_2$ in perfused bettong hindlimb (Fig. 4-3), which was also found in the perfused rat muscle (Colquhoun *et al.*, 1988; Hettiarachchi *et al.*, 1992), contrasts with the well-known stimulation of $\dot{V}O_2$ by β-adrenergic agonists *in vivo* in the rat (Rothwell, 1990) and in another marsupial rat-kangaroo the potoroo (Nicol 1978). This discrepancy might in part be explained by noting that in general, β-adrenergic excitation is known to elevate cardiac output and relax blood vessels *in vivo*. These effects could result in increased blood flow to skeletal muscle. Increased flow to skeletal muscle has been shown to increase $\dot{V}O_2$ by perfused skeletal muscle in the rat (Chinet & Mejsnar, 1989; Chapter 3). In addition, other thermogenic tissues, such as liver, might also contribute to β-adrenergic stimulation of the whole body metabolic rate (Ma *et al.*, 1987). Alternatively, β-stimulation leading to vasodilation *in vivo* may oppose a pre-existing pattern of vasoconstriction that inhibits $\dot{V}O_2$ as observed with either 5-HT or high concentrations of NE in the perfused rat hindlimb (Clark *et al.*, 1994). However, β-stimulation of $\dot{V}O_2$ was observed in the constant-pressure (recirculated) perfused rat hindlimb (Hardeveld *et al.*, 1980; Chapter 8). Chapter 9 will further discuss the different results obtained from these two models.

The action of the nitric oxide generator, nitroprusside to block both vasoconstriction and the changes in $\dot{V}O_2$ whether they be increased or decreased (Fig.

4-4), strongly suggests that vasoconstriction is tightly linked in some manner to these phenomena. Yet the mechanism for the vasoactive hormone-induced increase in $\dot{V}O_2$ is still controversial. Chapter 3 and 4 have discussed two possible mechanisms of vascular involvement in the vasoconstrictor-mediated increase in $\dot{V}O_2$: direct vascular $\dot{V}O_2$ and/or vascular-controlled muscle $\dot{V}O_2$. Both interpretations appear to explain vasoconstrictor-induced $\dot{V}O_2$ in the perfused hindlimbs of bettong and toad. Chapter 9 will further discuss various possibilities of the observations along with the results from other Chapters.

In summary, the present experiments show that vasoconstrictive hormones play an important role in controlling and/or contributing to $\dot{V}O_2$, lactate and glycerol efflux in perfused marsupial (bettong) muscle and clearly have the potential to act *in vivo*. There appears to be a greater contribution from the skeletal muscle vascular bed to the whole body thermogenesis in the bettong than in the eutherian in response to NE, which may *in vivo* compensate for its lack of BAT. The responses, however, do not relate in a simple way to the degree of vasoconstriction, because there are both increases and decreases in the metabolic parameters depending on the vasoconstrictor employed. Vasoconstrictor-mediated increases in muscle $\dot{V}O_2$ also takes place in ectothermic amphibians. The present findings on perfused bettong and toad muscle when taken together with other studies on perfused rat muscle raise the possibility of vasoconstrictor control of $\dot{V}O_2$ and metabolism as an entrenched and widespread physiological mechanism across ectothermic and endothermic species that has developed in the process of evolution.

CHAPTER 5

Vascular control of $\dot{V}O_2$ in the constant-flow perfused rat kidney, intestine and mesenteric artery

5.1 Introduction

As presented in Chapters 3 and 4, vasoconstrictors such as NE and VP stimulate both $\dot{V}O_2$ and increase perfusion pressure in the constant-flow perfused hindlimb of the rat, bettong, and toad. These data suggest that vasoconstrictor-mediated control of skeletal muscle $\dot{V}O_2$ appears to be universal across species, from ectothermic to endothermic animals.

The evidence supporting a controlling role for the vasculature includes observations where each of the vasoconstrictors alters $\dot{V}O_2$ as well as lactate and glycerol production by the hindlimb in conjunction with an increase in perfusion pressure (Colquhoun *et al.*, 1988; 1990) whereas the vasodilators, nitroprusside, nifedipine and Iso, which each act by different mechanisms, oppose vasoconstrictor-mediated changes in both $\dot{V}O_2$ and perfusion pressure (Colquhoun *et al.*, 1990; Chapter 44). The notion that the vascular tissue itself consumes O_2 during vasoconstriction (Chapter 3) is indirect and based principally on data where $\dot{V}O_2$ due to skeletal muscle contraction is additive to that produced by vasoconstrictors (Colquhoun *et al.*, 1990; Chapter 3) and on experiments where near maximal flow-induced $\dot{V}O_2$ by the hindlimb is further increased by NE and inhibited by nitroprusside (Chapter 3).

If vascular tissue is largely responsible for the increase in $\dot{V}O_2$ during vasoconstriction, rates of $\dot{V}O_2$ per unit mass would approach that of the vigorously contracting skeletal muscle or cardiac muscle. This would assign a much higher respiratory rate to vascular tissue than has previously been estimated using fragments of large vessels, whether incubated or cannulated (Paul, 1980). Moreover, vasoconstrictor-induced $\dot{V}O_2$ would be expected to similarly occur as a general feature in other perfused vascular beds, especially vascular tissues.

In the absence of direct information on $\dot{V}O_2$ by perfused vascular tissue in the presence of vasoconstrictors, the present experiments were undertaken to assess whether vasoconstrictors increase $\dot{V}O_2$ in other perfused organs in general. The kidney was chosen because of its unique and high vascular density, and the intestine was chosen for comparison between intestine and mesenteric blood vessels.

5.2 Methods

Male Wistar rats weighing 180-200 g were used for the perfusions of kidney, intestine and mesenteric artery. The perfusion was carried out at 25°C with carbogen (95% O_2 and 5% CO_2) saturated Krebs-Ringer buffer containing 2% BSA and 1.27 mM Ca^{2+} in a constant-flow (once-through) model. Further details are outlined in Chapter 2 (Section 2.2). For the perfused mesenteric artery, a careful dissection was conducted to separate blood vessels indicated by the infused Evans blue from the surrounding white fat and lymphnodes after perfusion. The measured blood vessel weight in this preparation accounted for 6% of the perfused mass.

Venous perfusate was collected for the determination of lactate and glycerol output. The tissue samples were freeze-clamped by a pair of aluminium tongs precooled in liquid N_2 at the end of perfusion. For comparison, normal (*in vivo* under anaesthesia) and ischemic (5 min at 25°C after decapitation) kidney and intestine were also clamped and assayed. Both lactate and glycerol were measured according to the method of Bergmeyer (1974). Adenine nucleotides and creatine compounds were determined by ion-paired reverse phase column in HPLC (LKB) as described by Shellevold et al. (1986). Further details are given in Section 2.3.

5.3 Results

5.3.1 The perfused kidney

5.3.1.1 Vasoconstrictor-mediated changes in $\dot{V}O_2$ and pressure

The perfusion flow for the kidney was set to maintain a physiological pressure of approx. 90 mm Hg at 25°C. This was achieved at 4.7 ml/min per kidney. Under these conditions, the basal $\dot{V}O_2$ of 70.3 ± 3.4 $\mu mol \cdot g^{-1} \cdot h^{-1}$ compares reasonably well with published values of 171-425 $\mu mol \cdot g^{-1} \cdot h^{-1}$ (Cohen & Pendleton, 1980; Franke *et al.*, 1976) obtained at 37°C and at higher flow rate (approx. 25 $ml \cdot min^{-1} \cdot g^{-1}$ kidney) and therefore higher arterial pressure (112-120 mm Hg). The temperature difference could be expected to at least double the rate and the pressure difference of between 80 and 120 mmHg could be expected to increase $\dot{V}O_2$ by a further 23% (Franke *et al.*, 1976).

Figure 5-1 shows dose-response curves for NE- and VP-mediated increase in $\dot{V}O_2$ and perfusion pressure development. Although dose-dependent, the increases in

$\dot{V}O_2$ and pressure did not reach maximal values for each agonist. However, it was apparent that changes in pressure and $\dot{V}O_2$ followed each other closely; the shapes of the dose-response curves were similar for a given agonist. The highest value of $\dot{V}O_2$ attained was 102 μmol/h per kidney, representing an increase of 45% over the basal values.

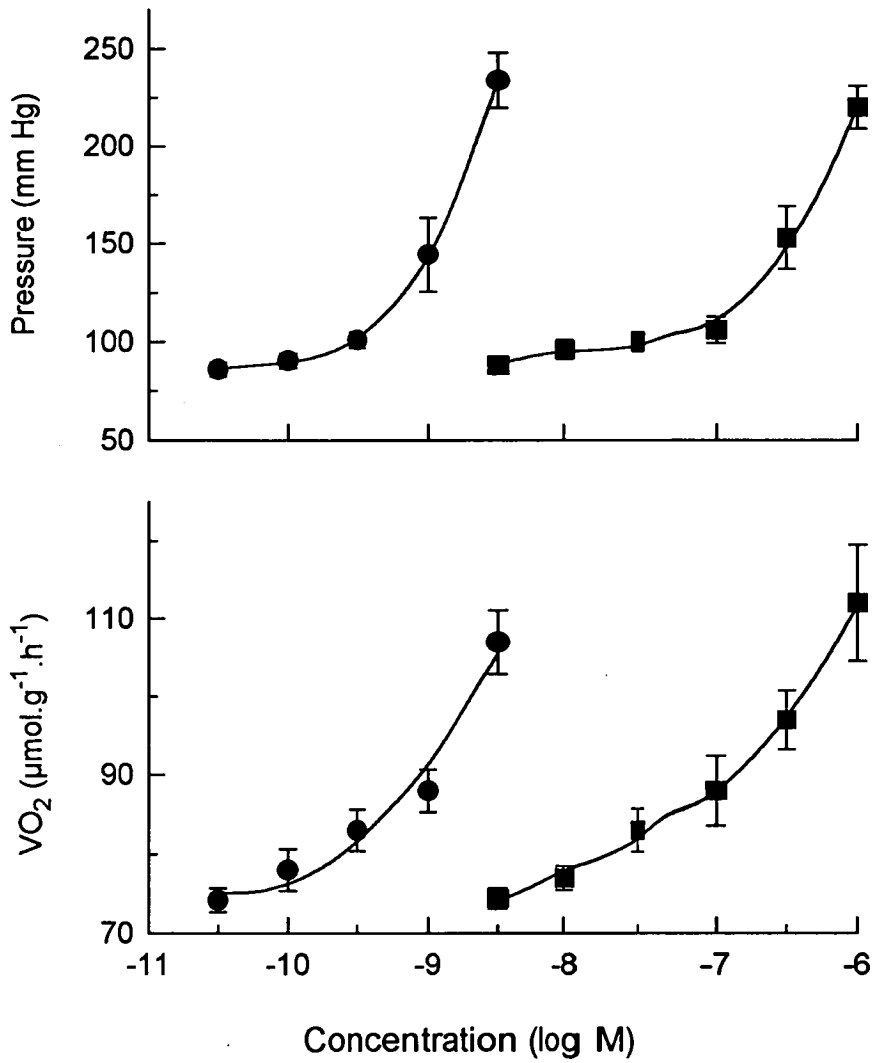

Fig. 5-1 Dose-response curves for VP and NE on perfusion pressure and $\dot{V}O_2$ in the constant-flow perfused rat kidney. The kidney (0.9 ± 0.1 g) was perfused at 25 °C. ●: VP; ■: NE. Data are means ± SE, n=6.

5.3.1.2 Effect of vasoconstrictors and nitroprusside *on pressure,* $\dot{V}O_2$*, lactate and glycerol*

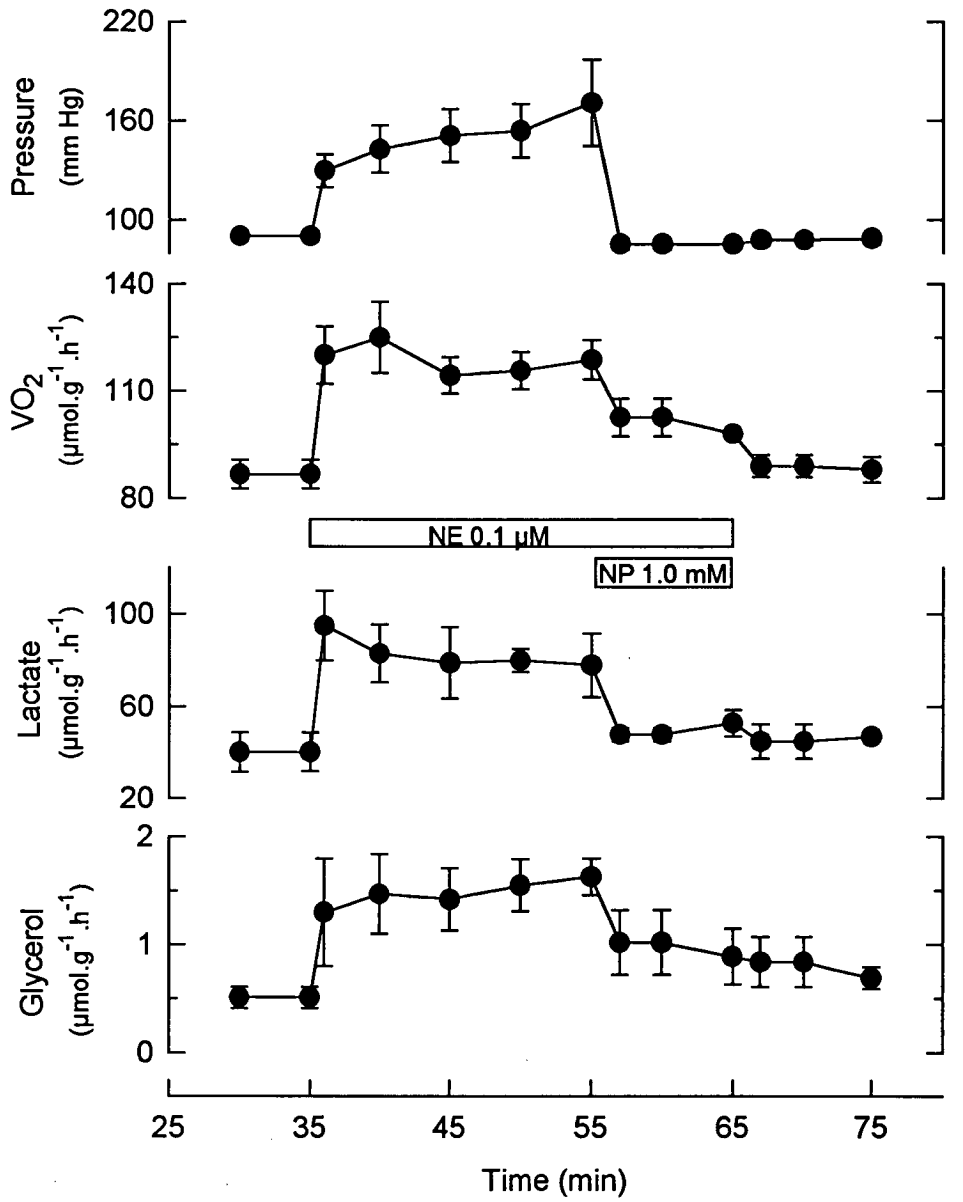

Fig. 5-2 Effect of nitroprusside on NE-induced pressure, $\dot{V}O_2$**, lactate and glycerol release in the constant-flow perfused rat kidney.** Perfusions were undertaken under the same conditions as shown in Fig. 5-1. NP: nitroprusside. Data are presented as means ± SE, n=4.

As illustrated in Figs. 5-2 and 5-3, both NE- an VP-induced $\dot{V}O_2$ and perfusion pressure of the kidney were sustained. NE-elicited increases in $\dot{V}O_2$ and pressure were accompanied by an increase in the release of lactate and glycerol (Fig. 5-2). VP also stimulated $\dot{V}O_2$ and lactate efflux in association with vasoconstriction. However, there was no significant change in glycerol efflux during the infusion of VP (Fig. 5-3). The infusion of nitroprusside significantly inhibited the changes in $\dot{V}O_2$, pressure, lactate and glycerol induced by NE or VP.

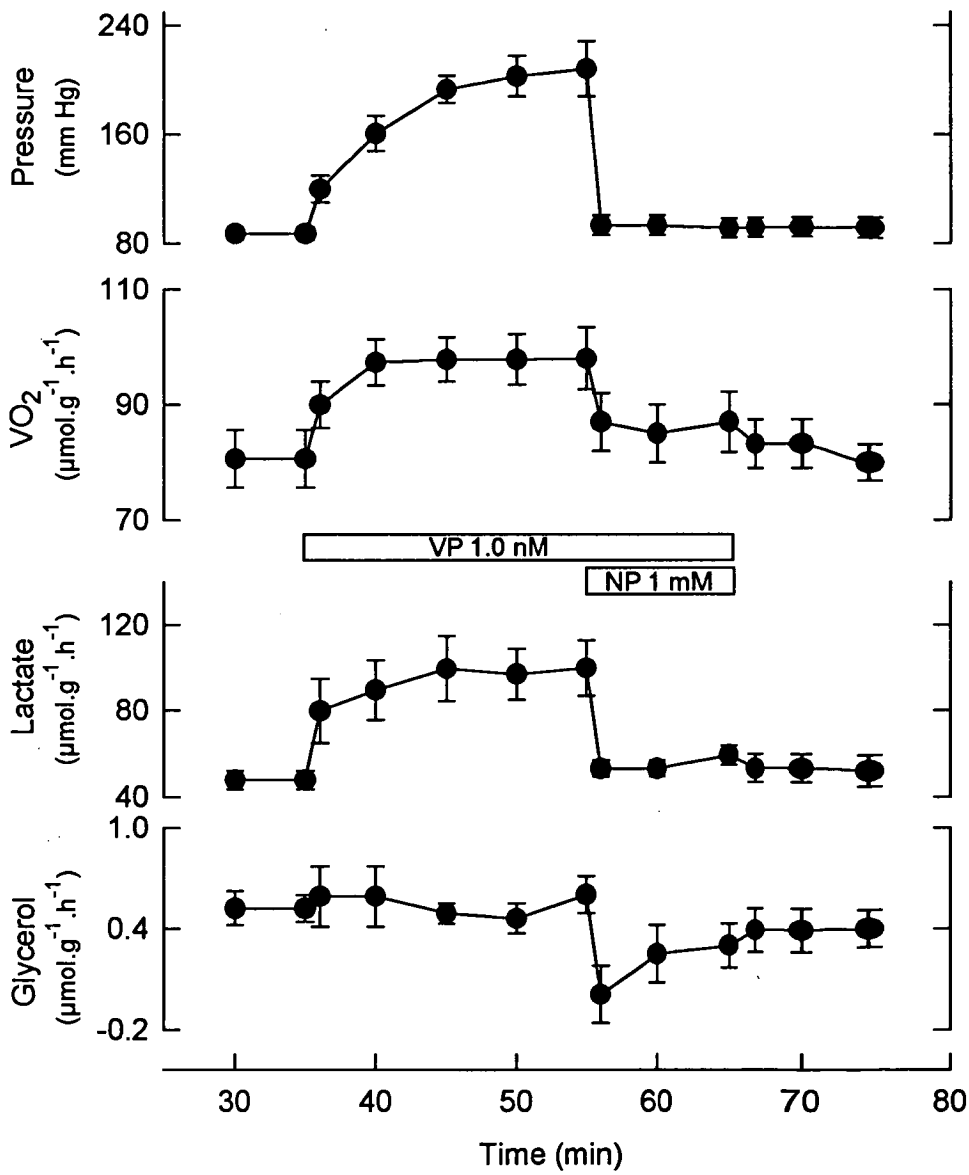

Fig. 5-3 Effect of nitroprusside on VP-induced pressure, $\dot{V}O_2$, lactate and glycerol in the constant-flow perfused rat kidney. Perfusions were conducted at the same conditions as shown in Fig. 5-1 (n=4). NP: nitroprusside.

Table 5-1 Metabolites of perfused rat kidney

	n	Lactate	Glycerol	Cr	CrP	Cr+CrP	AMP	ADP	ATP	ΣAD	EC	CrP/Cr	CrP/ATP
						μmol/g dry wt							
In vivo	5	7.03	0.24	13.35	18.10	31.45	0.13	1.73	10.89	12.75	0.92	1.47	1.69
		± 0.97	± 0.07	± 2.09	± 2.16	± 1.99	± 0.02	± 0.19	± 0.80	± 0.96	± 0.01	± 0.35	± 0.22
Perfused	4	7.12	0.29	12.54	15.70	22.60	0.23	2.12	11.03	13.39	0.90	1.27	1.60
(180 min)		± 0.75	± 0.08	± 0.75	± 0.75	± 5.74	± 0.05	± 0.42	± 0.2.26	± 2.62	± 0.01	± 0.08	± 0.31
Ischemia	5	22.09**	0.41	24.49**	5.37**	29.85	6.33**	2.54	1.38**	9.50*	0.23**	0.23**	5.77
(5 min)		± 0.63	± 0.11	± 1.35	± 0.78	± 0.79	± 0.60	± 0.79	± 0.25	± 0.71	± 0.02	± 0.05	± 2.60

Table 5-2 Metabolites of perfused rat intestine

	n	Lactate	Glycerol	Cr	CrP	Cr+CrP	AMP	ADP	ATP	ΣAD	EC	CrP/Cr	CrP/ATP
						μmol/g dry wt							
In vivo	4	19.74	1.24	14.24	16.02	30.25	0.09	1.38	8.33	9.80	0.92	1.13	1.92
		± 2.15	± 0.21	± 1.31	± 1.40	± 2.71	± 0.02	± 0.06	± 0.55	± 0.54	± 0.01	± 0.01	± 0.08
Perfused	8	11.43**	0.44**	12.62	15.19	27.81	0.17	1.70	6.94	8.81	0.89	1.19	2.18
(180 min)		± 1.04	± 0.08	± 1.04	± 2.54	± 3.26	± 0.05	± 0.30	± 0.45	± 0.70	± 0.01	± 0.15	± 0.32
Ischemia	5	53.19**	1.83	23.14**	2.39**	25.48	5.12**	3.22**	1.66**	9.84	0.33**	0.09**	1.22**
(5 min)		± 8.26	± 0.53	± 1.75	± 1.01	± 2.56	± 0.68	± 0.34	± 0.23	± 0.79	± 0.02	± 0.04	± 0.53

Note for Tables 5-1 & 5-2: ** P<0.01 vs *in vivo*. Statistical tests were not made between the perfused and ischemia (5 min at 25°C after decapitation).data are means ± SE.

5.3.1.3 Metabolites of the perfused kidney

Table 5-1 illustrates metabolites of the kidney *in vivo*, perfused for 180 min and ischemic for 5 min. The sensitivity of the kidney to ischemia (for 5 min) was demonstrated by marked decreases in CrP, CrP/Cr ratio, ATP and adenylate energy charge and increases in Cr. AMP and lactate. After perfusion, none of the parameters measured was significantly different from that obtained from kidneys *in vivo*. The calculated results did not show any significant difference in the EC and CrP/CR ratio of kidneys between the kidneys perfused and *in vivo*. These were totally different from the features following 5 min of ischemia where lactate and AMP markedly increased concomitant with a sharp fall in CrP, ATP, ΣAD, EC and CrP/Cr ratio.

5.3.2 The perfused intestine

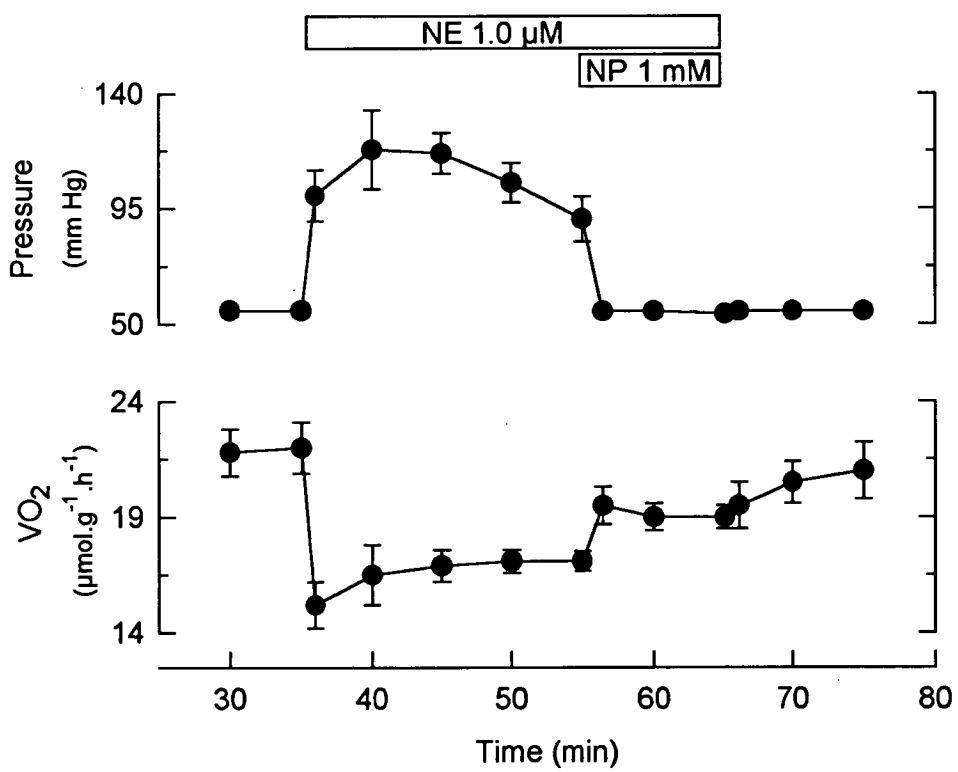

Fig. 5-4 Effect of nitroprusside on NE-induced pressure and $\dot{V}O_2$ in the constant-flow perfused rat intestine. The perfusion was conducted at 25°C at a constant flow of 8 ml/min (means ± SE, n=4). NP: nitroprusside.

5.3.2.1 Validity of the perfused intestine

Intestine is extremely sensitive to ischemia. A dramatic change in the profile of metabolites immediately after ischemia or hypoxia (Chapter 7). These changes, which are shown in Table 5-2 for the ischemic group, include sharp falls in CrP, ATP, CrP/Cr and energy charge as well as accumulations of lactate, AMP and ADP. The results from Table 5-2 demonstrate that the perfused intestine at a constant flow of 8 ml/min at 25°C is metabolically valid with no signs of ischemia. Thus the first part of intestine perfusions was carried out at 8 ml/min.

The infusion of either NE or VP immediately caused vasoconstriction as indicated by the increase in perfusion pressure (Figs. 5-4 & 5-5). But their effects on $\dot{V}O_2$ were completely opposite. NE inhibited $\dot{V}O_2$ whilst VP showed a positive stimulatory effect. Even though the changes in $\dot{V}O_2$ induced by NE and VP were totally opposite, nitroprusside infusion significantly reversed the changed $\dot{V}O_2$ when blocking the increased perfusion pressure (Figs. 5-4 & 5-5).

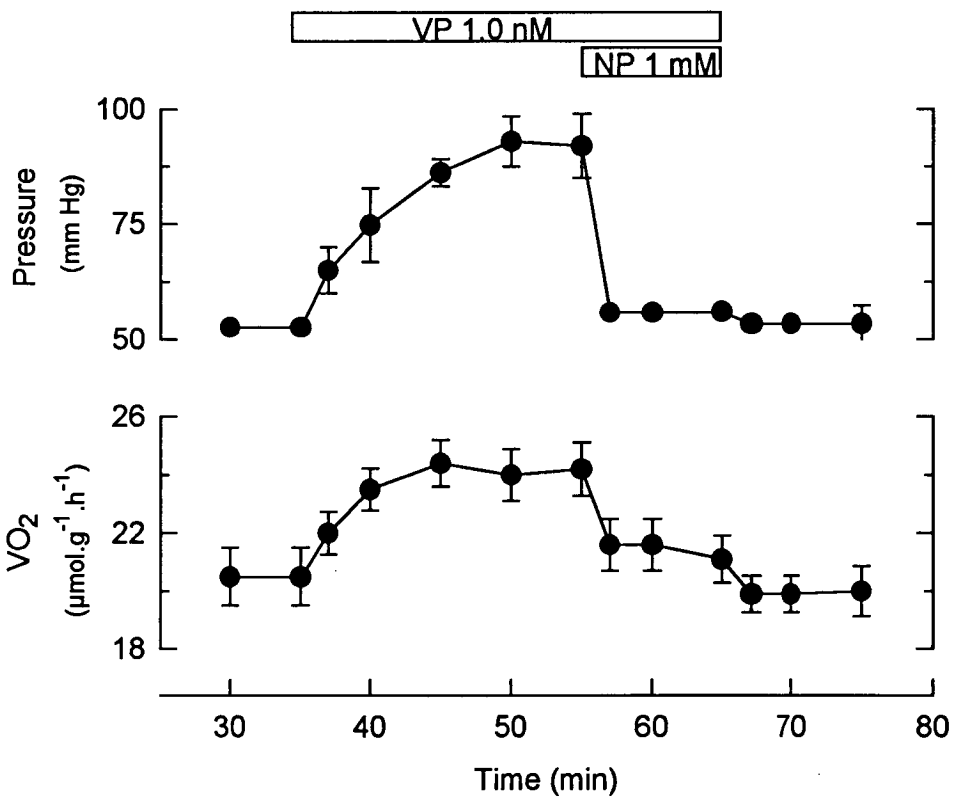

Fig. 5-5 Effect of nitroprusside on VP-induced pressure and $\dot{V}O_2$ in the constant-flow perfused rat intestine. The perfusion was conducted at 25°C at a constant flow of 8 ml/min (means ± SE, n=4). NP: nitroprusside.

5.3.2.2 Effect of vasoconstrictors and nitroprusside on pressure and $\dot{V}O_2$

Experiments shown in Figs. 5-4 & 5-5 were conducted at 8.0 ± 0.1 ml/min to observe the relationship between intestine $\dot{V}O_2$ and pressure produced by NE and VP, and the effect of the vasodilator, nitroprusside, on these changes. The intestine metabolites (Table 5-2) indicated sufficient O_2 was provided for the intestine at this flow rate. Under these perfusion conditions, the basal $\dot{V}O_2$ was 21.9 ± 1.3 $\mu mol \cdot g^{-1} \cdot h^{-1}$ and the basal perfusion pressure was 54 ± 2 mm Hg (n=9).

5.3.2.3 Vasoconstrictor-mediated changes in $\dot{V}O_2$ *and pressure at an increased perfusion flow rate*

In order to assess the effect of perfusion flow rate on vasoconstrictor-mediated changes in intestine, the flow was raised to allow maintenance of a pressure approaching the physiological value. To achieve an arterial perfusion pressure of 82 ± 2.4 mm Hg, the flow rate was maintained constant at 15.2 ± 0.1 ml/min per intestine. At this perfusion flow rate, the basal rate of $\dot{V}O_2$ increased from 21.9 ± 1.3 $\mu mol \cdot g^{-1} \cdot h^{-1}$ at 8 ml/min to 33.3 ± 1.0 $\mu mol \cdot g^{-1} \cdot h^{-1}$ ($P<0.01$, n=7, Fig. 5-6). Given a Q_{10} of 2.5, this latter value is comparable with values of 98.5 and 150.4 $\mu mol \cdot g^{-1} \cdot h^{-1}$ for fasted and fed rat intestine in vivo, respectively (Stevenson & Weiss, 1988) and 60 $\mu mol \cdot g^{-1} \cdot h^{-1}$ for autoperfused dog intestine (Granger *et al.*, 1982). As argued above (for kidney), the lower rate observed for intestine in the present study would largely be the result of the experiments being conducted at 25°C rather than 37-39°C. The slightly lower perfusion pressure (compared with 80-120 mmHg *in vivo*) could also be contributory.

The effects of vasoconstrictors on intestine $\dot{V}O_2$ was still maintained even though the basal value had been significantly elevated by increasing perfusion flow rate from 8 ml/min to 15 ml/min. Figure 5-6 shows dose-response curves for VP- and NE-mediated changes in $\dot{V}O_2$ and pressure development at a perfusion flow rate of 15 ml/min. VP is regarded as an intestinal vasoconstrictor (Richardson, 1984). It is important to note that $\dot{V}O_2$ increased uniformly over the dose range of 10 pM-3nM VP whilst pressure changes were relatively small at the lower doses and larger at the top end of the concentration range. The highest rate of $\dot{V}O_2$ attained was 39 $\mu mol \cdot g^{-1} \cdot h^{-1}$, representing an increase of 18% ($P<0.05$) over basal. By contrast, NE infusion resulted in a dose-dependent increase in perfusion pressure (+134%) which was accompanied by a decrease in $\dot{V}O_2$ (-24%, $P<0.01$).

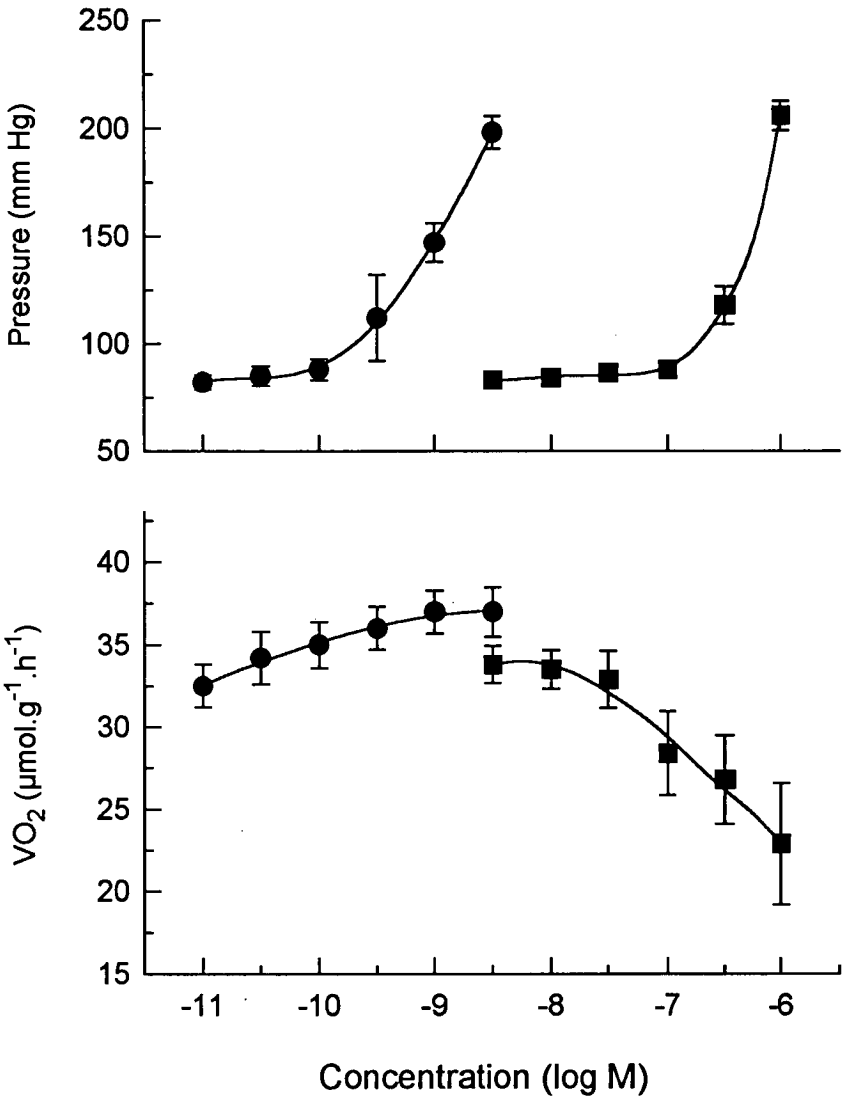

Fig. 5-6 Dose-response curves for VP and NE on perfusion pressure and $\dot{V}O_2$ in the constant-flow perfused rat intestine. ●: VP; ■: NE (means ± SE, n=3). Perfusions were conducted at a constant flow of 15 ± 0.1 ml/min at 25°C.

5.3.3 $\dot{V}O_2$ and pressure development by the perfused mesenteric artery network

The mesenteric artery network was perfused via the superior mesenteric artery. Major arteries of this network were severed close to the intestine which was excised and the entire network perfused in a sealed chamber so the effluent could be collected for PO_2 measurement. Because of the nature of the preparation it is likely that most capillaries and veins were not perfused as the intestine was removed.

Consequently the perfusion pressure was quite low at the relatively high flow rate of 2 ml/min per g artery network. Under these conditions the basal rate of $\dot{V}O_2$ was 8.2 ± 1.1 μmol·g^{-1}·h^{-1} (n=8).

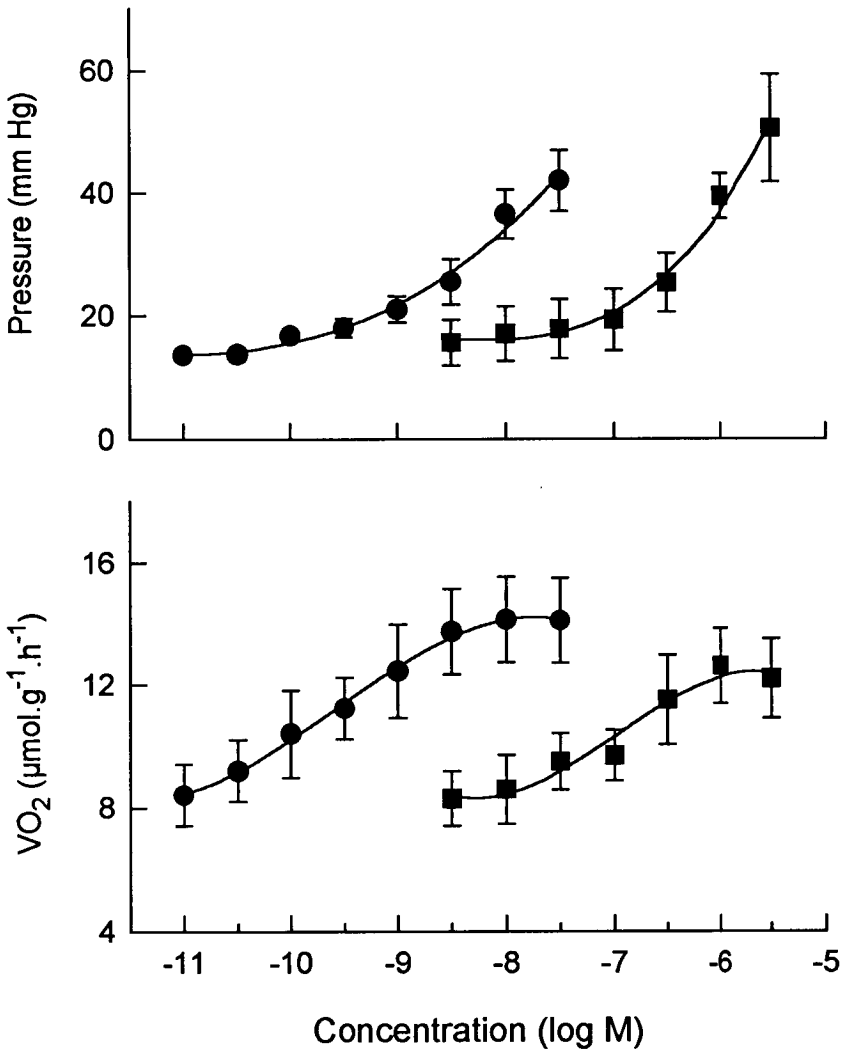

Fig. 5-7 Effect of VP and NE on pressure and $\dot{V}O_2$ in the constant-flow perfused rat mesenteric artery network. The perfused mesenteric artery network was 1.0 ± 0.1 g. ●: VP, n=3-4; ■ : NE, n=3-5. Data are presented as means ± SE.

Figure 5-7 shows dose-response curves for NE- and VP-mediated increase in $\dot{V}O_2$ and pressure development for the mesenteric artery preparation. NE infusion resulted in increase in $\dot{V}O_2$ with a half-maximal effect occurring at 0.3 μM. Pressure also increased in a dose-dependent manner but did not reach a maximal value and was

still increasing at the highest dose used of 3 μM NE. VP also resulted in a dose-dependent increase in $\dot{V}O_2$ and pressure development. Half-maximal dose occurred for both at approximately 0.3 nM VP. VP tended to have the greatest effect on $\dot{V}O_2$ and at maximal dose the rate of $\dot{V}O_2$ was 14.8 $\mu mol \cdot g^{-1} \cdot h^{-1}$, representing an increase of 80% over basal ($P<0.01$).

5. 4 Discussion

The study reported in this chapter was undertaken to address the possibility that vasoconstrictor-controlled $\dot{V}O_2$ was a general phenomenon in the perfused tissues. Two vasoconstrictors and one vasodilator were used and three tissues compared. In all cases except the perfused intestine, VP and NE increased $\dot{V}O_2$ in association with an increase in perfusion pressure. nitroprusside blocked or significantly inhibited the changes in $\dot{V}O_2$ and perfusion pressure induced by VP and NE.

Chapter 3 and 4 have shown that NE and VP each stimulates vasoconstriction and $\dot{V}O_2$ in the constant-flow perfused hindlimb. Both effects were blocked by vasodilators, eg. nitroprusside. The effect of vasoactive substances on $\dot{V}O_2$ is a separate mechanism from muscle contraction, thereby reflecting the component of NST of skeletal muscle. Vasoconstrictor-mediated control of skeletal muscle $\dot{V}O_2$ is supported by the evidence that $\dot{V}O_2$ by electrically stimulated skeletal muscle was not inhibited by nitroprusside (Chapter 3) and was additive to vasoconstrictor-induced $\dot{V}O_2$ (Colquhoun *et al.*, 1990). Tubocurarine does not affect NE-induced $\dot{V}O_2$ and perfusion pressure when blocking the $\dot{V}O_2$ produced by sciatic nerve stimulation (Chapter 3). When flow was increased (and therefore perfusion pressure) $\dot{V}O_2$ also increased and the addition of NE or nitroprusside increased or decreased, respectively, the $\dot{V}O_2$ at all flow rates (Chapter 3). Together, these findings led to the conclusion that vascular system plays a key role in the regulation of resting muscle thermogenesis (Chapter 3). Hence, it is proposed that blood vessels might be a major source for the increased $\dot{V}O_2$ mediated by vasoconstrictors in the perfused hindlimb (Colquhoun & Clark, 1991).

However, an inhibitory effect on $\dot{V}O_2$ occurs in the perfused hindlimb (Dora *et al.*, 1991) when 5-HT causes the increase in perfusion pressure. A similar inhibition in $\dot{V}O_2$ in the perfused hindlimb can be induced by NE at high concentrations. These data taken together led Clark *et al.* (1994) to hypothesize that there are two metabolically distinct vasoconstrictors: type A and type B. In the constant-flow

perfused rat hindlimb, type A vasoconstrictors, such as VP, AT II, and low dose NE, stimulate $\dot{V}O_2$, lactate and glycerol production whereas type B vasoconstrictors, including 5-HT and high dose NE, inhibit these parameters.

The results from the perfused kidney and intestine show vasoconstrictors mediated the control of metabolism in these tissues as well (Figs. 5-1 & 5-6) and these effects were generally opposed by nitroprusside (Figs. 5-2, 5-3, 5-4 & 5-5). In the perfused kidney, both VP and NE stimulated $\dot{V}O_2$ and lactate production whilst increasing the perfusion pressure. VP receptors are known to stimulate the absorption of water and sodium in the collecting tubule. A direct effect on the renal parenchymal tissues by these agonists can not be ignored. In fact, the direct effect of VP and NE is likely (Roobol & Alleyne, 1973) and may explain why nitroprusside failed to fully block some of the increased metabolism as it does in the perfused hindlimb (Colquhoun *et al.*, 1990; Chapter 4), in spite of the complete blockade of pressure. Nonetheless, the present results show that there is an obvious component of $\dot{V}O_2$ controlled by or associated with vasoconstriction. VP and NE clearly exert a positive control of metabolism in the kidney vascular bed.

In the intestine, vasoactive agents are considered to have either direct or indirect effects on oxidative metabolism (Kvietys & Granger, 1982) although the mechanisms are not clear. In contrast to its effect in the perfused hindlimb, NE inhibited $\dot{V}O_2$ thereby exerting a negative type B vasoconstrictor-controlled effect on the perfused intestine. In comparison, VP stimulated $\dot{V}O_2$ of the perfused intestine (Fig. 5-5). Both NE- and VP- mediated changes in $\dot{V}O_2$ were reversed by nitroprusside to various degrees when it blocked the increased perfusion pressure (Figs. 5-4 & 5-5).

In the perfused rat hindlimb, increasing perfusion flow alone elevates $\dot{V}O_2$ and the flow-induced $\dot{V}O_2$ can be further enhanced by NE (Chapter 3). Similarly, when the perfusion flow was increased from 8 ml/min to 15 ml/min in the perfused intestine, $\dot{V}O_2$ was almost doubled with no significant influences on its responses to VP and NE (Figs. 5-4, 5-5 & 5-6). The flow-induced $\dot{V}O_2$ in intestine was further augmented by VP. Fig. 5-6 shows that VP stimulated $\dot{V}O_2$ during vasoconstriction at a similar dose range as it does in the perfused hindlimb (Chapter 3).

The results of vasoconstrictor-elicited $\dot{V}O_2$ in the perfused mesenteric artery network is of particular interest in terms of vascular $\dot{V}O_2$. This preparation allows the separation of the direct $\dot{V}O_2$ from arterial system from the secondary $\dot{V}O_2$ utilized by the intestinal tissues *per se* as a result of changes in flow pattern or a messenger-metabolism communication between them. At the perfused organ level,

vasoconstrictors mediate either positive or negative regulation of $\dot{V}O_2$, supporting a similar but also different classification of type A and type B vasoconstrictors to that proposed by Clark *et al.* (1994). However, in the mesenteric artery preparation, they all show a type A effect. For instance, NE and 5-HT inhibit $\dot{V}O_2$ in the perfused intestine and hindlimb, respectively, but they all stimulate $\dot{V}O_2$ in the perfused mesenteric artery network (Fig. 5-7; Dora *et al.*, 1992). Anatomical vascular shunting has been suggested to exist in the intestine (Sherperd *et al.* 1976; Lundgren, 1984), thus the inhibitory effect of NE on $\dot{V}O_2$ in the perfused intestine may be due to vascular shunting as suggested by Sherperd *et al.* (1976).

The basal $\dot{V}O_2$ of 8.2 $\mu mol \cdot g^{-1} \cdot h^{-1}$ of the perfused mesenteric artery arcade at 25°C is calculated to be 20.5 $\mu mol \cdot g^{-1} \cdot h^{-1}$ at 37°C using a Q_{10}=2.5 (Bonen *et al.*, 1994). From the estimate based on the dissected blood vessels in this preparation, blood vessels, vein included, account for 6% of the perfused mass. Other tissues are white adipose and lymphnodes. Assuming $\dot{V}O_2$ by nonvascular tissues is negligible in this preparation, the vascular $\dot{V}O_2$ would be 342 $\mu mol \cdot g^{-1} \cdot h^{-1}$ at 37°C. Thus the maximal $\dot{V}O_2$ of 14-15 $\mu mol \cdot g^{-1} \cdot h^{-1}$ at 25°C in the presence of NE or VP would be equal to 583-625 $\mu mol \cdot g^{-1} \cdot h^{-1}$ at 37°C. Compared with most reported values in the literature (Paul, 1980), such an estimated value appears to be too high. However, it should be borne in mind that vascular metabolic rate in larger vessels is much lower than that in small and muscular vessels. For instance, $\dot{V}O_2$ in rat portal vein is 20 fold the value of porcine carotid artery (Paul, 1980). An even higher basal $\dot{V}O_2$ was observed in bovine cerebral microvessels and is calculated to be 288 $\mu mol \cdot g^{-1} \cdot h^{-1}$ in the presence of succinate (Sussman *et al.*, 1988). Indeed, this estimate seems to match the value obtained from the perfused rat tail artery (Colquhoun & Clark, 1991). Reports for rat heart $\dot{V}O_2$ at the perfusion pressure of 80–110 cm H_2O at 35°C range between 556 to 655 $\mu mol \cdot g^{-1} \cdot h^{-1}$ (Kojima *et al.*, 1993) and $\dot{V}O_2$ in contracting skeletal muscle (gastrocnemius-plantaris-soleus group) obtained in this laboratory is 119 $\mu mol \cdot g^{-1} \cdot h^{-1}$ at 37°C (Dora *et al.*, 1994). Therefore, vascular $\dot{V}O_2$ could be as high as working heart and vigorously-contracting skeletal muscle. When vasoconstrictors cause the blood to constrict in the constant-flow perfused mesenteric artery, the vascular wall tension is increased as a result of an increased perfusion pressure. Thus, more O_2 is consumed by the blood vessel in response to the increased work to produce and maintain vascular contraction or wall tension (Colquhoun & Clark, 1991). Such a correlation between vascular $\dot{V}O_2$ and vascular wall tension has been revealed (Paul, 1980). Indeed, the research in the rat tail vascular smooth muscle in this laboratory has shown that the mitochondrial content accounted for approximately 8.4% of the cell volume, which is same to or greater than red striated

skeletal muscle (Peng, personal communication). The high mitochondrial content in vascular smooth muscle is consistent with a high $\dot{V}O_2$.

The association between striated muscle and energy consumption is well established (Gibbs, 1978) and thus an analogous role for the vascular smooth muscle to use O_2 during constriction or resisting pressure seems reasonable. Paul and Peterson (1975) showed that vascular $\dot{V}O_2$ depends on the active wall tension of the smooth muscle. In the constant-flow perfused tissues, the vascular wall tension would be increased when vasoconstrictors increase the perfusion pressure. Accordingly, vasoconstrictor-stimulated increase $\dot{V}O_2$ in the perfused kidney, intestine and mesenteric artery may result from the increased active wall tension of the vascular smooth muscle. However, there are properties of the vascular smooth muscle itself, as well as of the vascular tree of complex organs such as the hindlimb, intestine or kidney which make a simple relationship between perfusion pressure and putative $\dot{V}O_2$ by the vascular tissue inappropriate. Firstly, vascular smooth muscle is considered to have the potential for "latch bridge" formation. These are noncycling or very slowly cycling cross bridges formed when myosin is dephosphorylated (Murphy *et al.*, 1983). Such "latch bridges" are envisaged as providing an internal resistance to shortening and thereby allow conservation of energy. Secondly vascular tissue is considered to undergo vasomotion (Wiedman, 1984) characterised by oscillatory constriction and dilation. Thirdly, if vasoconstrictors induced flow redistributions (Dora *et al.*, 1992), the vascular surface areas could be altered. This could in turn affect the entire working vascular tissue mass. Consequently, the mean arterial pressure of a vascular bed may not necessarily reflect the energy consumption of the vasculature. In addition, the results from the perfused mesenteric artery indicated that vascular tissue may be energetically more expensive than previously recognized.

Overall, these results together with the observations in the perfused hindlimbs of the rat, bettong and toad (Chapters 3 & 4) suggest that vasoconstrictor-mediated control of metabolism is a general feature in constant-flow perfused tissues across species. A positive vasoconstrictor effect on $\dot{V}O_2$ in one tissue is not necessarily positive in another. If these effects occur *in vivo*, vasoconstrictors will have important implications in the control of the facultative thermogenesis.

CHAPTER 6

Vascular control of $\dot{V}O_2$ in the constant-pressure perfused rat hindlimb

6.1 Introduction

Vasoconstrictors such as NE, VP, AT II and 5-HT all stimulate $\dot{V}O_2$ in the perfused rat mesenteric artery (Dora *et al.*, 1991; Hettiarachchi *et al.*, 1992; Chapter 5). However, in the constant-flow perfused hindlimbs from rats (Chapter 3; Clark *et al.*, 1994), bettongs and toads (Chapter 4) have reproducibly shown the effects of vasoconstrictive agents to either increase (type A) or decrease (type B) muscle $\dot{V}O_2$. The stimulation or inhibition by vasoconstrictors of $\dot{V}O_2$ was also observed in constant-flow perfused rat intestine, although a type A vasoconstrictor in one tissue may not necessarily to be type A in another tissue (Chapter 5). For example, NE stimulates $\dot{V}O_2$ in the perfused hindlimb and kidney but inhibits $\dot{V}O_2$ in the perfused intestine (Chapter 3, 4 & 5).

In vivo, the blood flow in skeletal muscle undergoes an autoregulation which tends to maintain a relatively stable flow to meet metabolic demands (Meininger, 1991). The arterial blood pressure is maintained at a relatively constant level under resting conditions by complex mechanisms, such as the well-known baroreflexes of the aortic arcade and carotid sinus. Hence, the findings obtained from the constant-flow perfused hindlimbs would only reflect the responses *in vivo* where blood flow to the tissue is not significantly changed.

However, when vasoconstrictors are released or administered, both the blood pressure and perfusion flow to skeletal muscle can be affected. Thus, it might be argued that the effects of vasoconstrictors on $\dot{V}O_2$ observed in the constant-flow perfusion may not necessarily represent the scenario *in vivo* if the perfusion flow is changed. Hence the aim of this Chapter is to examine the effects of vasoconstrictors on tissue $\dot{V}O_2$ in the constant-pressure perfused rat hindlimb whilst the perfusion flow was permitted to change freely. The results were compared with those obtained from constant-flow perfused hindlimbs to further evaluate the effect of vasculature on muscle thermogenesis.

6.2 Methods

Male rats (100-110 g) were used for the study. The hindlimb was perfused at 80 ± 0.5 mm Hg in a recirculated model at 37°C similar to that described by Hardeveld *et al.* (1980). The perfusate was Krebs-Ringer buffer containing 6% BSA and 2.5 mM Ca^{2+}. The details for the surgery and perfusion are described in Section 2.2.4. $\dot{V}O_2$ was measured by a in-line Clark-type O_2 electrode. The values were regularly checked by measuring collected arterial and venous samples with an O_2 analyzer (TasCon, Department of Physiology, University of Tasmania).

For the assay of lactate and pyruvate, both arterial and venous perfusate were collected simultaneously at the steady-state when the flow rate and PvO_2 were taken. Lactate and pyruvate were enzymatically measured as described in Chapter 2 (Section 2.3). The net production of lactate and pyruvate was calculated from the arteriovenous difference of the concentration in the perfusate and the flow rate.

Creatine compounds and adenine nucleotides in gastrocnemius-plantaris-soleus muscle group were determined by an ion-paired reversed phase column (Radial Pak, C18, 4 μm, 8 × 100 mm) with HPLC (Waters, see Section 2.3).

6.3 Results

6.3.1 Basal values of flow, $\dot{V}O_2$, lactate and pyruvate

The hindlimb perfusion was performed at a constant physiologically relevant pressure of 80 ± 0.5 mm Hg (mean ± SE, n=30). The pressure fluctuation was controlled by an overflow shunt and manual adjustment (when necessary) within 1.0 mm Hg in each perfusion. At such a physiological pressure, the perfusion flow was measured at 19.7 ± 0.7 ml/min (means ± SE, n=30) for the hindlimb with 7.5-9 g muscle (2.2-2.6 $ml \cdot min^{-1} \cdot g^{-1}$ muscle). A high basal flow rate was expected because of the low viscosity of the perfusate and a low vascular tone of the hindlimb. This flow rate is comparable with the observations obtained by Johnsson *et al.* (1991) in the constant-pressure perfused rat hindlimb with cell-free 4% Dextran-Tyrode solution, where a flow rate of 2.75 $ml \cdot min^{-1} \cdot g^{-1}$ muscle was recorded at 125 mm Hg.

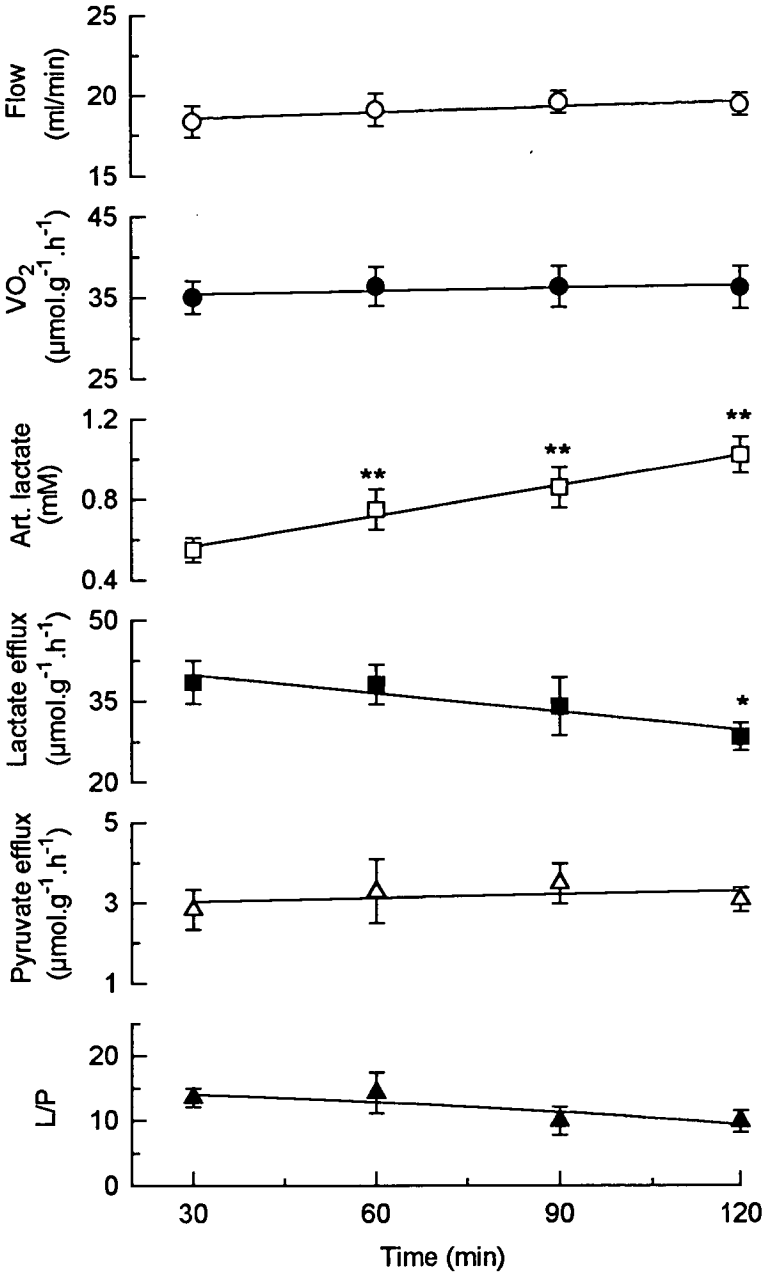

Fig. 6-1 Time course of flow, $\dot{V}O_2$, lactate and pyruvate efflux in the constant-pressure perfused rat hindlimb. The rat (100-110g) hindlimb was perfused at a pressure of 80 ± 0.5 mm Hg with Krebs-Ringer buffer containing 6% BSA and 2.5 mM Ca^{2+} at 37°C in a recirculating system. Art. lactate: arterial lactate concentration. Perfusion flow rate was permitted to change freely. Data are presented as means ± SE, n=5. *P<0.05, **P<0.0, vs the values at 30 min.(Paired *t* test).

Johnsson *et al.* (1991) found that in the constant-pressure perfused rat hindlimb blood vessel responsiveness could become "erratic" at a high flow rate for yet unknown reasons, sometimes showing only passive distension while a few preparations showed up to 50% resistance increases. In the present study, most of the hindlimbs showed a slight increase in flow rate after recycling the perfusate whereas a minority showed a slight reduction in flow rate. In general, it took 30 min to reach a steady state. Occasionally, a steady state flow rate could not be obtained. In these perfusions, there was always a dramatic fall in flow associated with deteriorating tissue edema within the equilibration time. In such cases, the experiment was abandoned.

Under these conditions, the basal values at 30 min of perfusion for $\dot{V}O_2$ and net release of lactate and pyruvate in the perfusate were 35.1 ± 0.5 (n=30), 36.5 ± 2.5 (n=22), and 3.01 ± 0.24 (n=22) $\mu mol \cdot h^{-1} \cdot g^{-1}$, respectively. The ratio of net lactate output to net pyruvate output in the perfusate was 12.0 ± 0.74 (n=22). The values of flow rate, $\dot{V}O_2$, and pyruvate remained steady for at least 120 min as demonstrated in Fig. 6-1. However, there was a gradual fall in lactate output when lactate concentration accumulated in the perfusate. Measurement of pH of the arterial perfusate at the end of perfusion showed a value between 7.35-7.40.

6.3.2 Effects of α-adrenergic agonists on flow, $\dot{V}O_2$, lactate, and pyruvate

NE started to have an effect on both flow and $\dot{V}O_2$ at a concentration of 1 nM (Fig. 6-2). Despite the decrease in perfusion flow, $\dot{V}O_2$ showed a biphasic response. NE stimulated $\dot{V}O_2$ in the range of 1 nM to 33 nM. The maximal increase in $\dot{V}O_2$ (29%) occured at 3 nM where the flow declined by 16%. As the concentration of NE reached 0.1 μM, the flow rate was reduced to 8.0 ml/min (60% of the basal value) and $\dot{V}O_2$ returned to the basal level with a trend of further decline. Lactate and pyruvate net production declined gradually (not corrected for the decline with time as shown in the control) associated with the increase in its accumulation in the perfusate. The ratio of lactate/pyruvate, however, was not significantly affected within the range of concentrations tested (1 nM-0.1 μM).

Phenylephrine is an α_1-adrenergic agonist. Compared with NE, phenylephrine-mediated changes in flow, $\dot{V}O_2$, lactate and pyruvate were similar, but the maximal $\dot{V}O_2$ was only 18% above the basal. However, the difference between the maximal $\dot{V}O_2$ induced by NE and phenylephrine was not statistically significant ($P>0.05$).

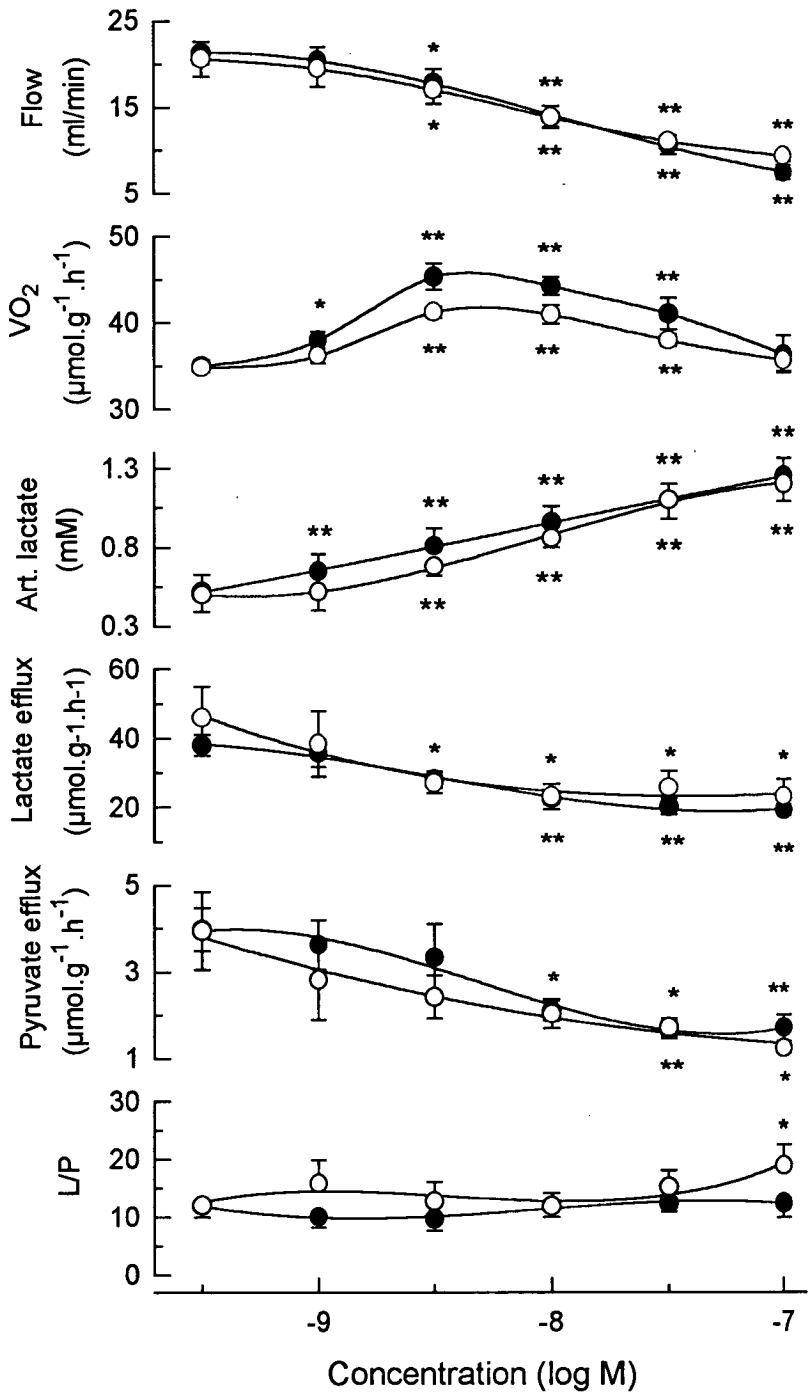

Fig. 6-2 Effects of α-adrenergic agonists on flow, $\dot{V}O_2$, lactate and pyruvate in the constant-pressure perfused rat hindlimb. The rat hindlimb was perfused under the conditions described in Fig. 6-1. ●: NE (n=7 for flow and $\dot{V}O_2$, n=4 for lactate and pyruvate); ○: phenylephrine (n=4). Art. lactate: arterial lactate concentration. Data are presented as means ± SE. * P<0.05, **P<0.01, vs the values before addition of agonists (Paired *t* test).

6.3.3 Effect of β-adrenergic agonists and antagonist

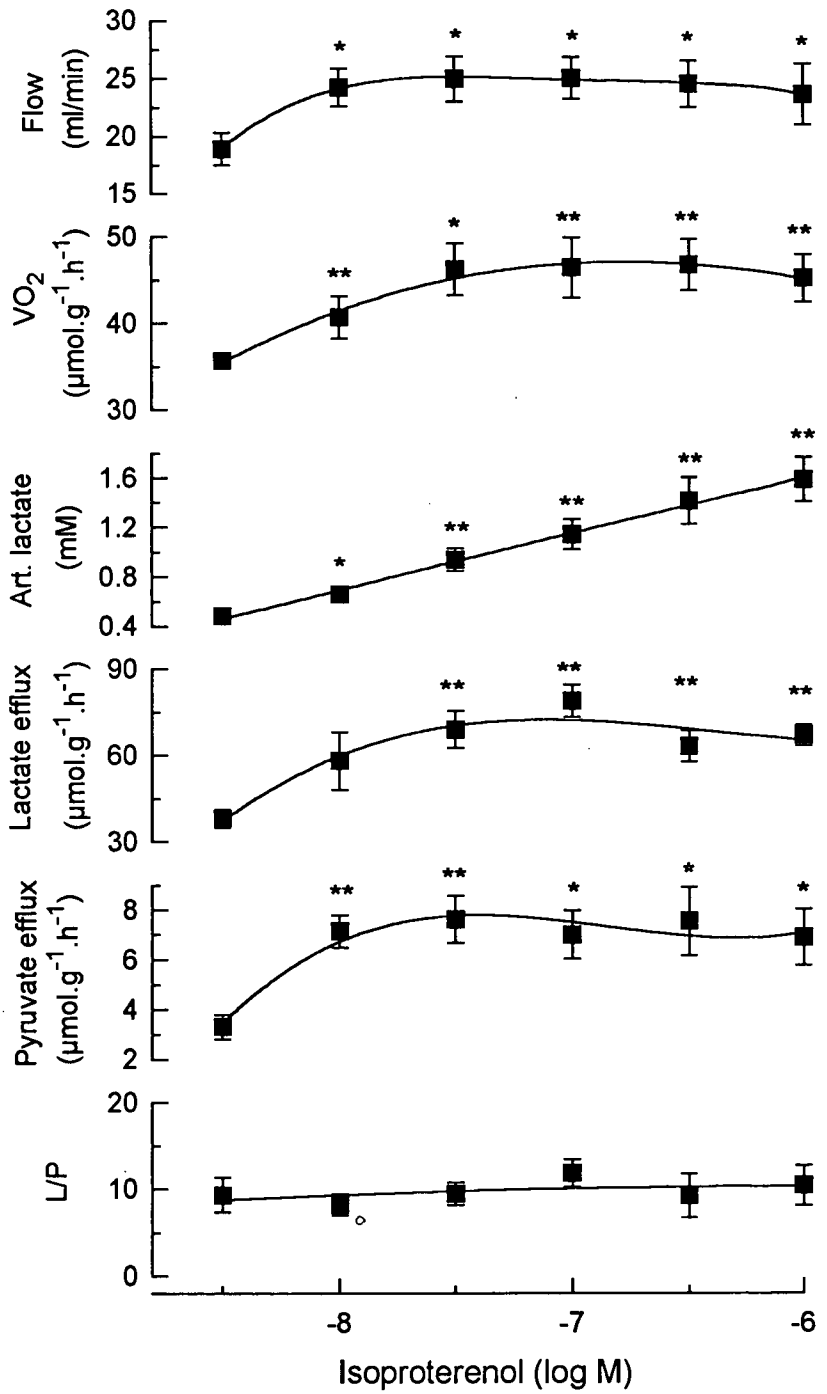

Fig. 6-3 Effect of isoproterenol on flow, $\dot{V}O_2$, lactate and pyruvate in the constant-pressure perfused rat hindlimb. The details for the perfusion were given in Fig. 6-1. Art. lactate: arterial lactate concentration. Data are means ± SE, n=4. *P<0.05, **P<0.01, vs the basal values.

In contrast to NE and phenylephrine, isoproterenol stimulated increases in $\dot{V}O_2$, lactate and pyruvate production in association with a marked increase in perfusion flow rate (Fig. 6-3). The maximal increases in flow, $\dot{V}O_2$, lactate and pyruvate were around 30%, 30%, 107%, and 130% over the basal levels, respectively. Fig. 6-4 shows the effect of propranolol, a β_1 and β_2 adrenergic antagonist, on isoproterenol-induced flow, $\dot{V}O_2$, lactate and pyruvate. Addition of propranolol (10 μM) blocked the increased flow, lactate and pyruvate induced by isoproterenol (1 μM). However, isoproterenol-increased $\dot{V}O_2$ was not significantly affected by propranolol (P>0.05).

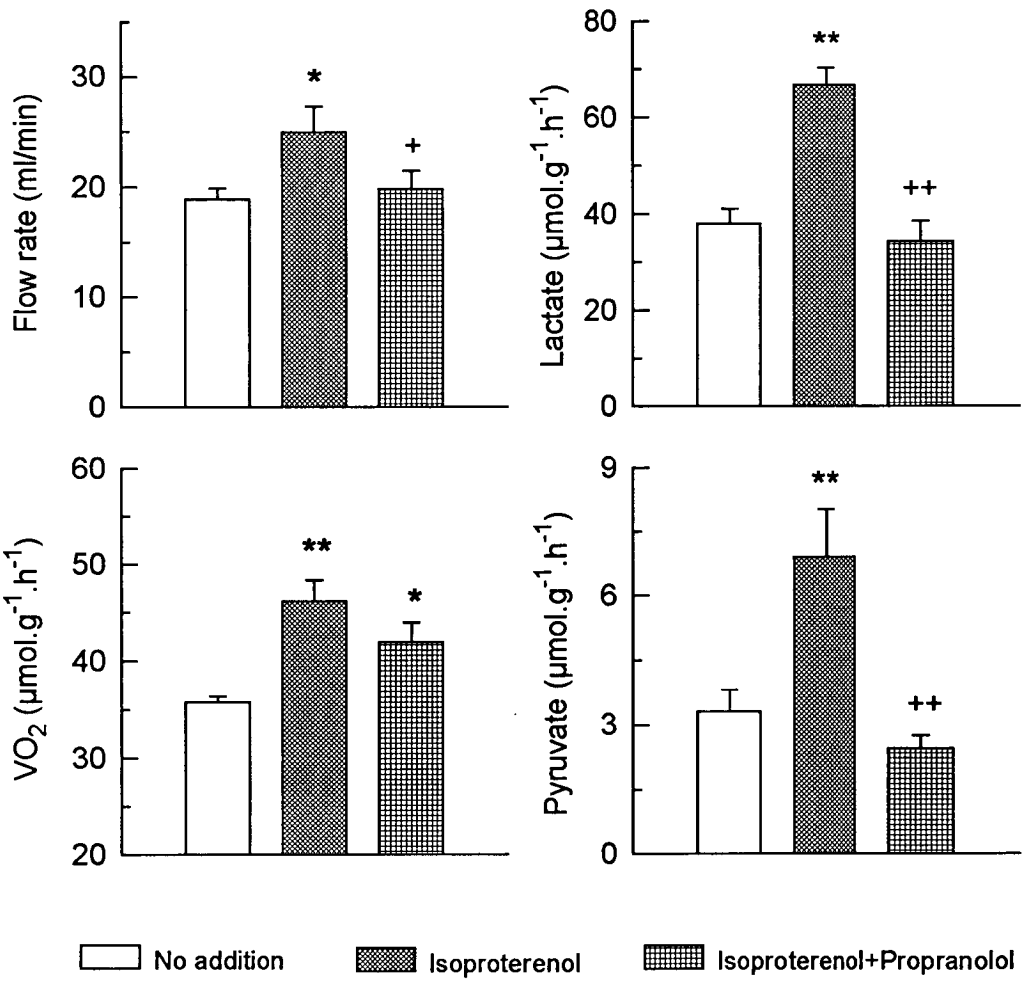

Fig. 6-4 Effect of propranolol on isoproterenol elicited flow, $\dot{V}O_2$, lactate and pyruvate in the constant-pressure perfused rat hindlimb. (DL)-propranolol: 10 μ M; isoproterenol: 1 μM. Data are means ± SE, n=3-4. *P<0.05, **P<0.01 vs the no addition group; + P<0.05, ++ P<0.01, vs isoproterenol alone (Paired *t* test).

BRL35135A is a β_3-adrenergic agonist (Cawthorne *et al.*, 1992) and was used in the present study to assess the effect of β_3-adrenergic receptors on muscle $\dot{V}O_2$. In order to prevent a possible cross reaction with other β-adrenergic receptor subtypes, 1 or 10 μM propranolol was added to the perfusate before the application of BRL35135A. Fig. 6-5 shows that in the presence of 1 or 10 μM propranolol BRL35135A still stimulated $\dot{V}O_2$. The maximal increase in $\dot{V}O_2$ was approximately 17% above the level noted in the presence of propranolol alone. Neither perfusion flow rate nor lactate release was significantly affected by propranolol alone or a combination of propranolol and BRL35135A.

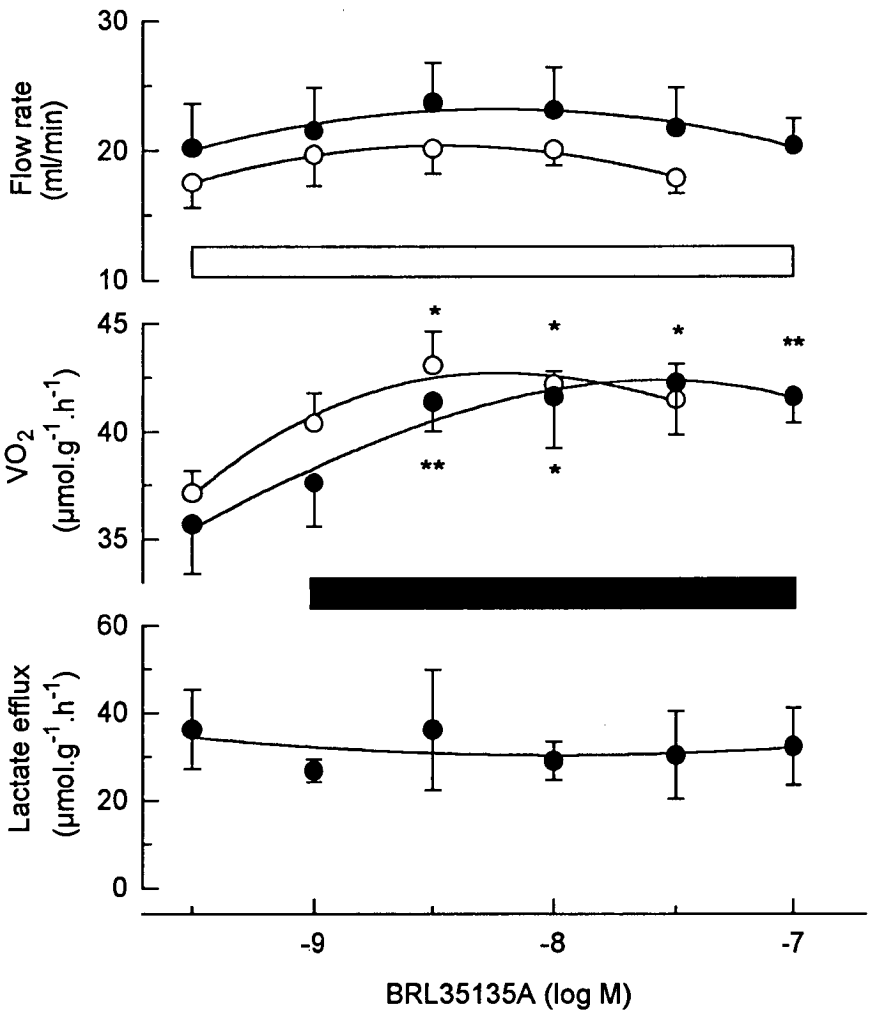

Fig. 6-5 Effect of BRL35135A on flow, $\dot{V}O_2$ and lactate in the presence of propranolol. The open bar represents the presence of propranolol in the perfusate and the filled bar represents the presence of BRL35135A in the perfusate. O: 1 μM DL-propranolol, ●: 10 μM DL-propranolol. Data are means ± SE, n=3. *P<0.05, **P<0.01 vs propranolol alone (Paired *t* test).

6.3.4 *Effect of VP on flow, $\dot{V}O_2$, lactate and pyruvate*

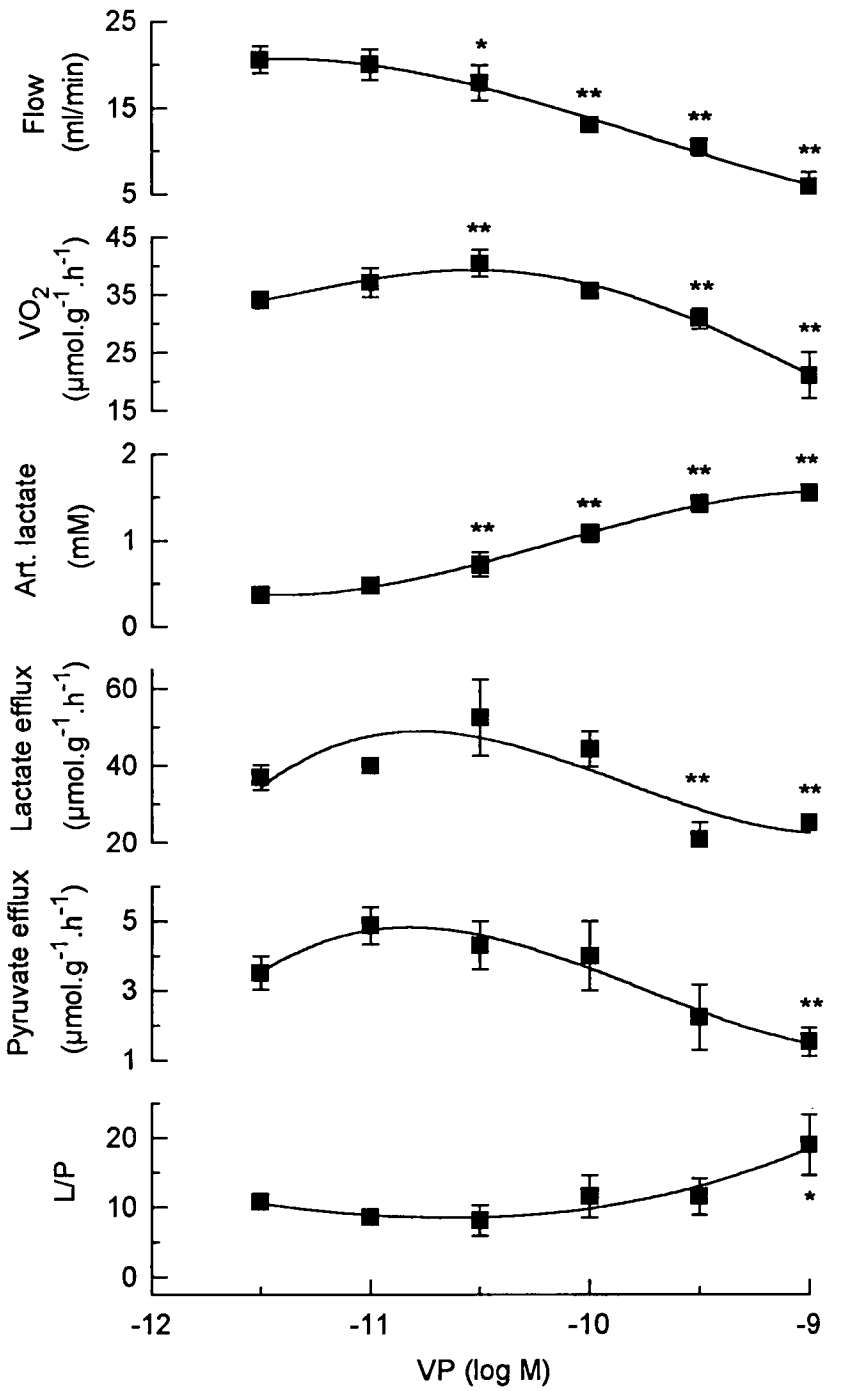

Fig. 6-6 Effect of VP on flow, $\dot{V}O_2$, lactate and pyruvate in the constant-pressure perfused rat hindlimb. Art. lactate: lactate concentration in the arterial perfusate. Data are means ± SE, n=3-4. *P<0.05, **P<0.01 vs the basal values (Paired *t* test).

VP mediated a biphasic change in $\dot{V}O_2$. At low doses, VP stimulated $\dot{V}O_2$ associated with a trend of increased lactate and pyruvate release (Fig. 6-6) despite a fall in flow rate. The maximal $\dot{V}O_2$ induced by VP reached 18% above the basal level ($P<0.01$) at 33 pM. At doses higher than 0.1 nM, VP began to inhibit $\dot{V}O_2$, lactate and pyruvate output. As the changes in lactate and pyruvate paralleled each other, the lactate to pyruvate ratio was not altered until the VP reached 1 nM (Fig. 6-6).

6.3.5 Effect of 5-HT on flow, $\dot{V}O_2$, lactate and pyruvate

Unlike NE and VP, 5-HT mediated a vasodilation at low concentrations before it caused vasoconstriction at higher doses. There were biphasic changes in $\dot{V}O_2$, lactate and pyruvate associated with the biphasic change in flow. At concentrations lower that 0.1 μM, 5-HT increased flow rate and $\dot{V}O_2$ associated with a dramatic reduction in lactate production (without a significant increase in lactate accumulation). During the vasodilation phase (as indicated by an increased flow), the maximal change was obtained at 33 nM 5-HT with increases in flow and $\dot{V}O_2$ of 22% and 30%, respectively, as well as a 64% decrease in lactate release. As a result of the decrease in lactate and the rising trend of pyruvate, the lactate/pyruvate ratio dropped markedly ($P<0.01$). At the concentrations higher than 0.1 μM, 5-HT reduced flow rate, $\dot{V}O_2$, lactate and pyruvate. At the turning point (0.1 μM) from vasodilation to vasoconstriction, there seemed to a rebound in lactate release.

6.3.6 Muscle metabolites

Table 6-1 shows the concentration of Cr, CrP, AMP, ADP, ATP and lactate in gastrocnemius-plantaris-soleus muscle group after constant-pressure perfusion. Following 120 min perfusion (perfusion control group), all of the measured metabolites were similar to those in the muscle sampled *in vivo*.

The muscle samples were also taken and measured for the metabolites after the dose-response curve for each vasoconstrictor. In the presence of NE at the dose (0.1 μM), which does not inhibit $\dot{V}O_2$ (see Fig 6-2), AMP and ADP were not significantly different from the control values whilst CrP/Cr, ATP and lactate showed a marked increase ($P<0.05$). Addition of phenylephrine showed similar results to those caused by NE except for ATP, which was not statistically different from the control. At a concentration of 1 nM where VP began to inhibit $\dot{V}O_2$, there was a marked decrease in CrP/Cr. Similarly, when 5-HT inhibited $\dot{V}O_2$ at 10 μM, CrP/Cr decreased significantly (Table 6-1).

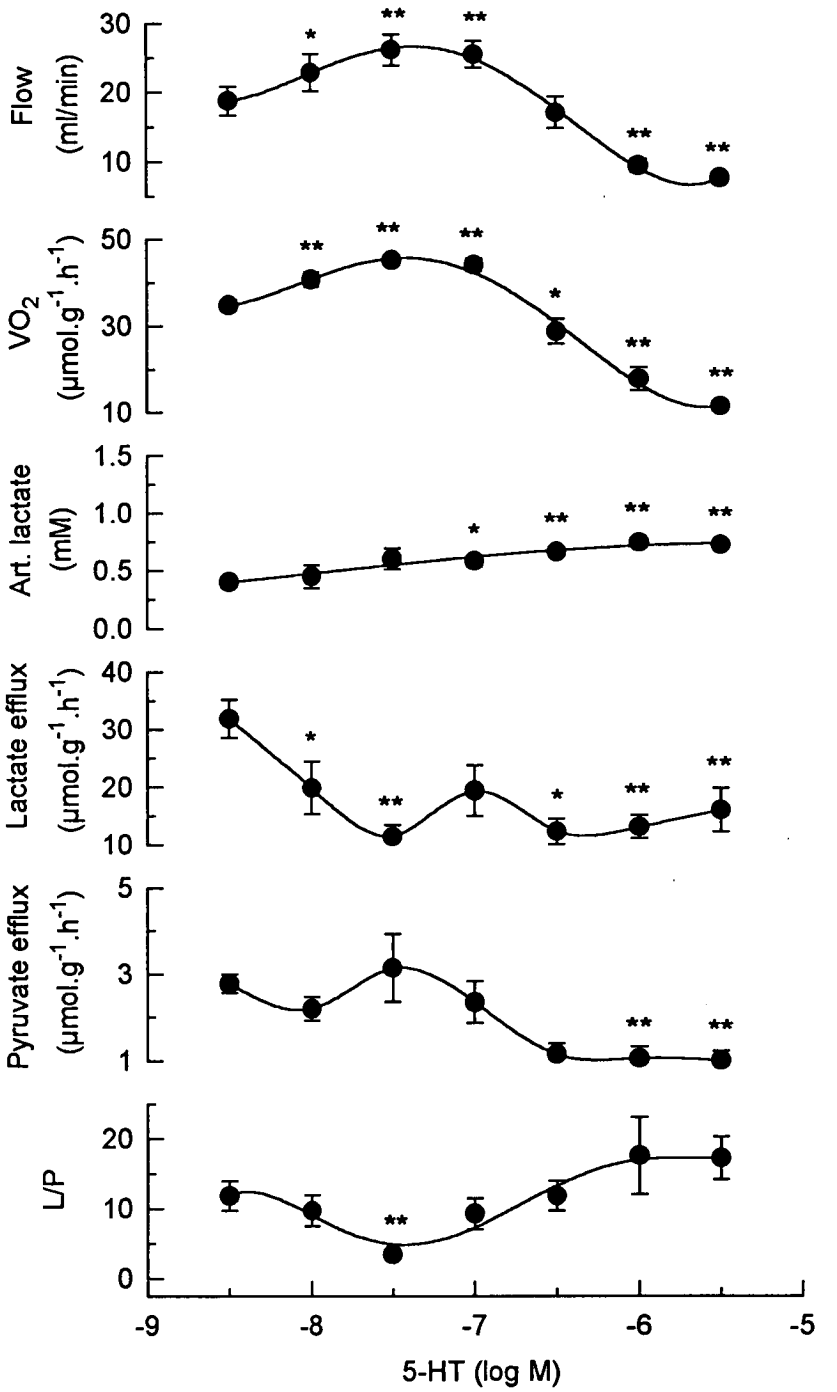

Fig. 6-7 Effects of 5-HT on flow, $\dot{V}O_2$, lactate and pyruvate in the constant-pressure perfused rat hindlimb. Art. lactate: lactate concentration in the arterial perfusate. Data are presented as means ± SE, n=4-5. * $P<0.05$, ** $P<0.01$, vs the basal values (Paired *t* test).

Table 6-1 Muscle metabolites in constant-pressure perfused rat hindlimb

	n	Cr	CrP	AMP	ADP	ATP	ΣAN	Lactate	EC	CrP/ Cr	CrP/ATP
		μmol/g dry wt.									
In vivo	5	111.6	119.3	0.23	2.69	28.18	31.10	9.31	0.952	1.07	4.24
		± 7.56	± 9.2	± 0 03	± 0.21	± 1.13	± 1.34	± 0.89	± 0.006	± 0.02	± 0.30
Perfused	5	114.8	111.0	0.22	2.04	28.05	30.30	9.02	0.960	0.97	3.96
(120 min)		± 4.86	± 3.54	± 0.03	± 0.13	± 0.46	± 0.44	± 0.93	± 0.003	± 0.03	± 0.14
NE	4	108.5	128.0	0.51	2.95	32.31 *	35.32*	16.40*	0.960	1.20*	3.98
(0.1 μM)		± 6.89	± 7.88	± 0.03	± 0.33	± 1.25	± 1.50	± 2.23	± 0.003	± 0.10	± 0.18
Phenyl.	4	105.5	115.6	0.22	3.04	29.59	32.84	18.89*	0.946	1.10*	3.94
(0.1 μM)		± 4.62	± 7.76	± 0.05	± 0.43	± 2.37	± 2.26	± 4.18	± 0.010	± 0.05	± 0.03
5-HT	4	126.4	101.0	0.15	2.34	29.19	31.52	7.41	0.957	0.86*	3.60
(3.3 μM)		± 2.72	± 0.25	± 0.03	± 0.10	± 0.81	± 0.92	± 1.36	± 0.001	± 0.03	± 0.03
VP	3	125.5	99.5	0.11	2.43	28.82	31.39	9.43	0.957	0.81*	3.45
(1.0 nM)		± 5.05	± 4.44	± 0.06	± 01.3	± 0.80	± 0.92	± 0.76	± 0.001	± 0.03	± 0.07

Gastrocnemius-plantaris-soleus muscle group was freeze-clamped by N_2-precooled tongs. The muscle *in vivo* was clamped under anaesthesia. Other muscle samples were taken at the end of the perfusion. No significant difference was found between the results from *in vivo* and control perfusion. Phenyl: phenylephrine; VP: vasopressin. Data are presented as mean ± SE. *: $P< 0.05$, vs control perfusion.

6.3.7 Comparison of flow, $\dot{V}O_2$, lactate induced by the vasoactive agents

Fig. 6-8 summarizes the changes in perfusion flow and $\dot{V}O_2$ mediated by NE, phenylephrine, isoproterenol, VP, and 5-HT. There were two patterns of relationships between flow rate and $\dot{V}O_2$ during vasoconstriction (Figs 6-8 & 6-9). At the early stages of fall in flow, NE, phenylephrine, VP stimulated $\dot{V}O_2$ before inhibiting. In contrast, 5-HT inhibited $\dot{V}O_2$ immediately when it reduced the flow.

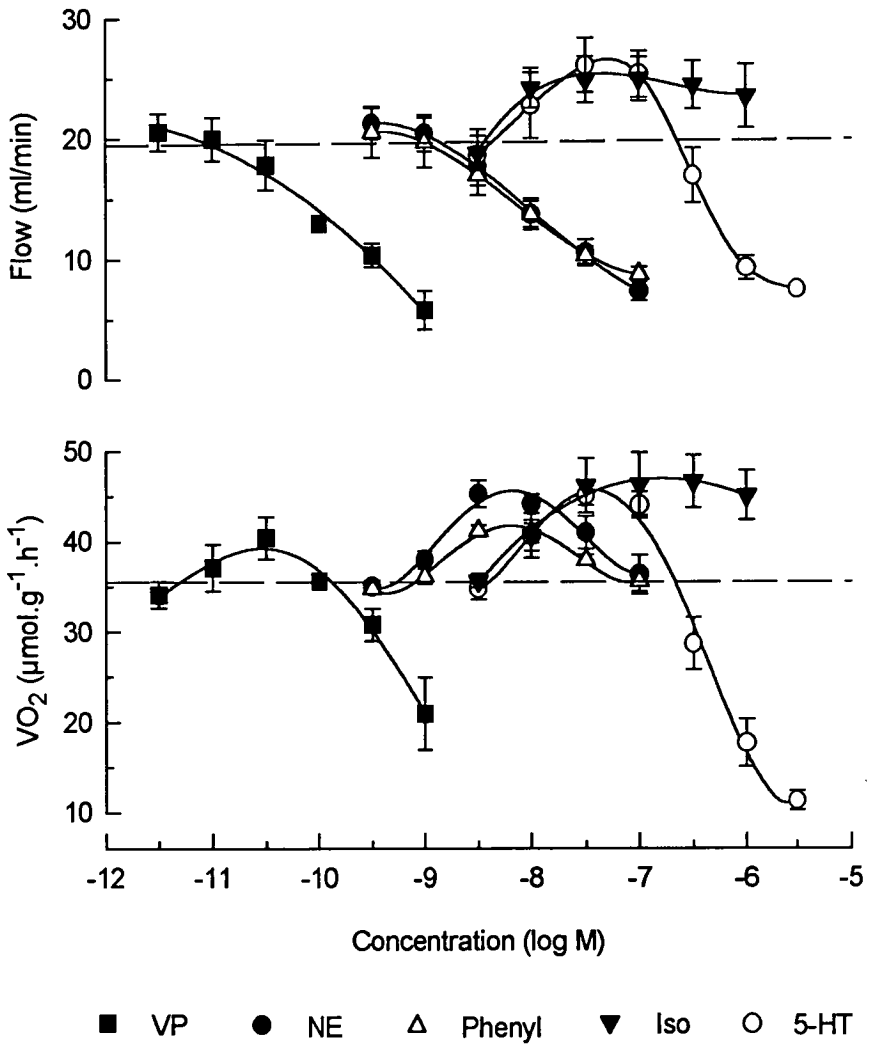

Fig. 6-8 Effects of VP, NE, phenylephrine, isoproterenol and 5-HT on flow and $\dot{V}O_2$ in constant-pressure perfused rat hindlimb. Combined data taken from perfusions in Figs.6-2, 6-3, 6-6 & 6-7. Phenyl: phenylephrine, Iso: isoproterenol.

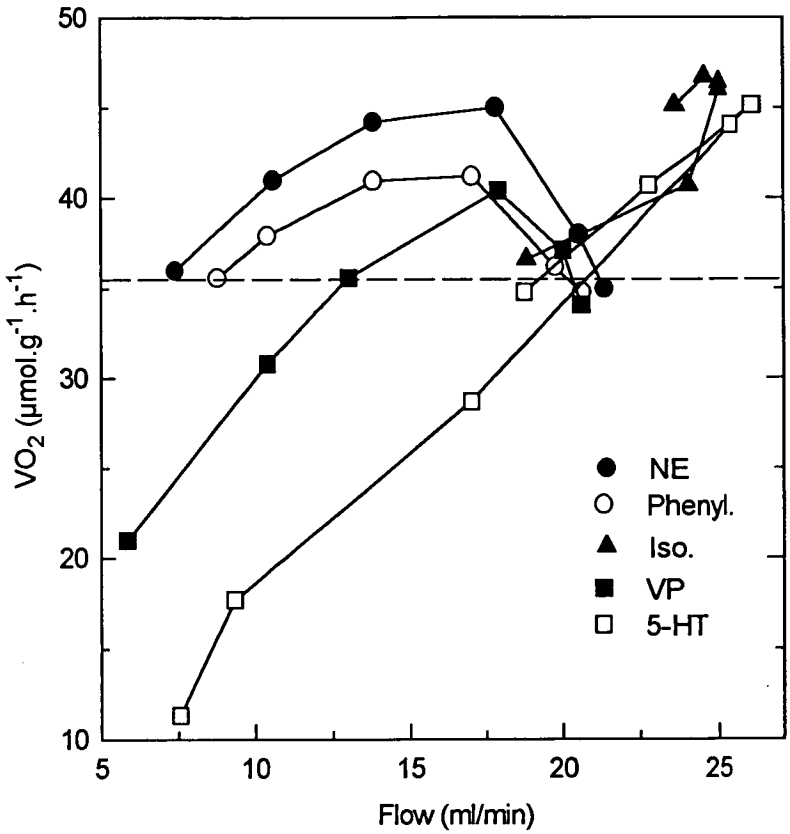

Fig. 6-9 $\dot{V}O_2$ as a function of flow induced by NE, phenylephrine, isoproterenol and 5-HT in the constant-pressure perfused rat hindlimb. Original data are presented in Fig 6-8.

6.3.8 Lactate accumulation and output

Figs. 6-2, 6-6 and 6-7 have shown that lactate efflux decreased with different magnitudes of increase in arterial lactate concentration. As lactate output also declined in the control perfusion when lactate concentration in the perfusate accumulated after recirculation (Fig. 6-1), the true change in lactate efflux in the presence of vasoconstrictors can not be clearly seen in those figures. Thus, Fig. 6-10 compares the effects of NE, phenylephrine, isoproterenol, VP and 5-HT on lactate accumulation and output after 120 min of perfusion. The results showed a trend of increased lactate accumulation in the presence of NE, phenylephrine and VP. These seem to correspond with a trend of decreased lactate output. Isoproterenol increased both the output and accumulation of lactate whilst 5-HT significantly inhibited them.

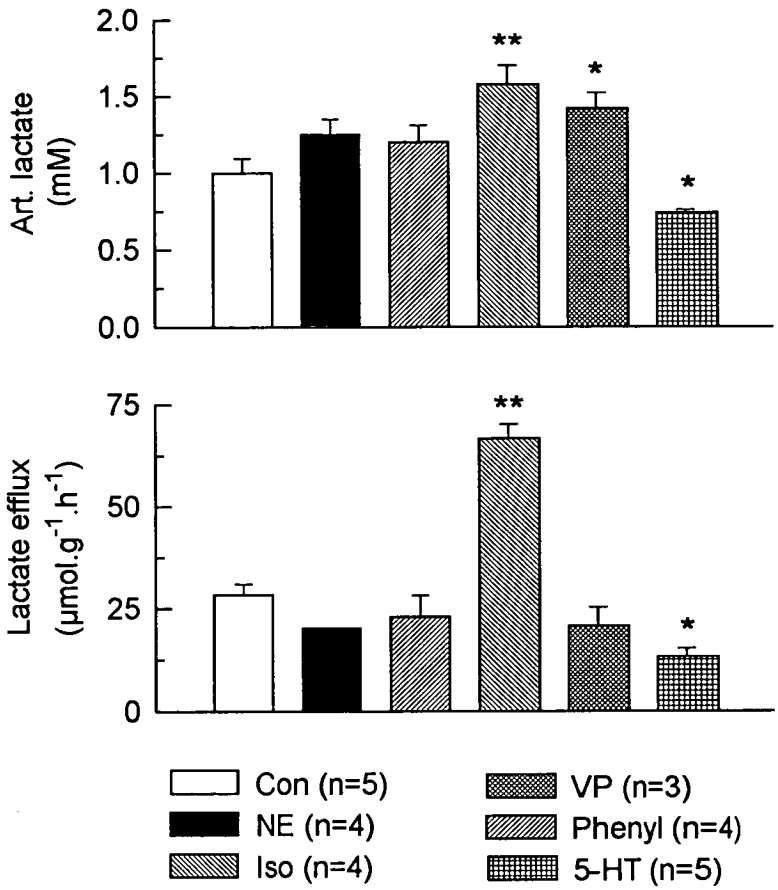

Fig. 6-10 Accumulation and efflux of lactate after 120 min of perfusion in the presence of vasoactive agonists. E (0.1 μM), phenylephrine (Phenyl, 0.1 μM), isoproterenol (Iso, 1 μM), VP (33 pM), 5-HT (1 μM). Art. lactate: lactate concentration in the arterial perfusate.

6.4 Discussion

In earlier Chapters, vasoconstrictors have been shown to exert either stimulatory (type A) or inhibitory (type B) effects on metabolism in the perfused hindlimb and intestine in a constant-flow perfused model (Chapters 3, 4 & 5). Under normal resting conditions, the systemic blood pressure *in vivo* is controlled by the neuro-humoral system at a relatively constant level although autoregulation at the organ or tissue level tends to maintain steady perfusion flow. Hence, the present study was designed to keep perfusion pressure at a constant and physiological level of 80 mmHg and allow the flow to change freely.

The basal $\dot{V}O_2$ of 35 $\mu mol \cdot g^{-1} \cdot h^{-1}$ in the present experiments is generally comparable with the value (16 $\mu mol.g^{-1}.h^{-1}$) observed in a constant-flow perfused rat hindlimb at 25°C at 18.2 ml/min (Chapter 3), given that Q_{10}=2.5 (Bonen *et al.*, 1994). This value is also similar to that (43.8 $\mu mol.g^{-1}.h^{-1}$) obtained in the rat hindlimb perfused at a constant pressure of 80 mm Hg with Krebs buffer containing erythrocytes (Hardeveld *et al.*, 1980). However, lactate (36 $\mu mol \cdot g^{-1} \cdot h^{-1}$) and pyruvate (3.3 $\mu mol \cdot g^{-1} \cdot h^{-1}$) appeared lower using the same estimate from data at 25° C where lactate and pyruvate outputs were 20.6 $\mu mol \cdot g^{-1} \cdot h^{-1}$ and 2.2 $\mu mol \cdot g^{-1} \cdot h^{-1}$, receptively. This discrepancy may result from the recirculation of the perfusate adopted in the present experiment.

As well as producing lactate, resting skeletal muscle is also able to utilize lactate (Mclane & Holloszy, 1979; Chin *et al.*, 1991; McDermott & Bonen, 1992). Lactate gradient between muscle and blood dictates whether lactate is taken up or released (Pagliassotti & Donovan 1990a; McDermott & Bonen, 1992). Data from Pagliassotti & Donovan (1990a) have clearly demonstrated that net lactate balance (lactate removal minus release) is proportional to arterial lactate concentration. When arterial lactate concentration exceeds 4-8 mM, skeletal muscle begins to remove rather than release lactate. It is possible that skeletal muscle takes up some lactate and pyruvate from the recycled perfusate, thereby inhibiting the net production of lactate and pyruvate. Nonetheless, the ratio of lactate/pyruvate of 12 is close to the value of approximate 10 obtained in the constant-flow perfused rat hindlimb at 25°C (Chapter 3). The metabolic parameters and perfusion flow remained steady for at least 120 min (Fig. 6-1) and muscle metabolites at the end of the perfusion showed similar results to those obtained from muscle taken *in vivo* (Table 6-1). These data indicate that a constant-pressure perfusion preparation of rat hindlimb has been established with valid physiological and metabolic characteristics.

Consistent with the findings in the constant-flow perfused rat hindlimb (Chapters 3 & 4), NE-stimulated increase in $\dot{V}O_2$ in the constant-pressure perfused rat hindlimb (Fig. 6-2). The similarities between NE and phenylephrine on flow, $\dot{V}O_2$, lactate and pyruvate would seem to indicate that α_1-adrenergic receptors are mainly responsible for the effects of NE. In contrast to constant-flow perfused rat hindlimb, both lactate and pyruvate tended to decline when $\dot{V}O_2$ was increased by NE and phenylephrine accompanied by a trend of greater accumulation of lactate in arterial perfusate (Figs. 6-2 & 6-10). Yet it is not known whether NE stimulates lactate uptake in the skeletal muscle. If so, when NE was added to the present recycling perfusion, the increased arterial lactate would probably impede the endogenous production of lactate in the muscle, thus inhibiting the net release of lactate. A similar

mechanism would possibly apply to the decrease in pyruvate during stimulation by NE.

In addition to α-adrenergic receptors, β-adrenergic receptors are also shown to be involved in NE-mediated increase in $\dot{V}O_2$ in the constant-pressure perfused hindlimb. β-Adrenoceptor-mediated increases in metabolism were first hinted at by the result that the maximal $\dot{V}O_2$ induced by NE appeared to be bigger (29%) than that (17%) induced by phenylephrine (Fig. 6-2) and was further supported by a direct stimulation of $\dot{V}O_2$, lactate and pyruvate net production by isoproterenol (Fig. 6-3). These results are consistent with the observations by Hardeveld *et al.* (1980) who found that isoproterenol stimulates $\dot{V}O_2$ (20%) and lactate production (approximately 400%) in a constant-pressure (80 mm Hg) perfused rat hindlimb with Krebs-Henseleit buffer containing erythrocytes.

Recent studies have revealed that β-adrenergic receptors can be classified into at least three subtypes: β_1, β_2 and β_3 (Challiss *et al.*, 1988; Emorine *et al.*, 1989; Roberts *et al.*, 1993). It has been suggested that β_3-adrenergic receptors are the major subtype responsible NE-mediated increase in thermogenesis in BAT (Arch & Kaumann, 1993) and possibly in skeletal muscle (Challiss *et al.*, 1988; Emorine *et al.*, 1989; Roberts *et al.*, 1993). Ligand binding studies on soleus muscle suggest the existence of β_3-adrenergic receptors in skeletal muscle (Roberts *et al.*, 1993; Silence *et al.*, 1993).

In order to assess the possible role of the putative β_3- (or atypical β) adrenergic receptors in $\dot{V}O_2$, the β_1, β_2-adrenergic antagonist, propranolol (Challiss *et al.*, 1988; Cawthorne *et al.*, 1992; Arch & Kaumann, 1993), was added in the presence of isoproterenol. The results in Fig. 6-4 demonstrated addition of that propranolol completely blocked isoproterenol-induced flow, lactate and pyruvate, but not $\dot{V}O_2$. These observations agree with the reports by Blaak *et al.* (1994) who found isoproterenol induced increases in blood flow, lactate and pyruvate fluxes from the forearm skeletal muscle of lean men and these increases were inhibited by the β_1-adrenergic antagonist, atenolol. Although they did not attribute isoproterenol-elicited $\dot{V}O_2$ to β_3-adrenergic receptors (Blaak *et al.*, 1993), administration of atenolol did not show a clear inhibitory effect on isoproterenol-increased skeletal muscle $\dot{V}O_2$. Interestingly, Fig. 6-4 shows that isoproterenol-stimulated $\dot{V}O_2$ was not significantly inhibited by propranolol, thus suggesting that atypical β-adrenergic receptors, possibly β_3-adrenergic receptors, are involved in mediating muscle $\dot{V}O_2$ in the constant-pressure perfused rat hindlimb.

Such a possibility is confirmed by the stimulation of $\dot{V}O_2$ by the β_3-adrenergic agonist, BRL35135A (Cawthorne *et al.*, 1992; Arch & Kaumann, 1993) in the presence of propranolol (Fig. 6-5), although the maximal $\dot{V}O_2$ produced by BRL35135A was not as big as that produced by isoproterenol. Coincidently, a similar increase in energy expenditure (10-23%) was also observed in a study of glucose-induced thermogenesis in men when BRL35135 was administered (Cawthorne *et al.*, 1992). Abe *et al.* (1993) reported BRL35135A stimulates glucose uptake by approximately 20 % in rat soleus muscle. Thus these observations together with the findings of atypical β- or β_3-adrenoceptor-mediated $\dot{V}O_2$ in constant-pressure perfused rat hindlimb suggest that skeletal muscle may contribute to the increased thermogenesis produced by putative β_3-adrenergic agonists.

Similar to α-adrenergic agonist, VP stimulated $\dot{V}O_2$ by 17% at low dose (33 pM) while it reduced perfusion flow rate in a dose-response manner (Fig. 6-6). At doses higher than 0.1 nM where the flow was reduced more than 40%, VP became inhibitory to $\dot{V}O_2$. The stimulation of $\dot{V}O_2$ by VP, NE and phenylephrine suggests that type A vasoconstrictors stimulate $\dot{V}O_2$ in the constant-pressure perfused rat hindlimb.

5-HT can have either vasodilatory or vasoconstrictive action in the human forearm dependent on the concentration (Bruning *et al.*, 1993). At low concentrations, 5-HT exerts a biphasic vasodilation whereas at high concentration it causes vasoconstriction (Blauw *et al.*, 1988). In the constant-flow perfused hindlimb of rats 5-HT inhibits $\dot{V}O_2$ (Dora *et al.*, 1991 & 1992) and skeletal muscle contraction during vasoconstriction (Rattigan *et al.*, 1994). A similar inhibition of $\dot{V}O_2$ was also observed in constant-flow perfused hindlimb of marsupial bettongs (Chapter 4). In these latter experiments, only a vasoconstrictive effect at high concentrations was observed.

In the present chapter, 5-HT (Fig. 6-7) showed not only the expected vasoconstrictive effect but also a clearly vasodilatory effect at doses lower than 0.3 μ M. Furthermore, there was an increase in $\dot{V}O_2$ in parallel with the increased perfusion flow (Fig. 6-7). In contrast to isoproterenol which also increased perfusion flow (Fig. 6-3), 5-HT significantly reduced lactate output and the lactate/pyruvate ratio (Fig. 6-7) when $\dot{V}O_2$ reached the maximum. This difference would seem to suggest that 5-HT increases $\dot{V}O_2$ during vasodilation in a mechanism different from β-adrenergic stimulation.

In incubated rat skeletal muscle, 5-HT does not inhibit contraction performance (Dora *et al.*, 1994) or glucose uptake (Rattigan *et al.*, 1994). However, when infused via vascular route in the constant-flow (once-through) perfused rat

hindlimb, 5-HT does inhibit exercised performance and glucose uptake (Dora *et al.*, 1994; Rattigan *et al.*, 1994). Therefore, it is suggested that 5-HT affects muscle metabolism by changing blood flow patterns. The enhanced $\dot{V}O_2$ by 5-HT at low doses with constant-pressure perfusions would appear to be related to the increase in flow rate. As shown in Chapter 3, an increased flow to muscle can induce a rise in $\dot{V}O_2$ by this tissue. Thus, the increase in $\dot{V}O_2$ induced by low dose 5-HT in the constant-pressure perfused rat hindlimb may be due to the increased flow. However, when perfusion flow is increased (Chapter 3) without restricting perfusion pressure, there is a parallel rise in both lactate and pyruvate without affecting their ratio. The decrease in lactate/pyruvate ratio suggests 5-HT-induced $\dot{V}O_2$ at low doses may also be associated with some changes in mitochondrial respiration.

In agreement with the observations in the constant-flow perfused rat hindlimb, 5-HT at concentrations higher than 0.1 μM caused both vasoconstriction as indicated by decreased flow and inhibition of $\dot{V}O_2$ (Fig. 6-7). The pattern of $\dot{V}O_2$ as a function of flow rate induced by 5-HT is different from that for type A vasoconstrictors such as NE, VP and phenylephrine (Fig. 6-9). Whilst type A vasoconstrictors stimulate $\dot{V}O_2$ at low doses during the initial stage of decrease in perfusion flow, 5-HT does not show any trend of stimulation of $\dot{V}O_2$ when it reduces flow rate below the basal level. Thus the distinction of their effects on $\dot{V}O_2$ by type A and B vasoconstrictors in the constant-flow perfused hindlimb (Clark *et al.*, 1994) appears to stand as well for the constant-pressure perfusion of hindlimb even though the effects are not identical.

The mechanism accounting for the increased $\dot{V}O_2$ mediated by type A vasoconstrictors in the constant-pressure perfused hindlimb may involve the energy cost of constricting blood vessels. As discussed earlier (Chapters 3, 4 & 5), $\dot{V}O_2$ of vascular smooth muscle is dependent on the vascular wall tension (Paul, 1980). According to the Laplace law of tension-radius relationship (Tension = Pressure/Radius), vascular wall tension is proportional to its radius. Since the pressure was controlled at a constant level in the present experiments, a decrease in perfusion flow would seem to reflect a decrease in the radius (Flow $\propto P/r^4$, according to Poiseuille's law). How could a decreased total vascular wall tension consume more O_2 if microvessels are responsible for the increased $\dot{V}O_2$?

It has been well established that the total vascular wall tension (Tt) consists of two components: active tension (Ta) and passive tension (Tp) (Paul & Peterson, 1975; Mulvany & Warshaw, 1979; VanBavel & Mulvany, 1994). Tp reflects the properties of the elastic elements in vascular wall whereas Ta is produced by vascular smooth muscle contraction (Mulvany & Warshaw, 1979; VanBavel & Mulvany,

1994). Paul & Peterson (1975) have demonstrated in bovine mesenteric vein that vascular $\dot{V}O_2$ is dependent on Ta rather than Tt. Consequently, the key issue for vascular $\dot{V}O_2$ lies on its Ta. It is also known that the relationship between vascular radius and Ta is opposite below or above the optimal point. Below the optimal radius (or circumference), Ta falls linearly with decreasing radius. Above the optimal radius, Ta also falls with increasing radius (Mulvany & Warshaw, 1979). Hence, when vascular radius is reduced to the optimal by an activating agonist such as NE, Ta increases in a reverse proportion to the fall of Tt (Mulvany, 1991). Indeed, vascular $\dot{V}O_2$ has been shown to match Ta very well below or above the optimal radius (Paul & Peterson, 1975).

In the present constant-pressure perfused hindlimb, a suprabasal radius beyond its optimal level is likely to be present in the resistance vessels despite the physiological pressure because of the following reasons. Firstly, the basal flow rate of 2.4-2.6 ml/min per g muscle is 9-10 fold the value *in vivo* (0.27 ml/min per g). Such a supraphysiological flow would substantially increase the shear stress to the vascular endothelium, thereby releasing EDRF (Bevan, 1991) to relax vascular smooth muscle. Secondly, the viscosity of the perfusate is lower than blood because of the lack of red blood cells. In the arterial trees, viscosity is higher in upstream resistance vessels than it is downstream (Folkow & Neil 1971). Since viscosity is proportional to vascular resistance and the effect of high flow, the transmural pressure would be expected to be higher in the downstream arterial tree and to expand the vascular radius. At a similar perfusion of rat hindlimb with 4% Dextran-Tyrode solution, a high capillary filtration pressure has been noted (Johnsson *et al.*, 1991). When type A vasoconstrictors elicit vasoconstriction (indicated as decrease in perfusion flow rate), Ta would be expected to increase in the early stages of vasoconstriction even though Tt decreases with the fall in flow. Accordingly, an increase in $\dot{V}O_2$ in the constant-pressure perfused hindlimb vascular bed would be expected before the optimal level or the decreased flow restricts the parenchymal metabolism. Such an interpretation seems in agreement with the fact that the maximal $\dot{V}O_2$ produced by NE, phenylephrine and VP all lies at an approximately similar flow rate of 17 ml/min (Figs. 6-4, 6-8 & 6-10).

Alternatively, if nutritive and nonnutritive capillaries exist in skeletal muscle (Renkin *et al.*, 1971; Clark *et al.*, 1994), the eventual $\dot{V}O_2$ would be dependent on the actual flow though nutritive capillary areas. Accordingly, a redirection of flow to these capillaries should increase $\dot{V}O_2$ no matter how the total flow changes within the range of sufficient supply. Hence, in analogy to the argument for constant-flow perfusion (Chapters 3, 4 & 5), NE, phenylephrine and VP can increase $\dot{V}O_2$ by

redistributing flow to the nutritive capillaries. Sufficient oxygen supply during the stimulation of $\dot{V}O_2$ by NE, phenylephrine and VP was supported by the normal ratio of lactate/pyruvate (Figs. 6-2 & 6-6). Although the present experiments did not show direct evidence of the recruit rate of capillaries, partial opening of the muscle capillaries under basal conditions is indicated by the improved high phosphate compounds in the presence of NE and phenylephrine (Table 6-1). Such an argument appears to agree with the observation (Fig. 6-7) that 5-HT at low doses was able to increase $\dot{V}O_2$ when it increased the flow rate, presumably increasing perfusion of nutritive capillaries as well. However, when the flow was further reduced by type A vasoconstrictors, $\dot{V}O_2$ was depressed due to the insufficiency of O_2 supply as shown by an increased perfusate lactate/pyruvate ratio (Fig. 6-6) and decreased muscle CrP/Cr ratio (Table 6-1) for VP at a flow rate of 4.8 ml/min.

In contrast to the effects of NE, phenylephrine and VP, the inhibition of $\dot{V}O_2$ by 5-HT (>0.3 μM) is probably due to the functional shunting of flow away from nutritive areas as suggested by Dora *et al* (1991). As shown in Table 6-1, CrP/Cr ratio is significantly inhibited by 5-HT although the flow rate, at which the muscle was sampled, was similar to that for NE or phenylephrine. Currently, it is not known how an increased flow to the nutritive capillaries would elicit skeletal muscle $\dot{V}O_2$ above a normal level in the resting state.

Similar to the classification of type A and type B vasoconstrictors, it could be postulated that each vasodilator may act on site specific receptors or utilize specific mechanisms so that the effect of vasoconstrictors on muscle metabolism would not be homogeneous. For example, NP relaxes vascular smooth muscle in general. 5-HT and isoproterenol vasodilate via acting on their specific receptors in blood vessels. This might explain why vasodilators such as NP inhibit $\dot{V}O_2$ (Chapter 3) whereas 5-HT (<0.3 μM) increases $\dot{V}O_2$.

Compared with the results obtained from constant-flow perfused hindlimbs in the same laboratory (Chapters 3 & 4; Colquhoun *et al.*, 1988; 1990; Dora *et al.*, 1991; 1992), several new findings emerge from the constant-pressure perfused rat hindlimb as follows:

1. Type A vasoconstrictors can stimulate $\dot{V}O_2$ even though perfusion flow is slightly reduced;
2. Both α and β-adrenoceptors are involved in NE-mediated increase in muscle thermogenesis. α_1-Adrenoceptor, the major α-adrenoceptor subtype responsible for the increased $\dot{V}O_2$, may mediate muscle thermogenesis via vascular constriction;

3. For β-adrenoceptors, atypical β-subtype (or putative β_3-subtype) is the major population to stimulate muscle $\dot{V}O_2$ independent of vascular effect. However, other subtypes may also be involved in β-adrenergic stimulation of muscle thermogenesis by increasing perfusion flow and lactate and pyruvate production;
4. 5-HT exerts a biphasic action on flow and $\dot{V}O_2$. It increases both perfusion flow and $\dot{V}O_2$ at lower concentrations (< 0.03 μM) but reduces $\dot{V}O_2$ and flow at high concentrations (≥ 0.3 μM);
5. In the present recycling perfusion at a constant pressure, an increased $\dot{V}O_2$ is not necessarily associated with parallel changes in lactate and pyruvate production.

Taken together, the present findings confirm the previous observations in a constant-flow model that vasoconstrictors control or contribute to skeletal muscle $\dot{V}O_2$.(Chapters 3 & 4). *In vivo*, the perfusion of skeletal muscle is at neither constant flow rate nor at constant pressure, vasoconstrictor such as NE is released or administrated (Foster and Frydman 1979; Kurpad *et al.*, 1994). While NE constricts blood vessels in the skeletal muscle, it also enhances the cardiac output (Foster and Frydman 1979; Liard, 1989) so that the actual blood flow to the skeletal muscle may not decrease dramatically to limit the $\dot{V}O_2$. Previous findings have revealed that $\dot{V}O_2$ is increased by type A vasoconstrictors associated with a rise in perfusion pressure when the flow remains unchanged (Chapters 3 & 4). The present study suggests that even though the perfusion flow is slightly reduced and the perfusion pressure remains unchanged, type A vasoconstrictors can still stimulate $\dot{V}O_2$, thus confirming the argument for the important role of the vascular system in skeletal muscle, and thereby in whole body thermogenesis.

CHAPTER 7

Bioenergetics of different muscle tissues during ischemia and its features in skeletal muscle during hyperoxemia

7.1 Introduction

High energy phosphate compounds (HEC) and lactate are well recognised as important metabolites of bioenergetics to evaluate the state of aerobic metabolism in living cells. In earlier Chapters, the mechanisms underlying vascular control of muscle thermogenesis have been discussed by using perfused hindlimbs, kidney and intestine. In those studies, bioenergetic metabolites have been repeatedly used to assess the validity of the perfused tissue preparations and the mechanism(s) involved.

In the perspective of NST, both skeletal muscle and BAT are important contributors (Jansky, 1971; Foster & Frydman, 1978; Astrup *et al.*, 1986). In BAT, NE elicits lipolysis in the brown adipocytes by β-adrenergic receptors. The released fatty acid subsequently activates uncoupling protein in the mitochondria to decouple oxidation of substrates from phosphorylation of ADP, thus producing heat. Both ATP and ADP are involved in regulating the function of oxidative-phosphorylation uncoupling (Himms-Hagen, 1990). It has been shown in this laboratory that adenine nucleotides are very sensitive to ischemia in BAT (Matthias *et al.*, 1994). Several studies suggest that decoupled oxidative phosphorylation may occur under certain conditions in skeletal muscle as a heat production mechanism (Barré *et al.*, 1986 & 1987b; Duchamp *et al.*, 1992, Herpin & Lefaucheur, 1992; Matthias *et al.*, 1993). A comparison between skeletal muscle to BAT in the aspects of bioenergetics would be informative of exploring the mechanism of muscle thermogenesis.

As shown in the perfused hindlimb as well as in intestine and in kidney, blood vessels play a key role in vasoconstrictor-induced $\dot{V}O_2$ (Chapters 3, 4, 5 & 6). The vascular tree appears to be an important source for vasoconstrictor-elicited production of lactate (Hettiarachchi *et al.*, 1992) and purine/pyrimidine breakdown metabolites (Clark *et al.*, 1990). However, little is known about the catabolic kinetics of bioenergetics in vascular tissue.

Ischemia of different periods is a useful model to observe kinetic changes in HEC and the intermediate metabolites. Thus the first purpose of this Chapter was to characterise the features of the bioenergetic kinetics in various muscle tissues during total ischemia. Based on the results, a second attempt was made to evaluate the

possible involvement of metabolites in controlling the respiratory rate by comparing them with those obtained from the resting muscle at a high $\dot{V}O_2$ state.

7.2 Methods

7.2.1 Ischemia model

Male rats weighing 180-200g were anaesthetised with an intraperitoneal injection of sodium pentobarbital (60 mg/k g body weight). Special care was taken to ensure that the animals were breathing normally with no signs of hypoxia before and during the surgery to sample the tissues. The ischemia model was produced by decapitating the animal with a guillotine under anaesthesia. The remaining carcase was wrapped in plastic bags to prevent evaporation of tissue water and kept at 37°C (or 25°C when indicated) before the samples were taken at the set intervals.

7.2.2 Hyperoxemia model

The high $\dot{V}O_2$ state of skeletal muscle was obtained from at 25°C with Krebs buffer that contained 4% BSA and 1.27 mM Ca^{2+}. The perfusate was saturated with 95% O_2 and 5% CO_2. Perfusions were performed at a constant flow of 18.5 ml/min for 20 min followed by an infusion of 50 nM NE for another 20 min. Previous data in the perfused rat hindlimb have shown that flow induces $\dot{V}O_2$ and NE augments this effect (Chapter 3). Hence the high resting skeletal muscle $\dot{V}O_2$ was produced by infusing NE to the high flow perfused rat hindlimb. Other details for the surgery and perfusion were described in Chapter 2 (Section 2.2.1)

7.2.3 Tissue sampling

All the tissue samples were freeze-clamped by liquid N_2-precooled tongs. Gastrocnemius-plantaris-soleus muscle group was taken for the measurement of skeletal muscle metabolites. The heart was exposed by cutting the diagram and ribs on both sides. Immediately after the exposure, the heart was freeze-clamped prior to removal. The aorta was sampled from the abdominal section. After evisceration of the intestines, the abdominal aorta was separated from the vena cava and the surrounding tissues. Immediately following the dissection, a segment between the bifurcation of iliac arteries and the left renal vessels was rinsed with filter paper and freeze-clamped

as mentioned above. The procedure from dissection to the freeze-clamp was completed within 10 seconds.

7.2.4 Determination of HEC, lactate and glycerol

The tissue samples were extracted by $HClO_4$ as described in Chapter 2 (Section 2.3). Creatine compounds and adenine nucleotides were determined simultaneously with ion-paired reverse phase chromatography (Shellevold *et al.*, 1986) by HPLC. The energy charge of adenylate pool was calculated according to the equation (Atkinson, 1968):

$$\frac{[ATP] + 0.5\,[ADP]}{[ATP] + [ADP] + [AMP]}$$

Lactate and glycerol were assayed enzymatically by a UV-spectrophotometer and spectrofluorimeter, respectively. Further details are outlined in Section 2.3.

7.3 Results

7.3.1 HPLC tracings of HEC in normal tissues

Fig. 7-1 shows HPLC tracings of normal HEC and related metabolites for skeletal muscle, heart (Fig, 7-1A), abdominal aorta, and small intestine (Fig. 7-1B). Cr, CrP, AMP, ADP and ATP were all observed at 214 nm wave length. Since creatine compounds are 3-4 fold that of ATP in skeletal muscle under non-ischemic conditions and eluted earlier than adenine nucleotides, a wide diversity in the peak heights appeared on chart paper for the compounds measured, causing difficulty in adjusting each peak at a measurable height within one scale. Therefore a second channel was set at 254 nm, where there was no absorption for creatine compounds, to detect adenine nucleotides at another suitable sensitivity.

As shown in Fig. 7-1, the compounds were well separated within 10 min. Each peak from samples was identified by comparing the retention time with the standard, and confirmed, when necessary, by addition of a known amount of the standard to the sample peak enrichment. The calculated contents of normal Cr, CrP, AMP, ADP, ATP, lactate, and glycerol was shown in Table 7-1. The energy charges were approximately 0.9 for each tissue except for aorta. These results together with high content of CrP indicate that a reliable sample processing had been achieved.

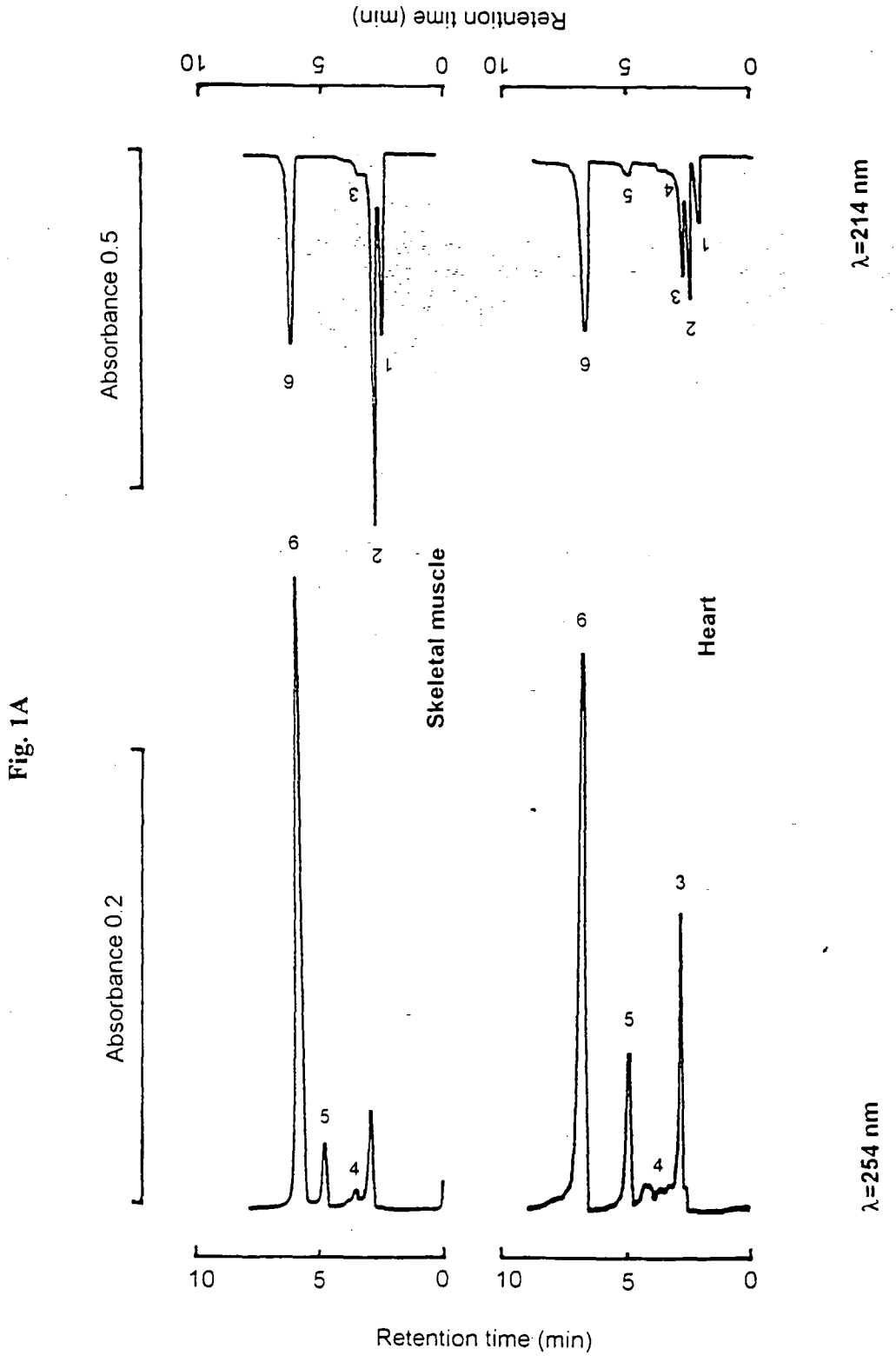

Fig. 1A

Fig. 7-1 HPLC tracings for high energy phosphate compounds for skeletal muscle, heart (A), intestine and aorta (B). 10 mV was set in the recorder for the full scale. An aliquot of 10-20 µl neutralized $HClO_4$ extract was injected into HPLC for each assay. For skeletal muscle and heart, AUFS was set at 2.0 (at 214 nm)

and 0.5 (254 nm). AUFS was set at 1.0 at both wave lengths for intestine, and 0.2 (at 214 nm) and 0.05 (at 254 nm) for aorta. Peaks: 1 (Cr), 2 (CrP), 3 (inosine), 4 (AMP), 5 (ADP) and 6 (ATP).

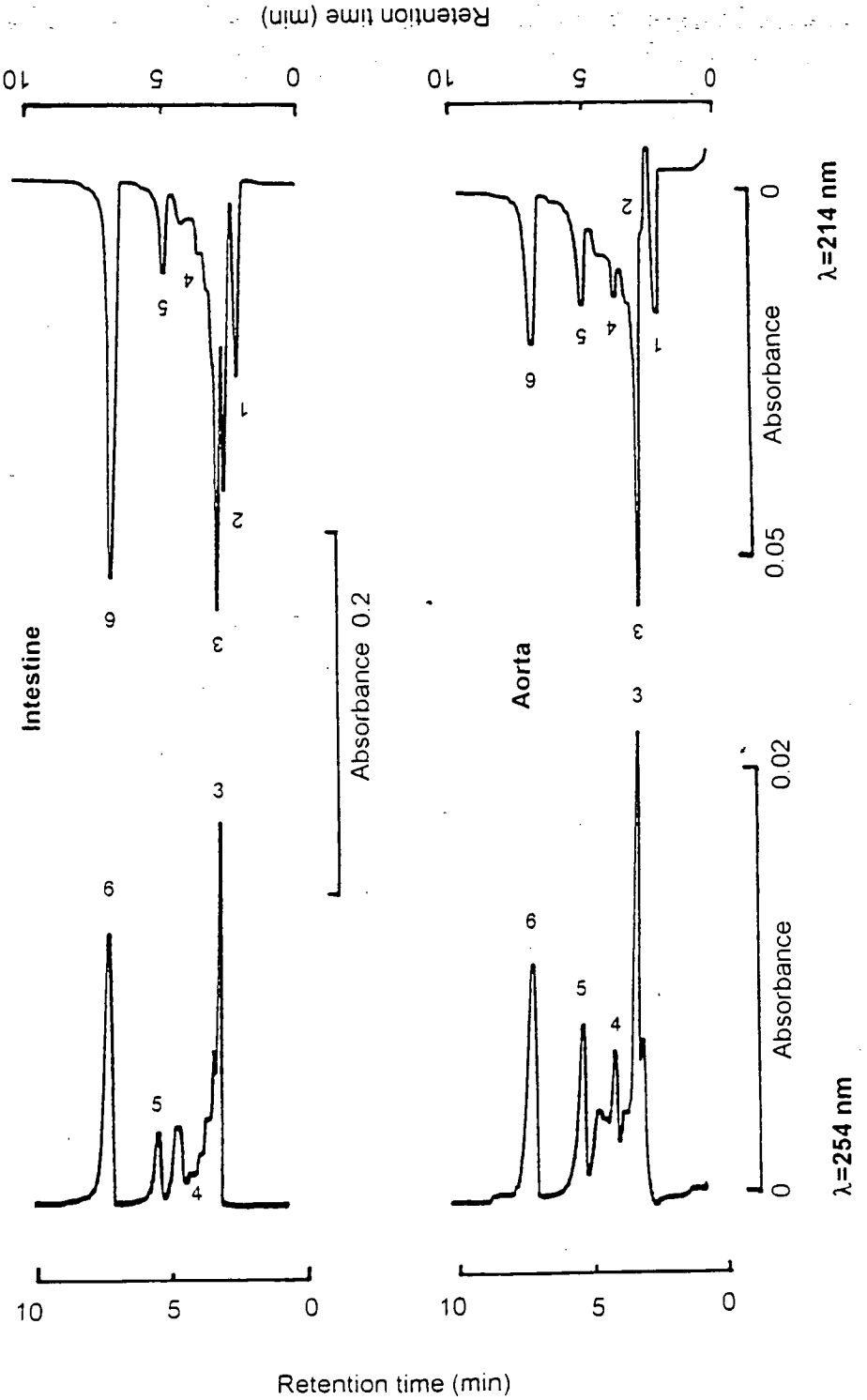

Fig. 7-1B

Table 7-1 Normal energetic metabolites of the rat skeletal muscle, heart, intestine and aorta

	n	Lactate	Glycerol	Cr	CrP	Cr+CrP	AMP	ADP	ATP	ΣAD	EC	CrP/Cr	CrP/ATP
		μmol/g dry wt											
S. muscle	5	9.31	1.90	111.60	119.27	230.86	0.23	2.69	28.18	31.10	0.95	1.07	4.24
		± 0.89	± 0.17	± 7.56	± 9.24	± 16.71	± 0.03	± 0.21	± 1.13	± 1.34	± 0.01	± 0.02	± 0.30
Heart	5	6.20	1.48	24.46	28.48	52.84	0.25	4.13	19.11	23.50	0.90	1.25	1.58
		± 0.83	± 0.20	± 3.76	± 2.11	± 4.61	± 0.07	± 0.54	± 2.15	± 1.94	± 0.01	± 0.15	± 0.11
Intestine	4	19.74	1.25	14.24	16.02	31.45	0.09	1.38	8.33	9.80	0.92	1.15	1.92
		± 2.15	± 0.21	± 1.31	± 1.40	± 1.98	± 0.02	± 0.06	± 0.55	± 0.54	± 0.01	± 0.15	± 2.60
Aorta	4-5	16.90	0.47	3.18	3.16	6.33	0.12	0.66	0.81	1.19	0.75	1.05	4.67
		± 1.97	± 0.19	± 0.39	± 0.12	± 0.48	± 0.04	± 0.31	± 0.14	± 0.0	± 0.04	± 0.11	± 1.13

Muscle tissues were freeze-clamped by a pair of tongs precooled in liquid N_2. $HClO_4$ extract was used for the measurements. EC: energy charge.

ΣAD: sum of adenine nucleotides.Data are presented as mean ± SE.

Comparison of the data reveals that the smooth muscles generally had a higher level of lactate but lower concentrations of CrP and ATP than the striated muscles under aerobic conditions. Skeletal muscle contained the highest phosphagens among all of these muscle tissues whereas aorta contained the lowest level HEC and glycerol.

7.3.2 Changes in creatine compounds during ischemia

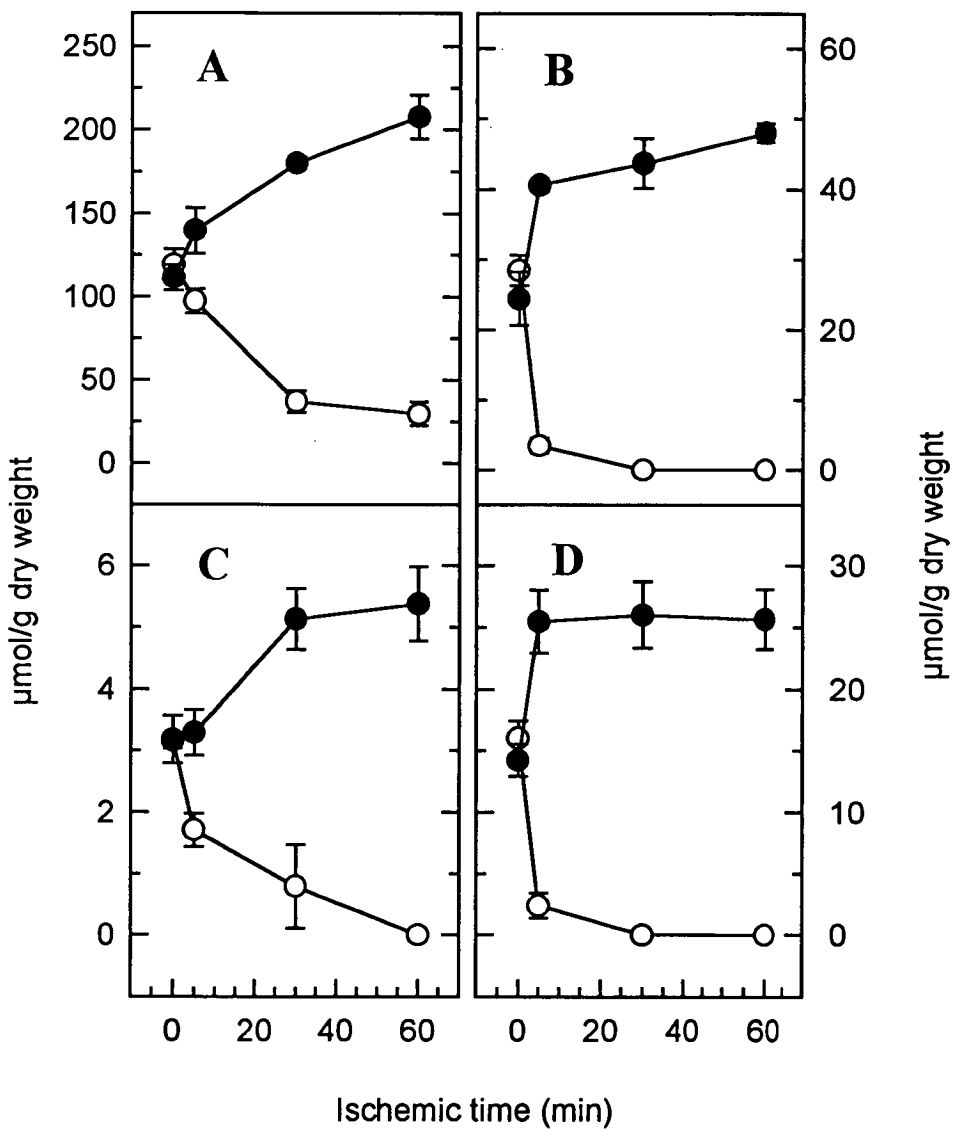

Fig. 7-2 Changes in creatine compounds in different muscle tissues in the time course of ischemia. Panel A: skeletal muscle (n=4-5); panel B: heart (n=4-6); panel C: aorta (n=4-5) and panel D: intestine (n=4-5). O: CrP, ● Cr. Data are presented as means ± SE.

As demonstrated in Fig. 7-2, after 5 min of total ischemia CrP decreased by 18%, 46%, 88% and 85% for the skeletal muscle, aorta, heart and intestine, respectively. CrP completely converted to Cr before 30 min of ischemia in the heart and intestine whereas there were still 30% and 33% left for skeletal muscle and aorta each with proportionally increased Cr.

7.3.3 Changes in adenine nucleotides during ischemia

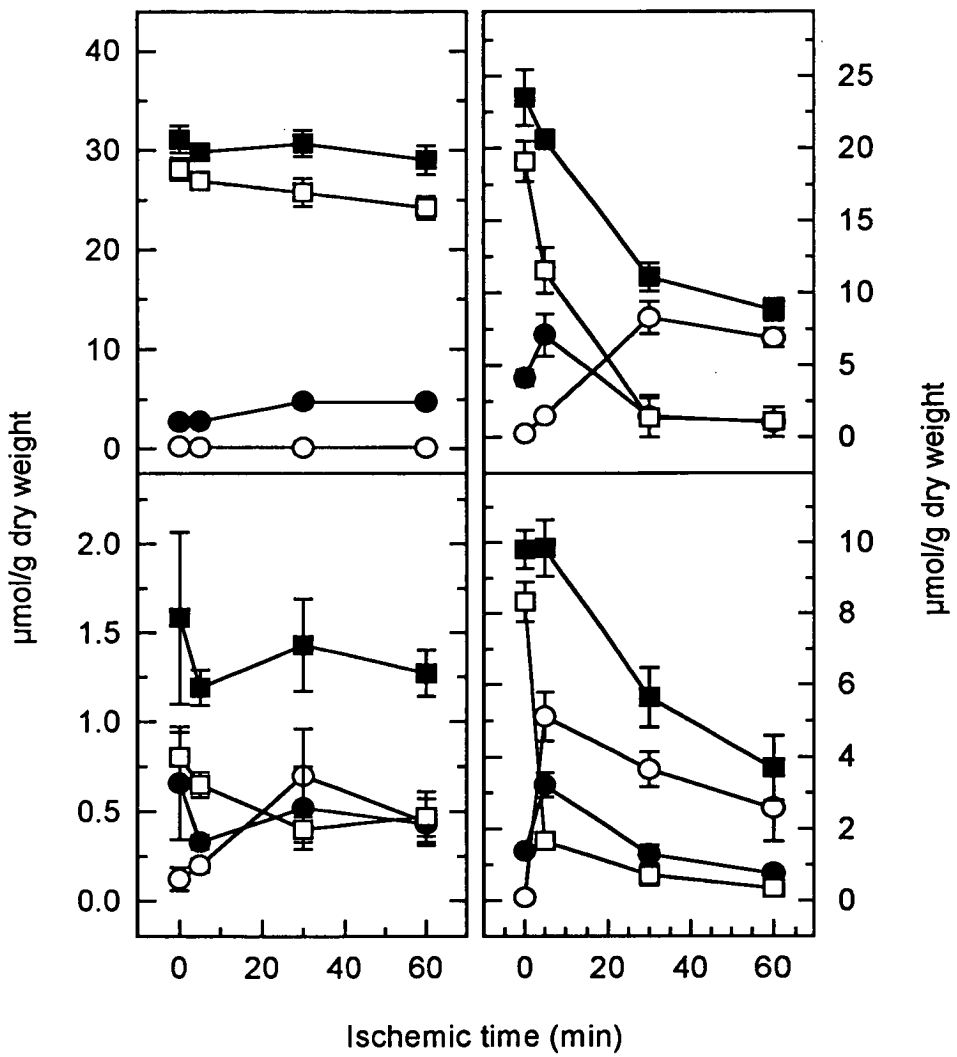

Fig. 7-3 Changes in adenine nucleotides of different muscle tissues during ischemia. Panel A: skeletal muscle (n=4-5); panel B: heart (n=4-6); Panel C: aorta (n=4-5) and panel D: intestine (n=4-5). ○: AMP; ● ADP; □: ATP and ■: ΣAN. Data are means ± SE.

In contrast to creatine compounds, adenine nucleotides were sustained much longer in skeletal muscle and aorta. In skeletal muscle (Fig 7-3A), ATP decreased by only 14% ($P<0.05$) of the initial level without significant reduction in $\sum AD$ ($P>0.05$) following 60 min of ischemia. ADP did not show a marked change after 5 min of ischemia but doubled at 30 min and thereafter ($P<0.01$). Interestingly, AMP did not alter appreciably during the period of ischemia examined. In aorta, ATP depleted faster than in skeletal muscle but more slowly than in the heart and intestine.

The four tissues varied significantly in their degradation rate and catabolic products when subjected to ischemia as illustrated in Fig. 7-3. In the heart which continued to beat for about 2 min, ATP dropped substantially immediately after ischemia and was almost depleted within 30 min. This was accompanied by a marked accumulation of AMP whilst ADP increased temporarily followed by a prolonged decline. When ischemia lasted to 60 min $\sum AD$ fell to 43% of the preischemic value.

Surprisingly, ATP depleted more quickly in the small intestine (where the auto-peristalsis was not controlled) than in the heart. About 80% ATP was hydrolysed after 5 min of ischemia whilst 40% ATP disappeared in the heart at the same time. Concomitant with the changes in ATP content, AMP also differed considerably with a greater and quicker accumulation in the intestine, although the profiles of ADP and $\sum AD$ appeared similar to those of the heart.

7.3.4 CrP/Cr ratio and EC

When CrP degraded, Cr was formed, leading to a larger change in CrP/Cr ratio than either CrP or Cr alone. After 5 min of ischemia, for example, CrP in skeletal muscle decreased by 18% ($P>0.05$, Fig.7-2), but the CrP/Cr ratio decreased 26% ($P<0.05$, Fig.7-4A). Calculated EC for the different muscle tissues (Fig.7-4B) together with the actual data (displayed in Fig. 7-3) indicated that the depletion rates of adenine nucleotides ranged in the order: intestine>heart>aorta>skeletal muscle (at rest). When compared to EC, the ratio of CrP/Cr altered earlier in each of tissues (Fig.7-4), especially in skeletal muscle. For instance, CrP/Cr ratio fell significantly ($P<0.05$) after 5 min of ischemia in skeletal muscle whereas none of the measured HEC metabolites showed a statistical difference from the initial levels. EC did not change appreciably in skeletal muscle until after 60 min of ischemia.

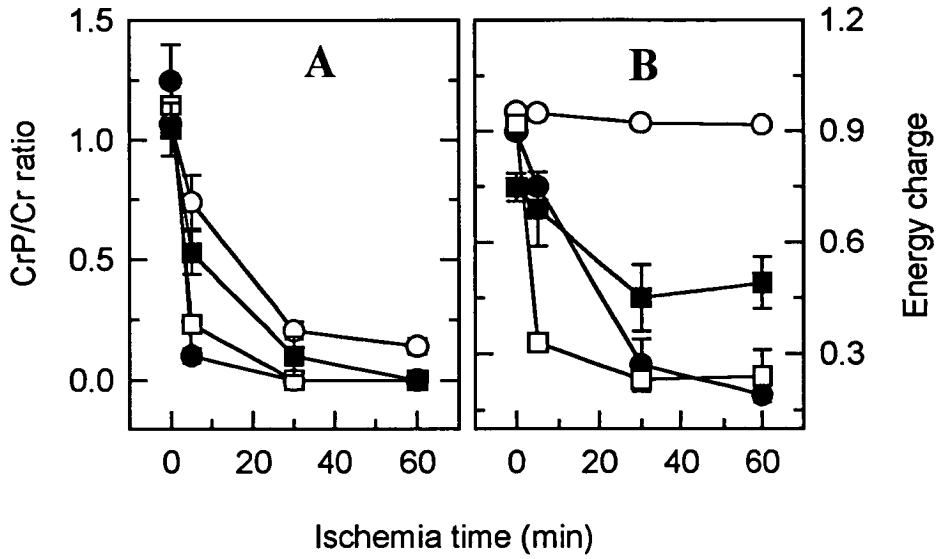

Fig. 7-4 Comparison between CrP/Cr ratio and EC in different muscle tissues during ischemia. Panel A shows CrP/Cr ratio; panel B shows EC. ○: skeletal muscle (n=4-5), ●: heart (n=4-6); □: intestine (n=4-6) and ■: aorta (n=4-5). Data are shown as means ± SE. The actual values were shown in Figs. 7-2 and 7-3.

7.3.5 Changes in lactate and glycerol during ischemia

Under *in vivo* aerobic conditions, lactate levels in striated muscles (9.31 ± 0.89 and 6.20 ± 0.83 μmol/g dry weight for skeletal muscle and heart, respectively) were about half of those in the smooth muscles, being 19.74 ± 2.15 and 16.90 ± 1.97 μmol/g dry weight for intestine and aorta, respectively, (Table 7-1). After ischemia, lactate accumulated at a wide diversity of rates (Fig.7-5A). In the intestine, lactate rose at the fastest rate among all the tissue tissues with 75% of the maximum occurring at 5 min of ischemia and reached the plateau (69.6 μmol/g dry weight, 3.5 times of the basal) between 10 to 30 min. Similarly, lactate also increased quickly and to a larger magnitude in the heart with an almost 15-fold increase over the basal at 5 min (55% of the maximum), and reached a plateau around 30 min with a value of 175 μmol/g dry weight, 28 times that of the nonischemic control.

In skeletal muscle, lactate increased to a large magnitude but at rather a slow rate. At 60 min of ischemia, it amounted to 97.3 μmol/g dry weight and the plateau of around 210 μmol/g dry weight (23 times of the basal) occurred at 120 min (Fig.7-8A), at which 95% ATP has been consumed (Fig.7-7A). In aorta, lactate accumulated

at a rather quick rate but to a lesser magnitude than the other three muscles mentioned above. Its maximal values amounted to about 30 μmol/g dry weight at 30 min, no more than twice the initial level.

Fig.7-5B shows the scenario of glycerol, known as a catabolic product of triglyceride, in the time course of ischemia. It seemed to transiently decrease a little in all tissues followed by a slow and prolonged rebound without reaching a maximum within the period observed. After 60 min of ischemia, glycerol amounted to 2.5 and 3.0 μmol/g dry weight for the heart and intestine, respectively. Within this period, glycerol did not seem to change markedly in either skeletal muscle or aorta ($P>0.05$) but prolonged ischemia eventually resulted in significant buildup in glycerol in the skeletal muscle ($P<0.05$ in Fig. 7-8B).

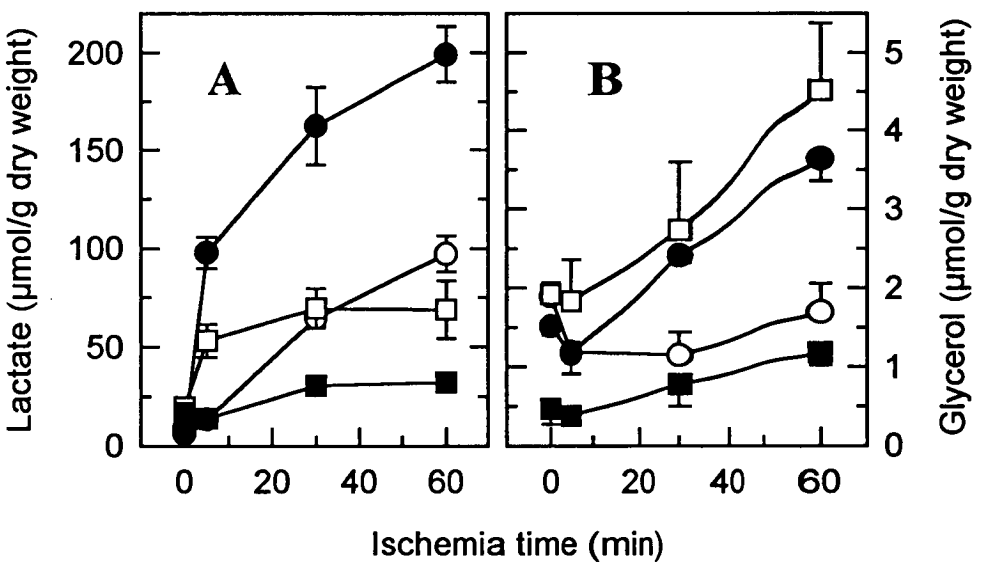

Fig. 7-5 Changes in lactate and glycerol of different muscle tissues during ischemia. Panel A: lactate; panel B: glycerol. ○: skeletal muscle (n=4-5), ●: heart (n=4-6); □: intestine (n=4-6) and ■: aorta (n=4-5). Data are means ± SE.

7.3.6 Effect of temperature on skeletal muscle metabolites during ischemia

Fig.7-6 illustrates the effect of temperature on creatine compounds in skeletal muscle during ischemia. Lowering temperature to 25°C led to a marked delay for the catabolism of CrP after 60 min of ischemia. The protective effect of lowering temperature on adenine nucleotides was even more evident at 2 hours of ischemia

(Fig.7-6). The accumulation of lactate and glycerol were also delayed at 25°C (Fig.7-7). However these delays at 25°C were no longer sustained at 5 hours of ischemia, except for glycerol. But the time at which 50% HEC depletion occurred could not be determined owing to the relative longer intervals set in the time course. It appeared that at least twice as long time was needed to reach the same degree of alteration. Clearly, each change observed at 37°C appeared similarly at 25°C only at a slower rate.

Throughout the period of ATP depletion, no substantial increment in AMP or adenosine was observed in skeletal muscle at either temperature. Associated with the degradation was a rise in inosine at 2 hours of ischemia and thereafter (37°C). The value of inosine (3.65 ± 0.2 μmol/g dry wt. at normal, n=5) started to increase after 1 hour of ischemia (4.95 ± 0.66 μmol/g dry wt., n=5, $P<0.05$) and attained plateau at 2 hour of ischemia (23.47 ± 1.70 μmol/g dry wt., n=4).

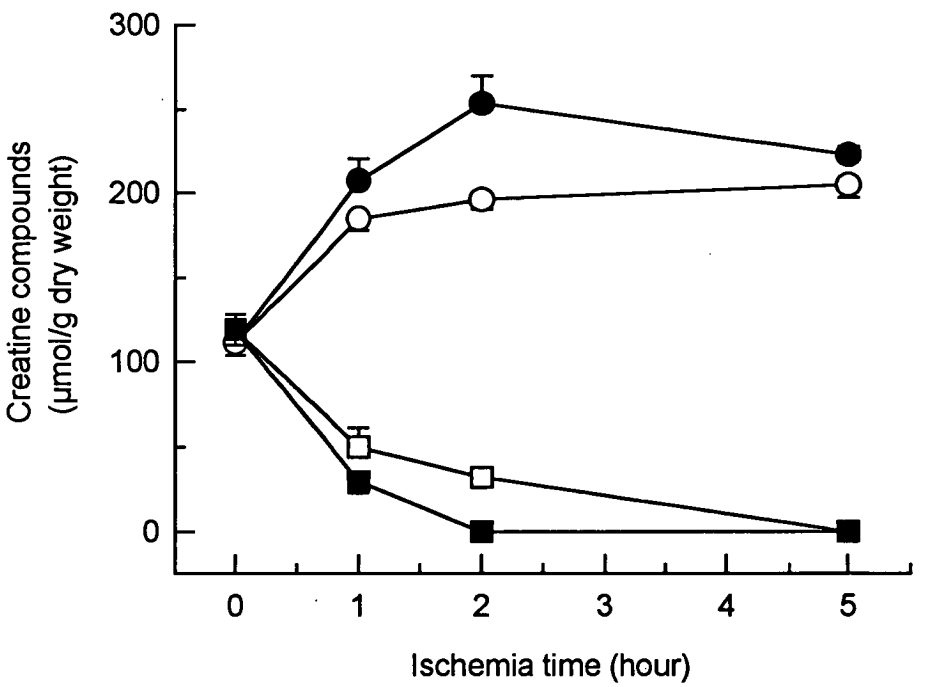

Fig. 7-6 Effect of temperature on the breakdown kinetics of CrP of skeletal muscle during ischemia. ●: Cr at 37°C, ○: Cr at 25°C, ■: CrP at 37°C, and □ CrP at 25°C. Data are means ± SE, n=4-6.

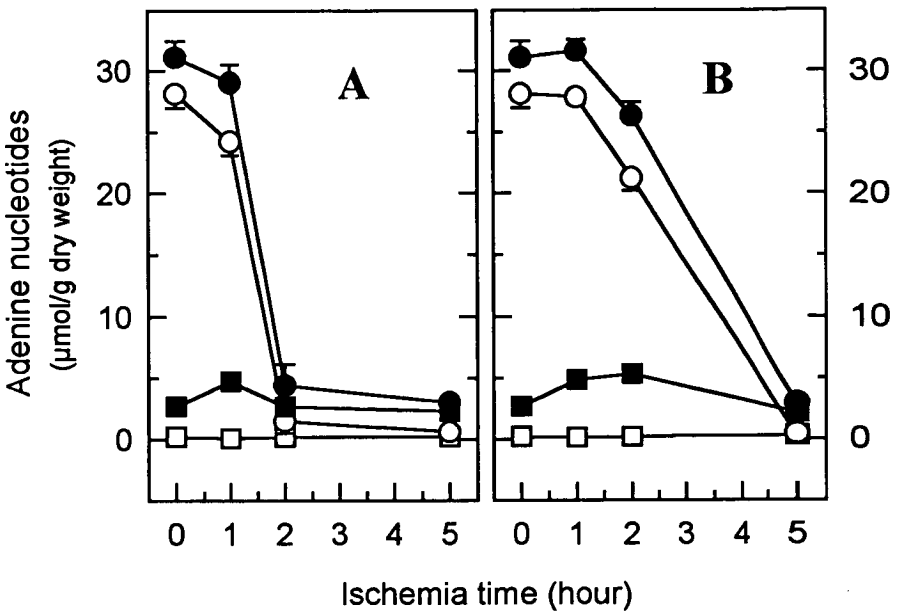

Fig. 7-7 Effect of temperature on the breakdown of adenine nucleotides of skeletal muscle during ischemia. Panel A: 37°C, and Panel B: 25°C. ●: total adenine nucleotides, ○: ATP; ■: ADP; □: AMP. Data are means ± SE, n=4-5.

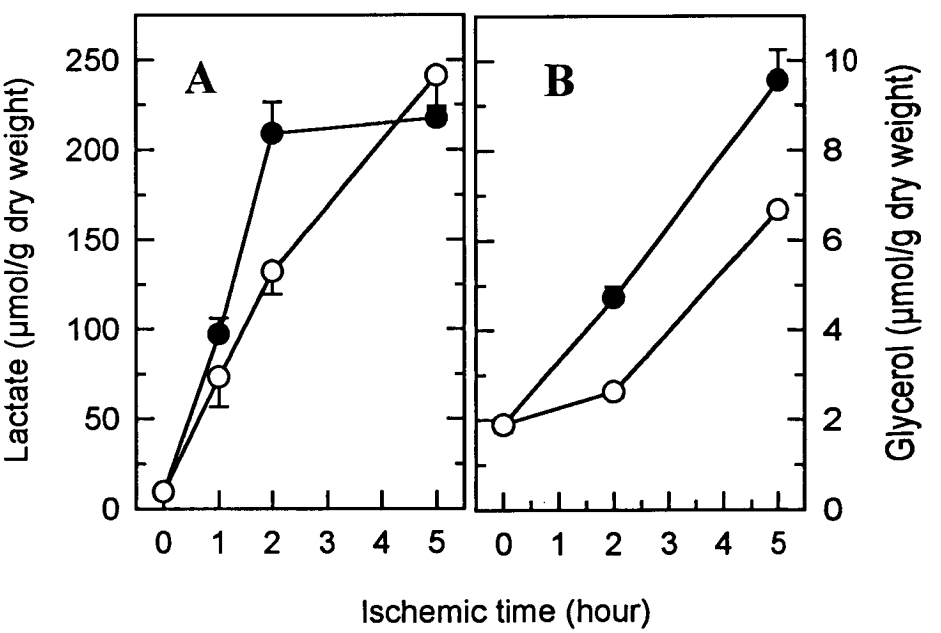

Fig. 7-8 Effect of temperature on the accumulation of lactate and glycerol of skeletal muscle during ischemia. ●: 37°C, and ○: 25°C. Data are means ± SE, n=4-6.

Table 7-2 Skeletal muscle metabolites in different $\dot{V}O_2$ status

	n	Lactate	Cr	CrP	Cr+CrP	AMP	ADP	ATP	ΣAD	EC	CrP/Cr	CrP/ATP
						μmol/g dry wt						
In vivo	5	9.3	111.6	119.3	230.9	0.23	2.69	28.2	31.10	0.95	1.07	4.24
		± 0.9	± 7.6	± 9.2	± 16.7	± 0.03	± 0.21	± 1.1	± 1.34	± 0.01	± 0.02	± 0.30
Ischemia	4	73.3**	199.1**	49.8**	248.9	0.15	4.69**	27.8	31.62	0.92	0.25**	1.79**
(60 min)		± 16.6	± 8.9	± 11.6	± 13.4	± 0.03	± 0.30	± 0.6	± 0.94	± 0.01	± 0.06	± 0.40
$\dot{V}O_2$ max.	4	3.1**	98.2	148.0*	246.0	0.21	2.97	30.4	33.6	0.95	1.51**	4.85
(20 min)		± 0.4	± 3.2	± 6.4	± 8.5	± 0.02	± 0.27	± 1.03	± 1.15	± 0.01	± 0.08	± 0.11

Both ischemia and maximal $\dot{V}O_2$ were performed at 25°C. The maximal $\dot{V}O_2$ was achieved by perfusing the hindlimb at 18.5 ml/min plus 50 nM NE.

EC: energy charge. ΣAD: sum of adenine nucleotides. Dta are presented as mean ± SE. $P>0.05$, ** $P<0.01$, vs *in vivo*.

7.3.7 Skeletal muscle metabolites during ischemia and hyperoxemia

This experiment was designed to assess the relationship between muscle thermogenesis and bioenergetic changes at rest. Data from 60 min ischemia at 25°C (no $\dot{V}O_2$) were compared with those from the muscle in the perfused hindlimb at the same temperature where $\dot{V}O_2$ was maximised, by a high flow plus NE, to 27.6 ± 1.2 $\mu mol \cdot g^{-1} \cdot h^{-1}$. After 60 min of ischemia, CrP dropped nearly 60 % with substantial accumulations in Cr, lactate and ADP (Table 7-2). However, AMP, ATP and ΣAD still remained at preischemic levels and EC was unaltered. For the muscle perfused at high flow plus NE, CrP elevated by approximately 24% above the value *in vivo* and was associated with a dramatic decrease in lactate. Cr tended to decline, hence leading to a significant increase in CrP/Cr ratio ($P<0.01$). As yet, none of the adenine nucleotides were significantly different from the *in vivo* values although ATP tended to slightly increase (Table 7-2).

7.4 Discussion

Creatine compounds and adenine nucleotides were simultaneously determined by HPLC in this experiment to compare the degradation features of HEC in four different muscle tissues. Lactate and glycerol were enzymatically measured to assess potential *de novo* synthesis of the energy compounds from glycolysis and lipolysis of triacylglycerol. In the heart and intestine, ATP immediately falls when blood flow to the tissues reduces (Zager, 1991; Canada *et al.*, 1993; Matherne *et al.*, 1993;). Since metabolites such as HEC (high energy phosphate compounds) and lactate are extremely sensitive to ischemia in the heart, this tissue was used as a positive control for other muscle tissues.

The results obtained from the normal tissues (Table 7-1) were comparable with reports for skeletal muscle of rats (Kushmerick *et al.*, 1992), rabbit (Pagliassotti & Donovan, 1990a), and dogs (Harris *et al.*, 1986) as well as human skeletal muscle taken by percutaneous needle biopsy technique (Harris & Hultman, 1992), for the rat heart (Shellevold *et al.* 1986; Janier *et al.*, 1993), and small intestine (Zager, 1991). The concents of HEC in the four tissues of muscles were in an order as follows: skeletal muscle>heart>intestine>aorta. Apart from aorta, which has been previously found to contain a lower level of ATP (Paul, 1980; Clark *et al.*, 1990), all other tissues showed a sound HEC profile with EC at 0.9 or above, suggesting that a

satisfactory sampling of heart, skeletal muscle and intestine has been achieved. The relative low ATP and CrP in aorta may result from the unavoidable time delay during sampling, but are comparable with the reports by blood vessels by Paul (1980).

The value of skeletal muscle CrP was higher than that previously measured with enzymatic methods (Colquhoun *et al.*, 1990; Chapter 3). There are apparently two reasons for this difference. Firstly, the methods employed were different. The procedures for the enzymatic assay of CrP usually took a longer time to complete and no recovery was used to correct the value. As the standard CrP in $HClO_4$ solution was found to decline approximately 10% in an hour (see Method), a longer procedure would be expected to result in a lower value. If no corrections for the time lapse had been applied, the value of CrP would be about 90 μmol/g dry wt. Secondly, the sampling procedure was improved by freeze-clamping *in situ* whereas the muscle was clamped after removal from the body in previous experiments (Chapter 3). As shown in Fig. 7-2A, CrP immediately decreased as a result of ischemia.

CrP immediately started to convert to Cr following the onset of ischemia in each tissue examined even though there were no significant changes in adenine nucleotides and lactate in some muscle tissues like skeletal muscle and aorta (Figs.7-2 & 7-3). In the heart and intestine, the fall in CrP was immediately followed by a drop in ATP and a rise in lactate ($P<0.05$ at 5 min) whereas in skeletal muscle and aorta, levels of adenine nucleotides fell more slowly. In skeletal muscle and aorta, neither ATP nor lactate were significantly different from the normal values after 5 min of ischemia ($P>0.05$). ATP was stable in skeletal muscle for at least 30 min following ischemia (Fig. 7-3A, $P>0.05$). Thus creatine compounds are more valuable measures to assess early ischemia in the tissues, as opposed to other HEC which are relatively imprecise measures of hypoxia in skeletal muscle and blood vessels. In heart and intestine all of the high energy metabolites and lactate are indicative of ischemia.

Among the normal muscle tissues, skeletal muscle contains highest levels of creatine compounds and ATP (Table 7-1) but the lowest basal metabolic rate (Paul, 1980; Chapter 4). Moreover, this high level of ATP is well conserved in a large diversity of metabolic conditions. An unchanged ATP content of skeletal muscle was found by ^{31}P nuclear magnetic (NMR) spectroscopy during 2 min electric stimulation following 6 min of ischemia even though more than 40 % of CrP was depleted (Blei *et al.*, 1993). In the present study, skeletal muscle showed a unique dynamic catabolism of adenine nucleotides. ATP dropped by only 14% from the initial level following the first hour of ischemia. This agrees with the observation in canine gracilis muscle, where ATP did not statistically change after 60 min of ischemia (Harris *et al.*, 1986), and in the rat hindlimb adductor muscle, where ATP levels were unaltered even after

90 min of abdominal aorta clamping (Yoshioka *et al.*, 1992). Unlike that in other tissues, AMP did not increase significantly during the degradation of ATP throughout the period (Fig.7-3A and 7-7A) observed. In parallel with the fall in ATP there was a substantial accumulation of inosine. These results are consistent with the data from Terjung's group (Arabadjis *et al.*, 1993) who showed that once AMP is formed in skeletal muscle it will almost exclusively be deaminated into IMP rather than be dephosphorylated to adenosine. In contrast to the heart and intestine where ATP is almost entirely depleted after 1 hour of ischemia, ATP still remained at 86% and 52% of the pre-ischemic level even in skeletal muscle and aorta, respectively.

Deamination rather than hydrolysis of adenine nucleotides of skeletal muscle may imply a physiological significance. For example, adenosine is a potent vasodilator which modulates local vascular function to meet the O_2 demand in most tissues. When coronary blood flow decreases, adenosine will rise as a result of adenine nucleotide breakdown. The increased adenosine will then act as a local metabolic factor to dilate the blood vessel to increase the coronary flow so that the heart will receive more O_2 and nutrient (Schrader & Deussen, 1988). In addition, recent studies have provided strong evidence that adenosine possesses protective effect on heart during ischemia by acting on its specific receptor (Janier *et al.*, 1993). However, the vasodilatory effect of adenosine as a local metabolic control of microcirculation in skeletal muscle is still doubtful (Mohrman, 1991). Adenosine is rarely seen in the effluent from the perfused rat hindlimb during ATP-induced release of adenine nucleotide downstream products (Richards *et al.*, 1993). These data together with the present results suggest that adenosine is unlikely to play an important role as a local metabolite to control blood flow in skeletal muscle vascular bed. So far, no report has been found in the literature which shows metabolites, such as IMP and inosine, from the deamination of adenine nucleotides have similar properties in skeletal muscle to those of adenosine in the heart. If the deaminated metabolites have vasoactive action or direct effect on skeletal muscle metabolism, they may influence muscle thermogenesis.

The relatively stable ATP content in the skeletal muscle during hypoxia may be attributable to its lower resting metabolic rate, stronger buffer by a high storage of CrP (the CrP/ATP ratio is 4.24 ± 0.30; Table 1), and lasting compensation from *de novo* synthesis via glycolysis as indicated by the large amount of lactate accumulation (Fig 7-5A and 7-8A). This argument was supported by the data in Fig 7-6 and 7-7A at 60 min interval that shows ATP level depleted quickly soon after the CrP/ATP fell to approximately 1.2 (as seen in normal heart and intestine, and diminished when glycolysis was exhausted at 120 min (indicated by the plateau of lactate accumulation in Fig 7-8A). The slower depletion of HEC and lower energy demand provide skeletal muscle with biochemical mechanisms for better tolerance to ischemia.

As expected, in the development of ischemia, lactate accumulated (Fig.7-5A), reflecting the capacity of glycolysis to generate HEC *de novo*. The accumulation of lactate amounted to a much higher level (20-30 fold of the basal) in the striated muscle tissues than in the smooth muscle tissues (2-3 fold of their basal) although lactate levels in the intestine and aorta were both similarly twice as high as those of the heart and skeletal muscle under non-ischemic conditions. The actual accumulation level of lactate in the plateau (ie. after 60 min of ischemia) was 4-7 times as high in the striated muscles as in the smooth muscle tissues (Fig. 7-5A). The maximal lactate in the present study is similar to the report (175 μmol/g dry wt) by Harris (1986) in ischemic skeletal muscle. In general, the accumulation of lactate paralleled well with the depletion of ATP and changed earlier in skeletal muscle and aorta. Indeed, after 5 min of ischemia, skeletal muscle lactate increased by 48% above the basal but did not reach statistical difference because of bigger standard deviation ($P>0.05$).

It is interesting to note that glycerol also gradually accumulated at a slow rate in the tissues during the period of ischemia after a transient decrease or unaltered period (Fig.7-5B). The transient decrease or unaltered glycerol might be due to the oxidation of this metabolite by the residual myoglobin-bound O_2 in the tissues through tricarboxylic acid cycle in mitochondria before new glycerol is formed. Similarly, an increase in glycerol release was shown in canine gracilis during reperfusion following two hours of ischemia (Harris *et al.*, 1986). So far, the mechanism for increased glycerol has not been fully understood. In the heart, hypoxia is thought to stimulate the release of endogenous catecholamines which increase glycerol production by activating lipolysis (Karwatowska-Krynska & Beresewiez, 1983). However, neither β -adrenergic antagonist nor pretreatment with 6-hydroxydopamine failed to completely inhibit hypoxia-stimulated glycerol production (Wardle & Riemersma, 1994). In the perfused rat hindlimb, infusion of lactate significantly increased glycerol release (Chin *et al.*, 1991). Furthermore, the vasoconstrictor-altered glycerol parallels the changes in lactate release (Clark *et al.*, 1994; Chapter 4). It is possible that the increased glycerol results from the enormous accumulation of lactate in ischemic tissues. This explanation is consistent with the evidence that an increase in glycerol is dependent on an intermediate of glycolysis, such as glycerol-3-phosphate, in the ischemic or hypoxic rat heart (Trach *et al.*, 1986 and Wardle & Riemersma, 1994). In addition, phospholipid breakdown may also contribute to the increase in glycerol after a long period of ischemia.

Surprisingly, the bioenergetic kinetics changed faster in the intestine than in the heart. It would *a priori* appear more reasonable to expect a reverse result based upon the their basal metabolic rates. Comparison of the lactate time course during ischemia revealed a great distinction between these two tissues. Lactate accumulated

very quickly in both tissues, but it reached the plateau earlier in the intestine than in the heart (Fig.7-5A). In addition, the maximal value was four times as much in the heart as in the intestine, suggesting a greater compensation for HEC by *de novo* synthesis through glycolysis in the heart during ischemia. In contrast to the skeletal muscle, ADP and AMP, in particular, elevated enormously in the heart and intestine when ATP declined.

The results in Table 7-1 show that aorta possessed the lowest concentrations of CrP, ADP and ATP, consistent with our earlier findings that shows lower concentration of ATP but higher UTP in the rat blood vessel (Clark *et al.*, 1990). These data suggest that blood vessels have very little energy buffer and are more reliant on an external nutrient supply. When subjected to ischemia, HEC, lactate and glycerol staged a rather smooth and gradual change at a rate between intestine and skeletal muscle. However, it should be borne in mind that the aorta was relieved from blood pressure stretch in the present ischemic model, whereas the heart and intestine were still working under the control of autorhythmic system.

Temperature has an important influence on metabolic rate and a Q_{10}= 2.5 has been widely used to estimate metabolic rate from those obtained at various temperatures (Schmidt-Nielsen, 1974; Bonen *et al.*, 1994). The results from Figs. 7-6, 7-7 & 7-8 show that HEC were depleted much slower at 25°C than at 37°C. The measured metabolites differed significantly after 2 hours of ischemia but not within the first hour: This is probably because these metabolites, especially CrP declined at a faster rate in the earlier stage of ischemia (Harris *et al.*, 1986) when the rat temperature had not cooled down. Nonetheless, these results indicate that at least twice as long a time is needed to degrade HEC at 25°C as at 37°C.

In terms of the mechanism of vasoconstrictor-induced muscle thermogenesis, the present experiment confirms the previous argument that the vasoconstrictors-mediated increase in $\dot{V}O_2$ in the perfused hindlimb and other tissues does not result from attenuating ischemia or hypoxia (Chapter 3). If there was hypoxia or ischemia in a perfused preparation a marked drop in CrP or rise in Cr would be expected. As to vascular oxygen consumption, the energetic metabolites seem to change rapidly in the early stage of ischemia and then subsequently maintained a rather slow rate of degradation (Figs. 7-2, 7-3, 7-4). In aorta, The poor energy charge of adenine nucleotides (due to the delay in sampling; Table 7-1) and earlier plateau in lactate accumulation (Fig. 7-5C) suggest that the energetic turnover of blood vessels is higher than that of skeletal muscle in resting conditions and could be expected to be even higher when stimulated.

Although the present experiment did not show direct evidence for the hypothesized oxidative phosphorylation uncoupling as the possible mechanism of skeletal muscle thermogenesis, it provides relevant information for further investigating the mechanism. Firstly, degradation kinetics of adenine nucleotides in the skeletal muscle behaves very differently from those in BAT reported by this laboratory (Matthias *et al.*, 1994) where ATP depletes very quickly associated with dramatic rises in both ADP and AMP. Therefore, if there is an uncoupling process induced by vasoconstrictors in skeletal muscle, the control mechanism might also differ from that in BAT. Secondly, in searching for the uncoupling or recoupling regulators, there should be certain criteria: (1) the candidate messenger should be able to either start or stop the reactions directly related to the uncoupling process; (2) the candidate messenger should exist in skeletal muscle; (3) it should increase or decrease to reflect the thermogenic status of the tissue in order to switch on or off the uncoupling process. Neither ATP nor AMP fits in the third criterion. Other metabolites, such as CrP, Cr, ADP or lactate, may have a role to play in regulating muscle thermogenesis because they are the inherent compounds on the metabolic pathway and responded to muscle energetic status (Table 7-2). This argument is supported by the data in Table 3-2 (Chapter 3) which show that lactate/pyruvate ratio decreases whilst CrP tends to increase when NE stimulates $\dot{V}O_2$ in the constant-flow perfused hindlimb.

Above all, the tolerance of resting skeletal muscle to ischemia seems to suggest that its energy demand could be strongly compensated for by the large amount of HEC or switched into a partial arrest state when blood flow to the tissue is restricted. On the other hand, skeletal muscle metabolic rate can be accelerated far beyond its basal demand by increasing perfusion flow or type A vasoconstrictors (Clark *et al.*, 1994; Chapters 3, 4 & 6) to generate heat. Skeletal muscle thermogenesis is somewhat independent of its energy charge, which normally exerts strong controls of the metabolic rate (Atkinson, 1968). Skeletal muscle is the largest tissue in the body, yet it contains the highest level of HEC. The tolerance to ischemia suggests that the energy demand of this tissue at rest is very low. Thus most of the increased $\dot{V}O_2$ by vasoconstrictors or/and flow rate (as shown in Chapters 3, 4 & 6) could be expected to contribute to NST. The degradation profiles of aorta, such as quickly declined energy charge and earlier plateau of lactate and CrP/CR ratio, suggest that blood vessel it is an energetically active tissue with a quick turnover rate of metabolites even under unstretched conditions. It can be anticipated that more muscular vessels such as smaller arteries will be more energy consuming at rest and even more so when stimulated.

Chapter 8

Effect of adrenergic agents on whole body thermogenesis of intact bettongs

8.1 Introduction

Vasoconstrictors have been shown to mediate control of $\dot{V}O_2$ in perfused hindlimbs of rats, bettongs and toads (Chapters 3, 4 & 6). Such a control also takes place in other perfused tissues such as the rat kidney, intestine (Chapter 5). These data suggest that the vasoconstrictor-mediated control of $\dot{V}O_2$ may play an important role in regulating whole body thermogenesis.

In the literature, sympathetic hormones have been shown to stimulate thermogenesis in humans (Lundholm & Svedymr, 1965; Astrup *et al.*, 1985; Blaak *et al.*, 1993), rodents (Foster & Frydman, 1978), rabbits (Szreder, 1991), bats (Hayward & Ball, 1968), and marsupials such as the potoroo, (Nicol, 1978). However, the increased thermogenesis has been attributed to either BAT (Flaim *et al.*, 1977; Foster, 1984, Rothwell, 1990), or to skeletal muscle and mediated by β-adrenergic receptors (Astrup *et al.*, 1984; 1985; 1989; Thurlby & Ellis, 1986; Blaak *et al.*, 1993).

Although Loudon *et al.* (1985) reported the presence of BAT in the pouched-young wallaby (*Macropus rufogriseus*), most studies indicate that marsupials (including the bettong) do not have histological BAT (Nicol, 1978; Hayward & Lisson, 1992). In addition, attempts to detect thermogenin (UCP) mRNA in this species have been unsuccessful (Hayward & Lisson, 1992). Thus, the potential lack of BAT makes the bettong a valuable animal model to study whole body thermogenesis derived from other tissues.

In the constant-flow perfused bettong hindlimb (Chapter 4), NE can mediate $\dot{V}O_2$ via α-receptors, similar to that in the constant-flow perfused rat hindlimb (Colquhoun *et al.*, 1988; 1990). Moreover, in the constant-pressure perfused hindlimb, both α- and β adrenergic receptors mediate $\dot{V}O_2$. In order to further assess vascular-mediated control of thermogenesis *in vivo* and the mechanism involved, this chapter examined the effect of NE on the metabolic rate in the conscious bettong. Both α- and β- adrenergic agonists were used to identify the adrenergic receptors involved. Comparisons were made with the rat to estimate muscle contribution to whole body thermogenesis.

8.2 Methods

A limited number of bettongs (body weight: 1.67 ± 0.10 kg) were obtained from the University's breeding colony by kind permission of Dr. R.W. Rose, Department of Zoology, University of Tasmania. Whole body heat production was calculated from O_2 uptake ($\dot{V}O_2$) measured at 25°C under conscious conditions by an indirect calorimeter according to the method of Weir (1949). This temperature is within the thermoneutral zone for both marsupials (Nicol, 1976, Wallis *et al.*, 1992) and the rat (Foster & Frydman, 1979, Maxwell *et al.*, 1987). Drugs were freshly made in sterile 0.9 NaCl solution. For the administration of drugs to the bettong, a venous indwelling cannula (I.D. 0.8 × 25 mm) was inserted into the tail vein with 0.9% NaCl solution continuously infused by a pump (LKB, Bromma) at a flow rate of 0.02 $ml \cdot kg^{-1} \cdot min^{-1}$. Usually, animals had settled down within 15 min, but 30 min was allowed to elapse before collecting data. The details of the methods were described in Chapter 2 (Sections 2.1 & 2.4).

For the calculation of heat production from $\dot{V}O_2$, the standard average (4.83) of energy (kcal) from mixed food per liter $\dot{V}O_2$ (4.83 kcal=1 liter $\dot{V}O_2$, Brown & Grengelmann, 1965) was used. Thus:

$$1\ kJ \cdot kg^{-1} \cdot day^{-1} = \frac{1{,}000\ O_2}{4.184 \times 4.83 \times 24}\ \dot{V}O_2\ ml \cdot kg^{-1} \cdot min^{-1}$$

$$= 0.0344\ \dot{V}O_2\ ml \cdot kg^{-1} \cdot min^{-1}$$

$$= 0.092\ \dot{V}O_2\ \mu mol \cdot g^{-1} \cdot h^{-1}$$

8.3 Results

8.3.1 Resting metabolic rate for the conscious rat and bettong

Under the resting state, the measured metabolic rates were 249 ± 21 $kJ \cdot kg^{-1} \cdot day^{-1}$ and 566 ± 39 $kJ \cdot kg^{-1} \cdot day^{-1}$ for bettongs (n=7) and rats (n=14), respectively. These values are comparable with the data reported in literature for the same or similar species (see Table 1). The calculated results in $kJ \cdot kg^{-0.75} \cdot day^{-1}$ show that the resting metabolic rate of the marsupial (bettong) was lower than eutherian mammals (as shown in Table 8-1).

Table 8-1 Comparison of the resting metabolic rates of the bettong and rat with the values reported for relevant species

Author	Species	Body weight	$\dot{V}O_2$ ($ml \cdot kg^{-1} \cdot min^{-1}$)	Heat production ($kJ \cdot kg^{-1} \cdot day^{-1}$)	Heat production ($kJ \cdot kg^{-0.75} \cdot day^{-1}$)
	Marsupials				
Present study	Bettong	1.7 ± 0.10 kg	8.6 ± 0.7	249 ± 21	285 ± 21
Wallis *et al.*, 1992	Bettong	1.1 ± 0.03 kg	9.6	279	283
Wallis *et al.*, 1992	Potoroo	1.0 ± 0.03 kg	8.4	245	241
Wallis *et al.*, 1992	Aepyprymn	3.0 ± 0.16 kg	7.1	205	270
Nicol, 1976	Potoroo	~ 1.12 kg	7.5 ± 1.2	218 ± 34	224 ± 35
Dawson *et al*, 1982	Kowari	115 ± 12.0 g	10.8 ± 1.0	313 ± 29	184 ± 15
	Eutherians				
Present study	Rat	250 ± 10.2 g	19.0 ± 1.3	566 ± 39	400 ± 28
Dawson *et al*, 1982	Mouse	79.8 ± 6.9 g	17.5 ± 8.3	509 ± 24	296 ± 29
Foster *et al.*, 1979	Rat	~ 370 g	16.5 ± 0.2	480 ± 6.0	374 ± 4.6
Maxwell *et al.*, 1987	Rat	~ 110 g	26.4 ± 4.2	769 ± 122	443 ± 70
Gonzalez *et al.*, 1971	Rabbit	2 ~ 4 kg	9.2 ~ 9.5	269 ~ 277	353 ~ 365
Liard, 1989	Dog	20 ± 0.9 kg	6.4 ± 0.7	186 ± 20	394 ± 40

Data are presented as mean ± SE.

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8.3.2 Effects of adrenergic agonists

The resting metabolic rate of the conscious bettong was generally steady and intravenous infusion of 0.9% NaCl did not cause any marked change (Table 8-2). The infusion of norepinephrine (NE) stimulated heat production markedly (Fig. 8-1, top). The response was rapid in onset and was sustained for 30-60 min after termination of the infusion. Administration of isoproterenol elicited a prolonged response in thermogenesis that was usually sustained longer after the removal of infusion (Fig. 8-1, middle). More interestingly, phenylephrine (α_1-adrenergic agonist) also elicited significant heat production (Fig. 8-1, bottom).

The dose range for intravenous infusion of NE or phenylephrine to stimulate metabolic rates of the conscious bettong were between 10-80 $\mu g \cdot kg^{-1} \cdot min^{-1}$ (Fig. 8-2). Beyond 80 $\mu g \cdot kg^{-1} \cdot min^{-1}$, some bettongs became unsettled. At the same doses, NE-mediated increase in thermogenesis appeared to be bigger than that produced by phenylephrine but the difference was not statistically significant within the number of bettongs tested. The peak values of heat production in response to the stimulation of NE and phenylephrine reached 49 ± 15%, and 32 ± 7% above the resting basal value, respectively (shown in Table 8-2).

Table 8-2 Maximal metabolic rates timulated by adrenergic agonists on the bettong

Basal (n=7)	NS (n=7)	NE (n=2)	Iso. (n=3)	Phenyl. (n=4)
249 ± 21	252 ± 5.6	373 ± 57#	355 ± 39**	342 ± 26**
		48 ± 15%↑	42 ± 15%↑	32 ± 6%↑

The drugs were infused intravenously at a rate of 80 $\mu g \cdot kg^{-1} \cdot min^{-1}$. 0.9% NaCl (NS) was infused as the control vehicle at a similar flow rate of 0.02 $ml.kg^{-1}.min^{-1}$. Phenyl: phenylephrine, Iso: isoproterenol. Metabolic rate is expressed as $kJ \cdot kg^{-1} \cdot day^{-1}$. Increment percentage is also shown. Data are mean ± SE. # $P<0.1$; ** $P<0.01$ vs 0.9% NaCl infusion, paired *t*-test.

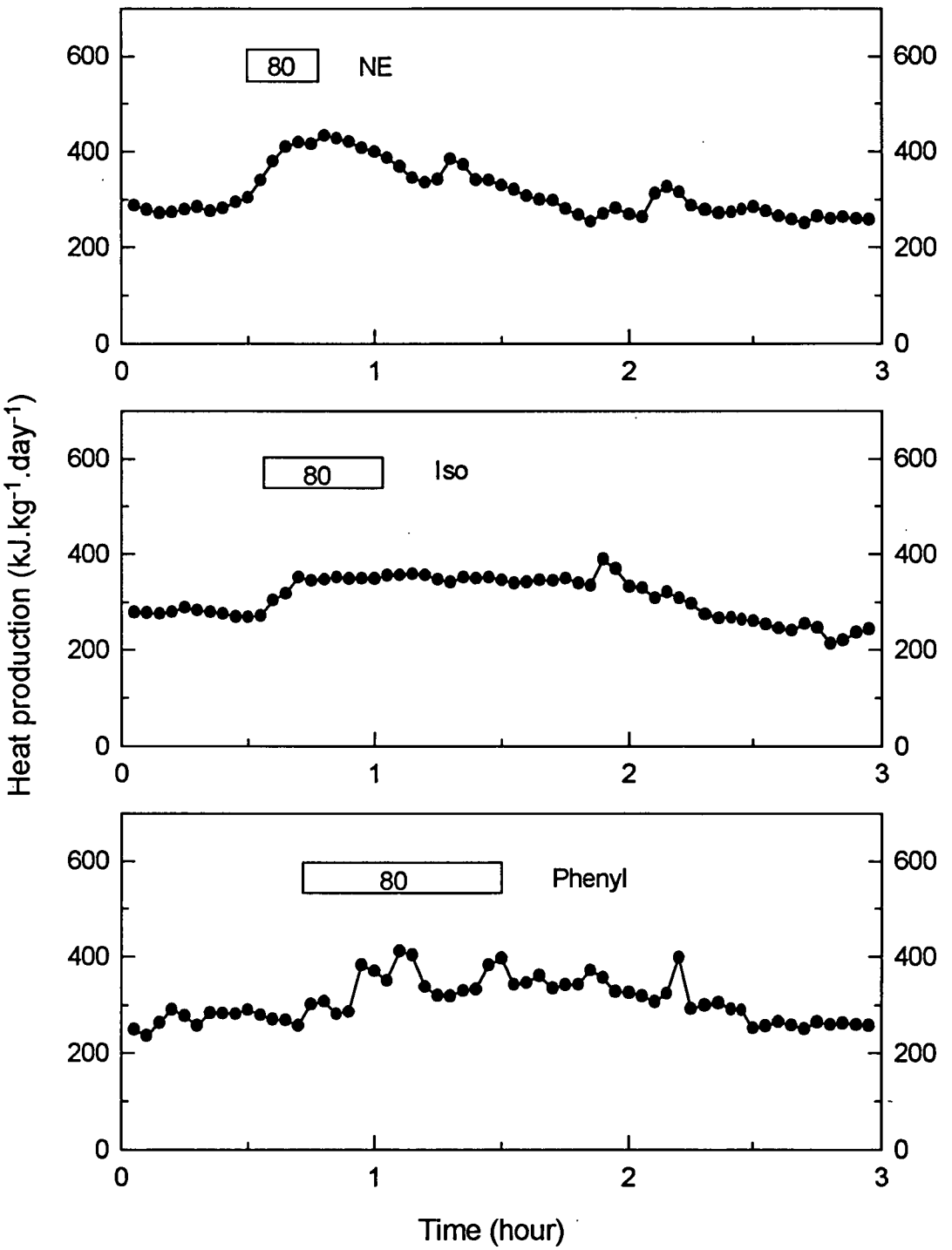

Fig. 8-1 Representative tracings for the of NE, isoproterenol and phenylephrine on heat production of intact bettongs. Each dot was the average value for 3 minute. The drugs were infused via an indwelling catheter in the tail vein. Iso: isoproterenol, Phenyl: phenylephrine. 80: the infusion rate of agonists ($\mu g \cdot kg^{-1} \cdot min^{-1}$)

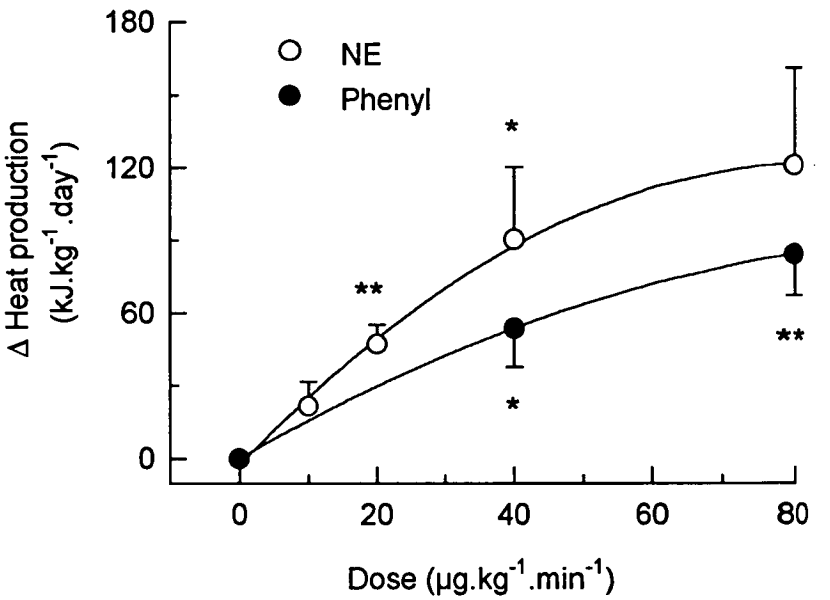

Fig. 8-2 Dose-response curves for the effect of NE and phenylephrine on heat production of intact bettongs. Data are means ± SE, n=2-4 for NE, n=3-4 for phenylephrine (Phenyl). * P<0.05; **P<0.01, paired *t* test.

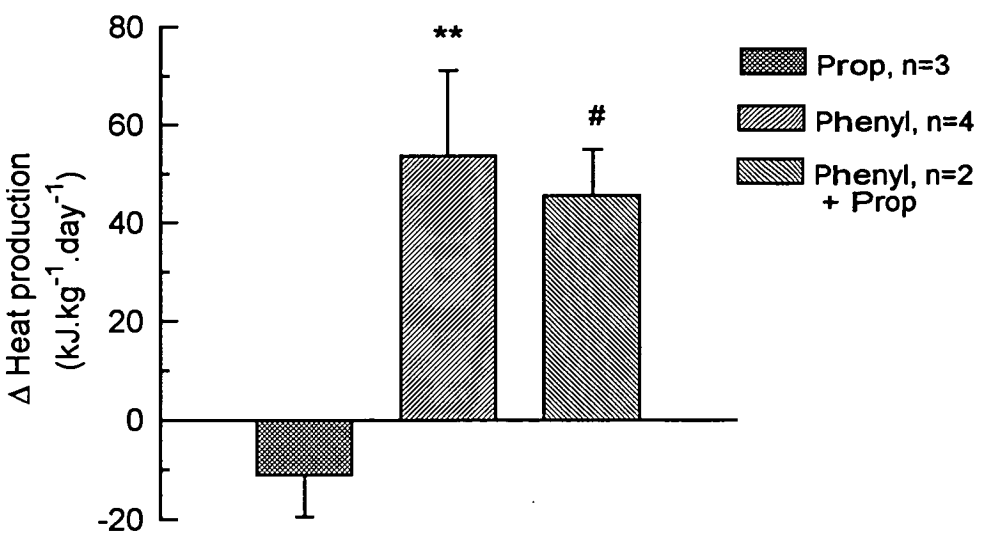

Fig. 8-3 Effect of phenylephrine on bettong heat production in the presence of propranolol. Phenyl (phenylephrine, 40 μg·kg^{-1}·min^{-1}), Prop (propranolol, 20 μg·kg^{-1}·min^{-1}) were infused from the tail vein. ** P<0.01, # P<0.1; paired *t* test, vs the control (0.9% NaCl).

8.3.3 Effects of adrenergic antagonists

Fig. 8-3 shows the effect of propranolol, a β-adrenergic antagonist, on the bettong heat production mediated by phenylephrine. The infusion of propranolol did not block phenylephrine-induced heat production. The α-adrenergic stimulation of heat production was also observed during the administration of naphazoline, an $\alpha_{1,2}$-adrenergic agonist (Foster, 1984). In contrast, the heat production mediated by either naphazoline or phenylephrine was significantly inhibited by the $\alpha_{1,2}$-adrenergic antagonist, phentolamine (Fig. 8-4).

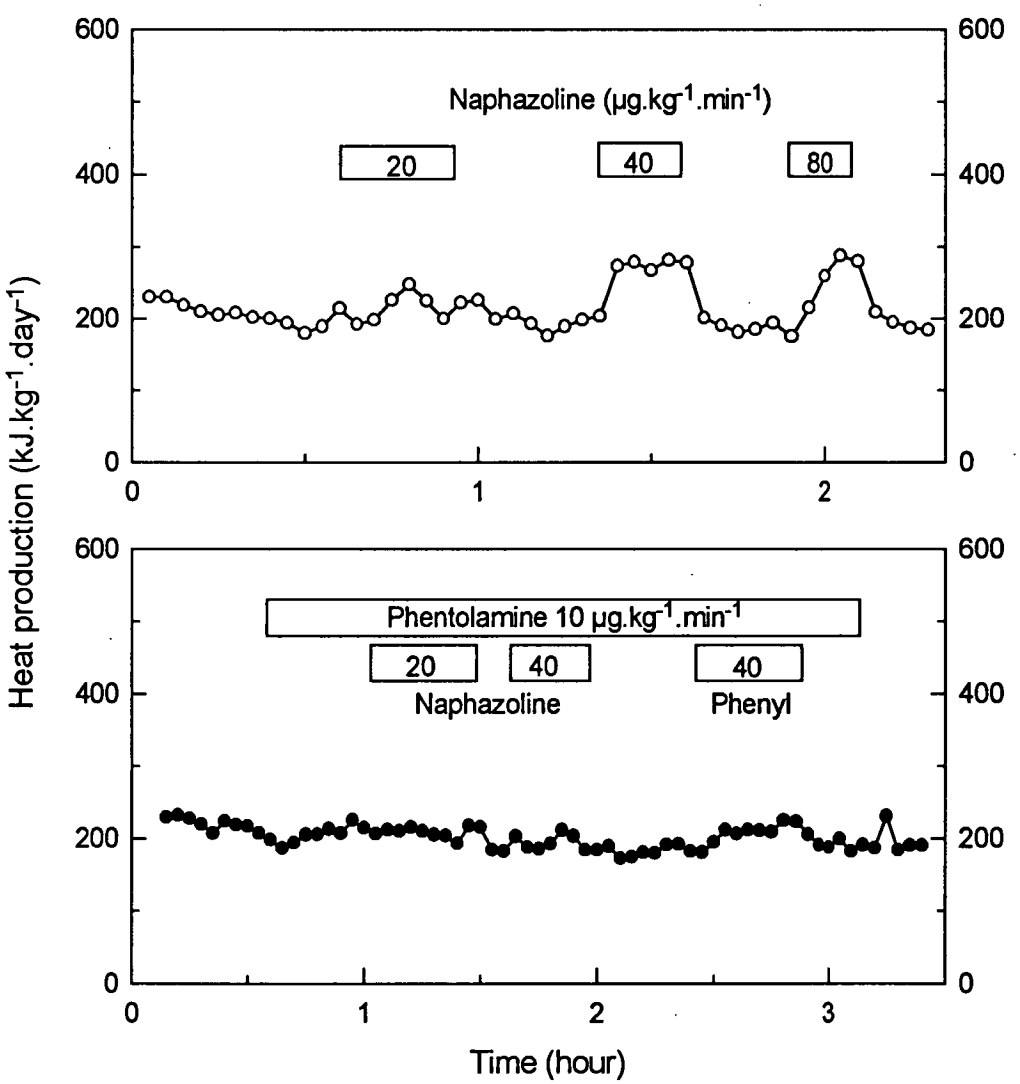

Fig. 8-4 Effect of naphazoline on bettong heat production and inhibition of phentolamine on the action of naphazoline and phenylephrine. Phenyl: phenylephrine. The numbers in the bars represent the infusion rate of drugs (μg·kg^{-1}·min^{-1}, n=1)

8.4 Discussion

Marsupials are capable of maintaining a stable body temperature in variable field temperatures. They have a thermoneutral zone around 20-25°C (Nicol, 1976). When exposed to the cold, the maximal heat production of marsupials can match or exceed that of the rodents of a similar size, although the resting metabolic rate of marsupials is lower (Dawson & Dawson, 1982). Careful histological analyses suggest that marsupials including the bettong are devoid of BAT (Hayward & Lisson, 1992). Molecular biology studies have not identified the expression of UCP in the bettong (Hayward & Lisson, 1992). An intensive search for UCP mRNA in bettong adipose tissues from the interscapular, periaortic, pericardial, peri-renal, and intestinal as well as from brain, heart, liver, kidney, intestine, and testis was unable to identify the existence of BAT in this species (Trayhurn, personal communication, 1994). It is obvious that other tissues must be responsible for the increased thermogenesis, and compensate for the contribution made by BAT in eutherian mammals.

The resting metabolic rates of 566 $kJ \cdot kg^{-1} \cdot day^{-1}$ for the rat and 249 $kJ \cdot kg^{-1} \cdot day^{-1}$ for the bettong were similar to the reports in the literature for the same or similar species (Table 8-1). A direct comparison in the unit of $kJ \cdot kg^{-1} \cdot day^{-1}$ shows that the resting metabolic rate of the bettong was only 44% that of the rat. When taking into account the body sizes by converting the unit into $kJ \cdot kg^{-0.75} \cdot day^{-1}$ as recommended by International Union of Physiological Sciences (Bligh & Johnson, 1973), the bettong resting metabolic rate was 285 $kJ \cdot kg^{-0.75} \cdot day^{-1}$, approximately 70% of the rat (400 $kJ.kg^{-0.75}.day^{-1}$). These results indicate that the resting metabolic rate of marsupial bettongs is also lower than most of the reports in literature for other eutherian mammals of a similar or bigger size, such as the rabbit (Gonzalez *et al*, 1979) and dog (Liard, 1989). These results are consistent with the earlier observations in dasyurid marsupials by Dawson & Dawson (1982) who found that the basal metabolic rate of small marsupials is lower than that of rodents of similar size.

It has been noted that the body temperature of marsupials is on average 1-2 ° C lower than eutherians (Schmidt-Nielsen, 1975; Hales & Rose, 1988). The lower metabolic rate, the lower body temperature and relatively lower muscle $\dot{V}O_2$ (compared with rats, Chapter 4) would appear internally consistent for the bettong. Thus, a close correlation appears to exist between muscle $\dot{V}O_2$ and whole body thermogenesis in this species.

In the perfused bettong hindlimb at 25°C, the measured basal $\dot{V}O_2$ was 4.2 $\mu mol \cdot g^{-1} \cdot h^{-1}$ (Chapter 4). Assuming that Q_{10}=2.5, this value would account for 14 μ

mol·g^{-1}·h^{-1} at 37°C. In the red and grey kangaroos skeletal muscle accounts for 52% of body mass and is proportionally more than that in other mammals (Tribe & Peel 1963). If this value (52% of the body mass as skeletal muscle) applies to the bettong and the muscle $\dot{V}O_2$ obtained from the perfused hindlimb (Chapter 4) represents the value of the entire muscle on average, the total muscle $\dot{V}O_2$ of the bettong would be equal to 12.2 mmol/h. This would account for 32% of the resting metabolic rate (whole body) of 249 kJ·kg^{-1}·day^{-1} (38 mmol/h) *in vivo*. By a similar estimate, 6.4 μ mol·g^{-1}·h^{-1} of rat skeletal muscle $\dot{V}O_2$ at 25°C (Colquhoun *et al.*, 1988 & 1990, Hettiarachchi *et al.*, 1992) would be equal to 27.7 μmol·g^{-1}·h^{-1} at 37°C. This value would make up to 2.98 mmol/h of $\dot{V}O_2$ for the whole body muscle provided that the muscle mass accounts for 43% body weight (Grubb & Folk, 1976). Thus 2.98 mmol/h of the whole body muscle $\dot{V}O_2$ would account for 23% of the whole rat resting metabolic rate of 566 kJ·kg^{-1}·day^{-1} (13 mmol/h) *in vivo*. Such a comparison suggests that skeletal muscle contributes more to the resting metabolic rate in the bettong than in the rat.

However, it should be pointed out that this estimate from the muscle $\dot{V}O_2$ *in vitro* is likely to discount the actual totality of muscle contribution to whole body thermogenesis *in vivo*. For instance, the circulating catecholamines and possibly other thermogenic hormones, such as thyroid hormones and insulin can increase the muscle $\dot{V}O_2$ *in vivo* even at rest. In addition, the respiratory movements and posture maintenance would not allow all the muscles in a fully relaxed state under conscious conditions. By contrast, the basal $\dot{V}O_2$ by perfused muscle was obtained in an entirely relaxed state with no circulating hormones (Colquhoun *et al.*, 1988 & 1990, Hettiarachchi *et al.*, 1992; Chapter 4). Accordingly, the actual muscle $\dot{V}O_2$ *in vivo* could be expected to account for a higher contribution of body metabolic rate than the calculated values above. Nonetheless the comparative estimates would be expected still to be valid under the same conditions for the bettong and the rat.

In the red kangaroo, the blood flow to the muscle varies considerably in response to the environmental temperature. The distribution of cardiac output to skeletal muscle increased 5 times at 7-10°C compared to its distribution at 33-37°C (Needham & Dawson, 1984). An increase in blood flow to the muscle would not only enhance muscle thermogenesis (Chapter 3), but would also provide more oxygen and nutrients to meet the increased metabolic demand and improve heat transport to other parts of the body (Trayhurn, 1994). Thus, these observations together with the results in the present study seem to suggest that skeletal muscle is relatively more thermogenic in marsupials than eutherians.

In constant-flow perfused hindlimbs of the rat and bettong (Colquhoun *et al.*, 1990; Hettiarachchi *et al.*, 1992; Chapter 4), NE-induced $\dot{V}O_2$ is mediated by α-adrenergic receptors. On the other hand, β-adrenoceptors were also found to mediate $\dot{V}O_2$ in the constant-pressure perfused rat hindlimb (Hardeveld *et al.*, 1980; Chapter 6). Consistent with the findings in the perfused hindlimb in earlier chapters (3, 4 & 6), both α- and β-receptors were found to be responsible for NE-stimulated heat production in the intact bettong, as shown by the stimulation of thermogenesis by isoproterenol and phenylephrine (Figs 8-1, 8-2; Table 8-2).

The effect of β-receptors in the NE-mediated elevation in heat production *in vivo* has been well established in the rat and human (Rothwell *et al.*, 1982; Astrup *et al.*, 1986, 1989; Himms-Hagen, 1990; Blaak *et al.*, 1993). β-Adrenergic stimulation of whole body metabolism has also been found in marsupial (Nicol, 1978). In comparison, the effect of α-receptors on whole body thermogenesis has not been well defined and is still controversial. Flaim *et al.* (1977) found that both phenylephrine and isoproterenol depolarized brown adipocyte membrane potential and raised interscapular brown fat temperature in intact rats. Foster (1984) reported an auxiliary effect of α-adrenergic stimulation on the positive thermogenesis of the rat BAT *in vivo* by β-adrenergic agonists. In unanaesthetized dogs an intravenous infusion of phenylephrine increases blood pressure associated with a significant rise in $\dot{V}O_2$ (Liard, 1989). Furthermore, α-adrenergic stimulation of whole body metabolism has strongly suggested by the evidence that phenylephrine is able to increase whole body thermogenesis in the rat (Rothwell, 1990). However, these investigators have attributed α-adrenergic mediation of whole body thermogenesis to BAT (Flaim *et al.* 1977; Foster, 1984; Rothwell, 1990). Hence, direct evidence for α-adrenergic stimulation of skeletal muscle heat production *in vivo* is still ambiguous.

Most studies in the human forearm have so far failed to show a conclusive effect of α-adrenoceptors on $\dot{V}O_2$ (Astrup *et al.*, 1989; Kurpad *et al.*, 1994). In the marsupial potoroo Nicol (1978) reported that NE-stimulated thermogenesis was blocked by intramuscular injections of propranolol but not phenoxybenzamine. Consequently, a question arises: does the α-receptor-mediated increase in $\dot{V}O_2$ observed in the perfused hindlimb (and other perfused tissues such as the kidney) have an influence on whole body energy expenditure?

The results in the present experiments (Figs. 8-1 & 8-2, Table 8-2) clearly demonstrate a marked increase in heat production in the conscious bettong during the intravenous infusion of phenylephrine (α_1-adrenergic agonist). The maximal rise in heat production reached 32% above the basal value. The stimulation of α-adrenergic receptors in whole body thermogenesis is strongly supported by the data in Fig. 8-3

which shows that propranolol did not block phenylephrine-elicited heat production. Further support came from the fact that phenylephrine- or naphazoline- mediated increments in heat production were inhibited by phentolamine, a general α-adrenergic antagonist (Fig. 8-4). However, this latter observation needs to be repeated when the animal becomes available again.

Since there is no detectable BAT in marsupials including the bettong (Nicol, 1978; Hayward & Lisson, 1992, Trayhurn 1994 personal communication), the α-adrenergic stimulation of whole body thermogenesis observed in the present study must derive from other tissues. In the perfused bettong hindlimb $\dot{V}O_2$ is increased during the stimulation of α-adrenoceptors (Chapter 4), thus skeletal muscle is likely to be one of the major tissues accounting for the α-adrenergic-mediated increase in heat production in intact bettongs.

The mechanism(s) for α-adrenergic agonists (and perhaps some other vasoconstrictors) on the heat production is still a matter of debate. Since α-adrenergic receptors are located predominantly on the vascular smooth muscle and responsible for NE-induced vasoconstriction (Martin *et al.*, 1990), the present results in the intact bettong imply that whole body thermogenesis is closely linked to the functions of the vascular system *in vivo*, possibly analogous to the mechanism discussed for the perfused hindlimb (Chapters 3, 4, 5 & 6). In fact, the effect of vasoconstrictors on oxygen metabolism was found in the conscious dog in which intravenous infusion of phenylephrine and angiotensin II increased both $\dot{V}O_2$ and arterial blood pressure (Liard, 1989). NE-mediated increases in metabolic rate and blood pressure are both blocked by either prazosin (α-adrenergic antagonist) or the vasodilator, NP, in rabbits (Szreder, 1993; Korolkiewicz *et al.*, 1993).

Overall, the present study suggests that not only β-adrenergic receptors but also α-adrenergic receptors are responsible for NE-stimulated thermogenesis in the marsupial bettong. The α-adrenergic stimulation of whole body thermogenesis probably originates from skeletal muscle or mircovessels. Combined with the data from the perfused hindlimbs of the rat and the bettong (Chapters 3 & 4), calculated estimates are that the skeletal muscle contributes significantly more to the body metabolic rate in the marsupial than in the eutherian mammals. This might be an alternative thermogenic strategy to compensate for the lack of BAT in evolution.

CHAPTER 9

Final discussion

9.1 Approaches for the present study

Vasoconstrictors such as norepinephrine, vasopressin, phenylephrine and 5-HT together with vasodilators (ie. nitroprusside) were used to assess vasoconstrictor-mediated control of $\dot{V}O_2$ in different perfused tissues, especially in the hindlimb. In the constant-flow perfusions, observations were first made in the rat hindlimb followed by the hindlimb(s) of the bettong (an endotherm without BAT) and cane toad (an ectothermic animal). The experiments were then extended to kidney, intestine and mesenteric artery of rats. The effects of the vasoconstrictors on muscle thermogenesis were further evaluated in the constant-pressure perfused rat hindlimb. In addition, the degradation of high energy phosphate compounds of skeletal muscle was characterized by comparing muscle tissues like the heart, intestine and blood vessels during ischemia. Skeletal muscle energy metabolites were compared in different oxygenation status (ischemia vs hyperoxemia). Finally, the role of vascular control of thermogenesis was examined in whole body via intravenous infusion of norepinephrine, phenylephrine, naphazoline, and isoproterenol in a marsupial bettong, an endotherm without detectable BAT.

9.2 Major findings

Increases in $\dot{V}O_2$ as well as lactate and pyruvate in perfused rat hindlimb were induced by increasing perfusion flow rate alone. The flow-mediated increment in $\dot{V}O_2$ (thus thermogenesis) was augmented by vasoconstrictors such as norepinephrine and vasopressin but inhibited by vasodilators such as nitroprusside (Chapter 3). Vasoconstrictor-mediated increases in $\dot{V}O_2$ in association with vasoconstriction also occurred in the perfused hindlimbs of other species such as bettongs and cane toads. The effect of vasoconstrictors on muscle metabolism in the perfused bettong hindlimb (Chapter 4) generally agree with those observed in the rat hindlimb (Clark *et al.*, 1994): norepinephrine and vasopressin (type A) stimulate $\dot{V}O_2$, lactate and glycerol release whereas 5-HT (type B) inhibits these metabolisms during vasoconstriction. In the constant-flow perfused hindlimb, norepinephrine-stimulated metabolism was mediated by α-adrenergic receptors (Chapter 4). The changes in metabolism and

pressure induced by both type A and B vasoconstrictors were reversed by NP (Chapter 4). The vasoconstrictor-mediated increase in $\dot{V}O_2$ in the perfused rat hindlimb was entirely distinguishable from muscle contraction-induced $\dot{V}O_2$ by using tubocurarine (Chapter 3).

Vascular control of $\dot{V}O_2$ is not an unique feature of skeletal muscle, and it occurs in other tissues as well, such as kidney, intestine and mesenteric artery although the stimulatory or inhibitory effects of a vasoconstrictor on $\dot{V}O_2$ may differ in different tissues (Chapter 5). In the constant-flow perfusion model, norepinephrine stimulates $\dot{V}O_2$ in perfused hindlimb but inhibits $\dot{V}O_2$ in the perfused intestine whereas 5-HT exerts the opposite effects (Chapters 3, 4, & 5). In the perfused rat kidney and intestine, nitroprusside markedly inhibited either the increased or decreased $\dot{V}O_2$, lactate and glycerol induced by vasoconstrictors when blocking the increased perfusion pressure. However, the inhibition of $\dot{V}O_2$ is not complete (Chapter 5).

In the constant-pressure perfusion model type A vasoconstrictors norepinephrine and vasopressin also stimulated $\dot{V}O_2$ even though the perfusion flow was slightly reduced from the initial level whilst a type B vasoconstrictor, 5-HT (at high concentrations), inhibited $\dot{V}O_2$ during vasoconstriction (Chapter 6). These results are consistent with the findings from the constant-flow perfusions (Clark *et al.*, 1994; Chapter 4). In addition, in the constant-pressure perfusion, β-adrenergic agonists, isoproterenol and BRL35135A were found to stimulate $\dot{V}O_2$ independent of perfusion flow in the presence of propranolol. At low concentrations, 5-HT increased both perfusion flow and $\dot{V}O_2$.

Skeletal muscle was unique in preserving adenine nucleotides during ischemia (Chapter 7). In contrast to the findings in other types of tissues such as the heart, intestine and aorta as well as the kidney (Chapter 5) and BAT (Matthias *et al.*, 1994) adenine nucleotides were not immediately altered during the early stages (60 min) of dramatic changes in O_2 supply (Chapter 7). The energy status, as indicated by the energy charge, was not significantly different among the normal, perfused (high flow plus norepinephrine) and ischemic (for 60 min) muscles, suggesting that rat skeletal muscle can be shifted to metabolic arrest state when O_2 supply is restricted. Among the energy metabolites measured, CrP and Cr were most sensitive to ischemia and differed in the three oxygenation states. Accordingly, they are more valuable in evaluating the muscle energetic status.

Similar to the basal metabolisms in the perfused hindlimb, the bettong BMR was low lower than that of the rat (Chapter 8). Norepinephrine and isoproterenol were shown to elicit heat production in the conscious bettong. More importantly, α_1-

adrenergic agonists, phenylephrine and naphazoline were also found to stimulate heat production.

9.2.1 Muscle is a thermogenic effector partly controlled by vasculature

Despite the highest storage of high energy phosphate compounds (Chapter 7), the basal metabolic rate of skeletal muscle is very low compared with most other tissues such as intestine, kidney, mesenteric artery (Chapter 5), heart and liver. Even if subjected to ischemia, the degradation kinetics of its high energy phosphate compounds is much slower than that of other muscle tissues (Chapter 7). A comparison of the estimated skeletal muscle contribution to BMR in the human, rat, bettong the toad is shown in Table 9-1. These indicate that skeletal muscle contributes to a high percentage of BMR in the species with no BAT.

If the value of 6.4 $\mu mol \cdot g^{-1} \cdot h^{-1}$ (Colquhoun *et al.*, 1988; 1990; Dora *et al.*, 1991; Hettiarachchi *et al.*, 1992) reflects $\dot{V}O_2$ at 25°C at the same flow (0.27 $ml \cdot min^{-1} \cdot g^{-1}$) as that *in vivo* (Armstrong & Laughlin, 1985) represents the basal $\dot{V}O_2$, the value of 27 $\mu mol \cdot g^{-1} \cdot h^{-1}$ at 18 ml/min in the presence of 50 nM norepinephrine would suggest that the resting skeletal muscle metabolism can be increased 400% by increased flow and norepinephrine stimulation together. This estimate would imply a huge potential of skeletal muscle for NST.

Table 9-1. Estimated muscle contribution to BMR in the rat, bettong and toad.

	Mass	% BMR	BAT	Source of the data
Rat	43%	23	yes	(Chapter 8)
Marsupial	52%	32	no	(Chapter 8)
Amphibians	35%	60-77	no	Flamigan, 1991

It might be argued that the vasoconstrictor-mediated increase in $\dot{V}O_2$ in constant-flow perfused hindlimb is less representative since the perfusion flow can be reduced *in vivo* when vasoconstrictors take action. A reduced flow would offset the stimulatory effect of $\dot{V}O_2$. The dose-response curves of norepinephrine, phenylephrine and vasopressin (Chapter 6) showed that vasoconstrictors are able to elicit $\dot{V}O_2$ at low doses before the perfusion flow is substantially decreased. The maximal $\dot{V}O_2$

elicited by norepinephrine reached 30% above the basal even though this basal value has been significantly elevated by a high basal perfusion flow rate as indicated in Chapter 3.

Thus, in light of the criteria proposed by Trayhurn (1994, see Section 1.4), the present study provides further support for vascular controlled skeletal muscle to be operationally thermogenic system in the following two points. Firstly, resting skeletal muscle as a whole is able to produce considerable amount of heat far beyond its basic requirements. Secondly, this thermogenic process can be rapidly turned on or off by vasoconstrictors such as norepinephrine.

9.2.2 The role of adrenergic receptors in muscle thermogenesis

Skeletal muscle has both α- and β- adrenergic receptors (Rattigan *et al.*, 1986) and α-receptors are prevalent on small arteries (Martin *et al.*, 1990). However, the role of adrenergic receptors in muscle thermogenesis is still controversial. A number of researchers (Rothwell & Stock, 1980; Blaak *et al.*, 1993; Astrup *et al.*, 1994) have shown β-adrenergic-mediated rise in muscle thermogenesis. There is also evidence that suggests the presence of β_3-adrenergic receptors in skeletal muscle (Emorine *et al.*, 1989; Roberts *et al.*, 1993, Sillence *et al.*, 1993). Conversely, most of the studies in the constant-flow perfused hindlimb demonstrated α-adrenergic mediation of muscle thermogenesis (Grubb & Folk, 1977; Richter *et al.*, 1982b; Colquhoun *et al* 1990; Hettiarachchi *et al.* 1992). In fact, some studies *in vivo* also showed α-adrenergic stimulation of thermogenesis in rats (Foster *et al.*, 1984; Rothwell, 1990), dogs (Liard, 1989) and rabbits (Szreder *et al,* 1991). However, this has been attributed to an auxiliary effect on BAT thermogenesis (Foster *et al* 1984; Rothwell, 1990).

The present study also examined the involvement of α- and β-adrenergic receptors in muscle thermogenesis. In the constant-flow perfused hindlimb, norepinephrine-stimulated $\dot{V}O_2$ is mediated by α-adrenergic receptors as shown by the blockade of the increased $\dot{V}O_2$ by phentolamine but not propranolol (Chapter 4). The α-adrenergic stimulation of muscle $\dot{V}O_2$ was similarly demonstrated by the enhancement of $\dot{V}O_2$ by phenylephrine in the constant-pressure perfused hindlimb. More interestingly, both phenylephrine (α_1 agonist) and naphazoline (α_1, α_2 agonist) were able to elicit heat production in the bettong (Chapter 8). The lack of detectable BAT using histological or molecular biology techniques (Hayward & Lisson, 1992; Trayhurn, personal communication) makes it impossible to attribute the α-adrenergic

mediated increase in heat production to BAT, strongly suggesting the α-adrenergic stimulation of vascular-associated muscle thermogenesis *in vivo*.

The experiments in this thesis also showed β-adrenergic stimulation of muscle thermogenesis in the constant-pressure perfused rat hindlimb (Chapter 6) and the whole body metabolic rate study in bettongs (Chapter 8), but not in the constant-flow perfused hindlimb (Chapter 4). A lack of β-adrenergic stimulation of $\dot{V}O_2$ has also been found by other researchers using constant-flow perfused rat hindlimbs (Colquhoun *et al.*, 1989; Hettiarachchi *et al.*, 1992; Dora *et al.*, 1994). The reason for the disparity of the results of β-adrenergic stimulation of muscle $\dot{V}O_2$ between the constant-flow (once through) and constant-pressure (plus recirculated) perfusions is not clear and may be related to the methods.

One of the major differences between the two perfusion models is the basal flow and pressure. For the constant-flow perfusion, both basal perfusion flow rate (0.27 ml/min.g) and pressure (25 mm Hg) were much lower than those (2.4-2.6 ml/min.g and 80 mm Hg) in the constant-pressure perfusion. Another important difference is the circulation models adopted. The constant-flow perfusion was performed in an one-through model whilst the perfusate was recirculated in the constant-pressure perfusion. It is known perfused hindlimb releases various substances such as metabolites [lactate, pyruvate, glycerol (Chapters 3, 4 & 5)], purine and pyrimidine compounds (Clark *et al.*, 1990), and possibly vasoactive substances (Chapter 6). Consequently, another possible reason for the contradictory results might be the involvement of the released substance(s) in β-adrenergic stimulation of $\dot{V}O_2$ in the constant-pressure perfused hindlimb. Indeed, β-adrenergic stimulation of $\dot{V}O_2$ and lactate production has been found in both constant-flow (Richter *et al.*, 1982b) and constant-pressure (Hardeveld *et al.*, 1980) perfused rat hindlimbs in a similar recirculated model.

Nonetheless the results of β-adrenergic mediation of muscle metabolism are probably more representative of *in vivo* because blood is circulated and blood pressure is around 80-100 mm Hg *in vivo*. Other supportive evidence for β-adrenergic meditation of muscle thermogenesis came from the increase in heat production in the intact bettong induced by intravenous infusion of isoproterenol (Chapter 8). Further studies on β-adrenergic mediated increase in muscle thermogenesis revealed that $\dot{V}O_2$ is mainly mediated by β_3- or atypical β receptors but other β-subtypes may be involved in other changes such as flow rate and lactate and pyruvate production (Chapter 6). Fig. 9-1 summarizes a proposed mechanism for β-adrenergic stimulation of muscle thermogenesis *in vivo*.

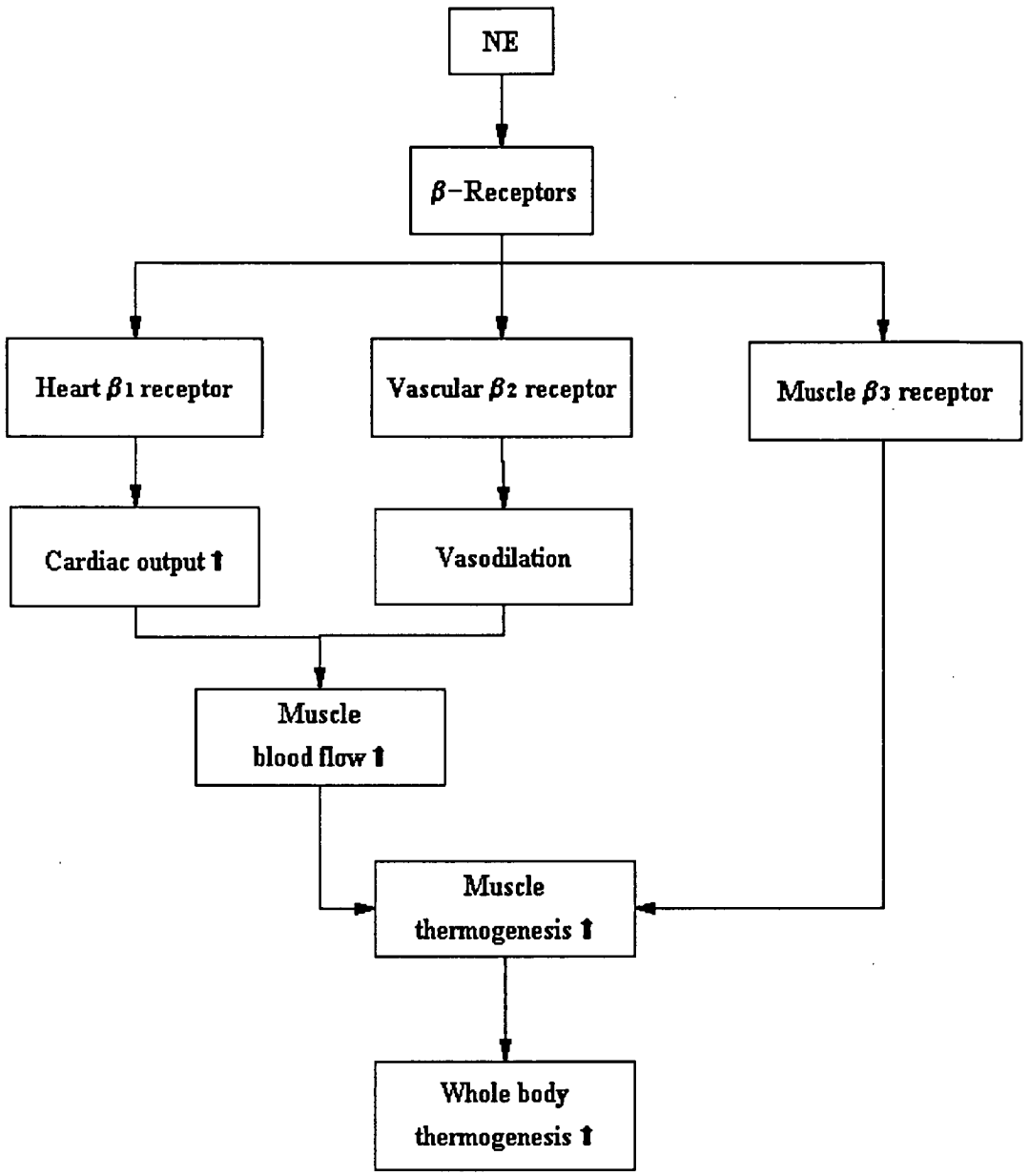

Fig. 9-1 Proposed mechanism for β-adrenergic mediation of muscle thermogenesis *in vivo.*

9.2.3 Vascular control of $\dot{V}O_2$ in other tissues

Table 9-2 compares sensitivity of metabolic response to norepinephrine infusion of the perfused rat hindlimb, kidney and intestine. The hindlimb (thus skeletal muscle) is the first to be affected by norepinephrine. When norepinephrine increases to

the level necessary to influence $\dot{V}O_2$ in the kidney and intestine, the $\dot{V}O_2$ has already risen to, or above, the half maximal value in skeletal muscle. If this relationship stands *in vivo*, norepinephrine-inhibited $\dot{V}O_2$ in intestine would affect very little of the norepinephrine-stimulated thermogenesis derived from other tissues such as skeletal muscle.

Although the response of $\dot{V}O_2$ to norepinephrine is shifted to the left in the constant-pressure perfusion, the dose response curve (Fig. 6-2) shows norepinephrine does not become inhibitory at 100 nM at which the maximal $\dot{V}O_2$ is reached in the constant-flow perfusion (Table 9-2). Hence, *in vivo*, the maximal effect of norepinephrine on muscle thermogenesis would probably be achieved at concentrations between 10-100 nM in the rat. Such an extrapolation seems to match the rat plasma catecholamines measured *in vivo*. For example, a carefully controlled study using both HPLC-EC (electrochemical detector) and radioimmunoassay revealed that the plasma norepinephrine and epinephrine in the resting rat are 1.8 and 1.1 nM, respectively (Eriksson & Persson, 1982). Cold exposure could easily increase these values more than four times (Chin *et al.*, 1973).

Table 9-2 $\dot{V}O_2$ response to norepinephrine concentration in the perfused rat hindlimb, kidney and intestine

	$\dot{V}O_2$ start	$\dot{V}O_2$ 50%	$\dot{V}O_2$ 100%	Original data
Constant-flow				
Hindlimb[a]	~ 1 nM	~ 10 nM	~ 100 nM	Fig. 3-5
Kidney[a]	~ 10 nM	~ 100 nM	~ 1000 nM	Fig. 5-1
Intestine[b]	> 30 nM	> 100 nM	> 1000 nM	Fig. 5-6
Constant-pressure				
Hindlimb[a]	~ 1 nM	~ 1.5 nM	~ 3-10 nM	Fig 6-2

Note: [a] stimulation; [b] inhibition.

It is rather interesting that vasoconstrictors other than norepinephrine also alter muscle thermogenesis associated closely with vasoconstriction (Chapters 3, 4 & 6). This is true in other perfused tissues such as the rat kidney and intestine (Chapter 5). Hence, vascular control of $\dot{V}O_2$ seems to be a general feature of the perfused

tissues. These data would also appear to indicate that there might be a common mechanism underlying the vasoconstrictor-mediated changes in $\dot{V}O_2$ in different tissues.

9.2.4 Vascular control of $\dot{V}O_2$ in the hindlimbs of other species

Table 9-3 summarizes $\dot{V}O_2$ in perfused hindlimb(s) of rats, bettongs and toads in response to norepinephrine at different concentrations. Of these three species, the rat hindlimb is most sensitive to the norepinephrine stimulation, and toad hindlimbs appear to need the highest concentration. So far, no data are available for plasma catecholamine concentrations for the bettong. The values obtained from a small carnivorous marsupial (*Antechinus stewartii,* Bradly, 1994 Personal communication), the measured plasma catecholamines (Table 9-2) are approximately four times as high as those of the rat. The sum of plasma norepinephrine and epinephrine is 2.9, 14.8 and 15.1 nM for the rat, the marsupial (*Antechinus stewartii*) and toad, respectively. The hindlimb $\dot{V}O_2$ in response to norepinephrine stimulation in these species (Table 9-2) appears match their plasma catecholamine levels *in vivo*. Furthermore, these observations taken together indicate that catecholamines have already started to exert a marginal effect on muscle thermogenesis under resting conditions. Any rise in plasma catecholamines would immediately have a significant impact on muscle thermogenesis.

Table 9-3 Norepinephrine concentrations to stimulate $\dot{V}O_2$ in perfused hindlimbs and its plasma levels in the rat, marsupial and toad at rest

	Plasma NE	Plasma E	$\dot{V}O_2$ start	$\dot{V}O_2$ 50%	$\dot{V}O_2$ 100%
Rat	1.8 nM[a]	1.1 nM[a]	1 nM	10 nM	100 nM
Bettong	10 nM[b]	4.8 nM[b]	10 nM	50 nM	1000 nM
Toad	2.1 nM[c]	13 nM[c]	30 nM	100 nM	1000 nM

a: Eriksson & Persson, 1982; b: Bradley, 1994 (Personal communication) for a carnivorous marsupial (*Antechinus stewartii*); c: Withers et al., 1988. Other data are quoted Figs. 3-5, 4-1 & 4-3). NE: norepinephrine, E: epinephrine.

9.3 Mechanisms underlying vasoconstrictor-mediated changes in $\dot{V}O_2$ in skeletal muscle.

Vasoconstrictor-mediated increases in $\dot{V}O_2$ in skeletal muscle are clearly distinguishable from muscle contraction-induced $\dot{V}O_2$. The close association between perfusion pressure and $\dot{V}O_2$ strongly suggest that vasoconstriction is tightly linked in some manner to this metabolism. Yet the mechanism for the vasoconstrictor-induced $\dot{V}O_2$ is still controversial. A *priori,* to explain the increased $\dot{V}O_2$, there must be an increased hydrolysis and subsequent synthesis of ATP, or a stimulated mitochondrial uncoupling within the hindlimb analogous to that occurring in BAT. Four general possibilities are apparent that could explain the vasoconstrictor-mediated control of such processes in the perfused hindlimb.

Firstly, the vasoconstrictors may act directly on skeletal muscle receptors to cause the above metabolic changes independent of the increases in perfusion pressure since some vasoconstrictor hormones have receptors on skeletal muscle. However, the failure to observe a stimulatory effect of norepinephrine, angiotensin II on $\dot{V}O_2$ by incubated or perifused muscles *in vitro* (Hannon *et al.*, 1963; Eaton 1964; Dubois-Ferrière *et al.*, 1981; Hettiarachchi *et al.*, 1992) or homoganate (Hannon *et al.*, 1963) does not support this argument.

The second possibility is that the rise in perfusion pressure associated with vasoconstriction may redistribute the perfusate within the hindlimb to previously under-perfused zones and thus by relieving regions of hypoxia, stimulate increased $\dot{V}O_2$. This is unlikely because, as discussed earlier, there is no evidence of regional hypoxia as Cr, CrP and lactate are normal (Chapters 3, 4, 5 & 6). As shown in Chapter 7, a decreased CrP together with an increased Cr would be expected if skeletal muscle is ischemic. Besides the PvO_2 remained above 200 mm Hg for all of the perfused hindlimb experiments which is a partial pressure greater than are achieved *in vivo*. The results in Fig. 6-2 showed that both norepinephrine and phenylephrine at low concentrations stimulate $\dot{V}O_2$ even though perfusion flow rate is decreased at a constant pressure of 80 mm Hg. It would seem more unlikely that some regions are still underperfused at physiological pressure plus supraphysiological flow and that a fall in flow would improve 'underperfused' regions. Furthermore, such a pressure-induced redistribution of flow to relieve hypoxia, does not accord with the inhibition of $\dot{V}O_2$ associated with the vasoconstriction induced by 5-HT (Figs. 4-1 and 4-5) in the perfused bettong hindlimb.

The third possibility is that arterial vasoconstriction is energetically expensive and uses the observed $\dot{V}O_2$. Colquhoun & Clark (1991) proposed that vascular

thermogenesis might be one of the mechanisms accounting for vasoconstrictor-induced $\dot{V}O_2$ in the perfused rat hindlimb. The relationship between active vascular wall tension and $\dot{V}O_2$ has been noted by Paul & Peterson (1975) and discussed in Chapters 3 & 6. The evidence for this hypothesis includes: 1) the close correlation between $\dot{V}O_2$ and perfusion pressure induced by vasoconstrictors in the constant-flow perfused hindlimb,

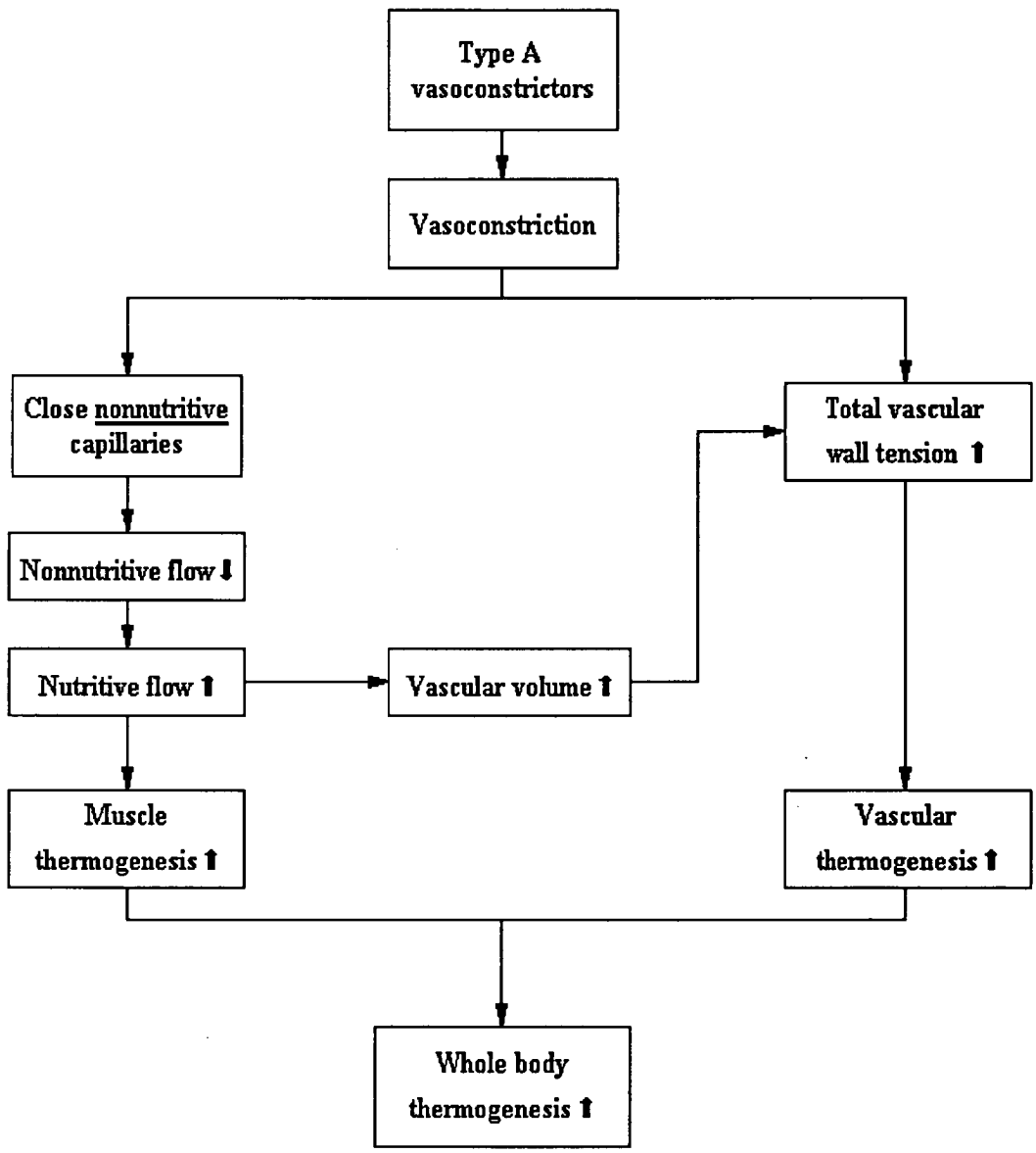

Fig. 9-2 Proposed mechanism of type A vasoconstrictor-mediated increases in muscle thermogenesis *in vivo*.

kidney, intestine and mesenteric artery (Chapters 3, 4 & 4); 2) the blockade or inhibition of the vasoconstrictor-mediated increase in $\dot{V}O_2$ in constant-flow perfused hindlimb, kidney and intestine by vasodilators when relaxing vasoconstriction (Chapters 4 & 5); 3) flow (thus vascular stretch)-induced $\dot{V}O_2$ and the inhibition by a vasodilator in the step- and constant-flow perfused hindlimb (Chapter 3); and 4) vasoconstrictor-stimulated increases in $\dot{V}O_2$ during small vasoconstriction in the constant-pressure perfused hindlimb.

Thus the key issue for working vascular thermogenesis seems to be a matter of how-much. Literature values for $\dot{V}O_2$ obtained by Warburg techniques of various vascular smooth muscle fragments incubated *in vitro* at 37°C (Paul, 1980) ranged from 1.8-3.0 $\mu mol \cdot g^{-1} \cdot h^{-1}$. However, the calculated $\dot{V}O_2$ for canine femoral artery (26 $\mu mol \cdot g^{-1}\ h^{-1}$) is more than 10-fold greater than incubated segments [2.4-3.6 $\mu mol \cdot g^{-1}\ h^{-1}$, (Kosan *et al.*, 1966)], which may reflect the effect of pressure loading on $\dot{V}O_2$. Isolated unstimulated, nonperfused brain microvessels have an endogenous rate of $\dot{V}O_2$ of 65.4 $nmol \cdot h^{-1} \cdot mg^{-1}$ protein at 37°C (Brendel *et al.*, 1974). At 150 mg protein/g wet wt. this corresponds to 96 $\mu mol \cdot g^{-1}\ h^{-1}$. Such vessels can further increase their $\dot{V}O_2$ threefold when given succinate as a substrate (Sussman *et al.*, 1988) and presumably would use even more when pressure loaded. The calculated value from the perfused mesenteric artery (Chapter 5) was 342 $\mu mol \cdot g^{-1} \cdot h^{-1}$. A high $\dot{V}O_2$ has also been found in the bovine microvessels [288 $\mu mol \cdot g^{-1} \cdot h^{-1}$ (Sussman *et al.*, 1988)] and perfused rat tail artery (Colquhoun & Clark, 1991).

It is not readily apparent how vascular thermogenesis could explain the inhibition of $\dot{V}O_2$ and lactate efflux by 5-HT that accompanies a rise in vascular resistance in perfused hindlimbs (Figs. 4-1 & 6-7). However when infused into the isolated perfused rat mesenteric artery, 5-HT (as well as norepinephrine and vasopressin) caused vasoconstriction and increased $\dot{V}O_2$ (Dora *et al.*, 1991; Chapter 5). These observations led to the idea that 5-HT initiates a state of functional shunting of perfusate within the muscle vascular bed away from actively metabolising cells (Dora *et al.*, 1991). If so, the areas of microvessels subjected to the increased pressure would also decrease, thereby reducing the total mass of working microvasculature and $\dot{V}O_2$.

The fourth possibility is that vasoconstrictors by selective actions redirect flow to particular thermogenic skeletal muscle cells or other thermogenic cells in the hindlimb Clark *et al.*, (1994). Type A vasoconstrictors (norepinephrine, vasopressin, angiotensin II) act on site-specific receptors on the arterioles to shut down the flow to nonnutritive capillaries hence redistributing flow to the areas or cells that are metabolically more active. In contrast, type B vasoconstrictors (ie. 5-HT in muscle

and norepinephrine in intestine) close down nutritive capillaries to direct flow to nonnutritive areas. Consequently, the metabolism is inhibited. According to this model, flow-induced $\dot{V}O_2$ (Chapter 3) could be explained as a result of an increase in nutritive flow. Similarly, the increased muscle metabolism produced by isoproterenol and 5-HT in the constant-flow perfused hindlimb (Chapter 5) may be partially attributable to an increased flow to nutritive capillaries via dilating their arterioles. So far, such specialised muscle cells have only been described in billfish, in which blood going to

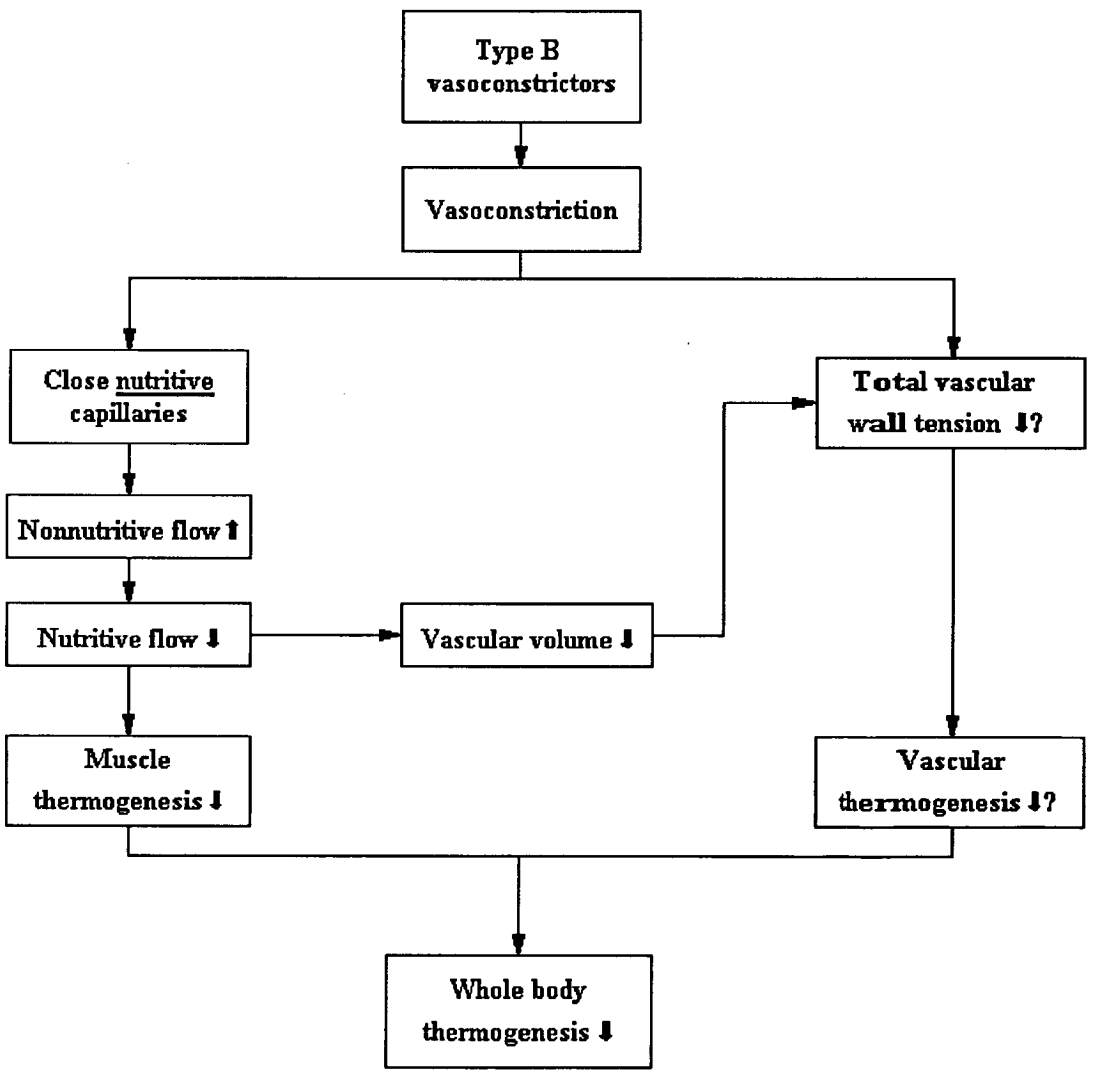

Fig. 9-3 Proposed mechanism of type B vasoconstrictor-inhibited muscle thermogenesis *in vivo*.

the retina and brain is heated by a specialized orbital muscle by presumptive calcium ion cycling (Block, 1994). Other biochemical bases for this argument have not yet been identified at the cellular level although some observations in muscle suggest controlled oxidative phosphorylation uncoupling might be possible (Barré *et al.*, 1986; Duchamp *et al.*, 1992; Herpin & Lefaucheur, 1992; Matthias *et al.*, 1993).

However, data from the present experiments, or data from the literature thus far, can not discriminate between these two mechanisms of controlling or contributing for the involvement of blood vessels in the vasoconstrictor-altered $\dot{V}O_2$ in the perfused hindlimbs. They may not be necessarily mutually exclusive. Fig. 9-2 illustrates the proposed mechanisms (Colquhoun & Clark, 1991; Clark *et al.*, 1994) for the vasoconstrictor-mediated increase in muscle thermogenesis for type A vasoconstrictors if they do not significantly inhibit the cardiac output. In comparison, type B vasoconstrictors inhibit muscle thermogenesis in the mechanism illustrated in Fig. 9-3.

9.4 Considerations for future studies

Both vasoconstrictors and vasodilator hormones are involved in controlling vascular functions *in vivo*. The effects of vasodilators in muscle thermogenesis have not been the focus of the thesis. There were some contradictions on the role of isoproterenol and 5-HT in muscle metabolism between constant-flow (Chapter 4) and constant-pressure (Chapter 6) perfusions. Likewise, discrepancies exist between the effect of β-adrenergic receptors on $\dot{V}O_2$ between the constant-flow perfused hindlimb (Chapter 4) and the intact animal study (Chapter 8). It is important to examine whether vasodilators fall into metabolically positive (type A) or negative (type B) categories as proposed for vasoconstrictors.

In addition to distinct haemodynamics, another important difference between these two models was once-through perfusion vs recirculation perfusion. As discussed in Chapter 6, recirculation could affect the results of lactate efflux. Hence, it would be interesting to further compare effects of vasoconstrictors on muscle thermogenesis in constant-flow and constant-pressure perfusion models using a once-through perfused skeletal muscle bundle/group. It would also be interesting to compare the results obtained at 37°C with those obtained at 25°C.

The findings from this thesis (Chapter 6) and other works (Clark *et al.*, 1994)) have demonstrated that all vasoconstrictors stimulate or inhibit muscle metabolism in a dose-dependent manner. Whether a vasoconstrictor such as norepinephrine,

phenylephrine or vasopressin stimulates $\dot{V}O_2$ or not depends on their concentration (Chapter 6). For some vasoconstrictors such as norepinephrine and vasopressin, their effect on muscle $\dot{V}O_2$ can be totally opposite at different concentrations (Chapter 6; Clark *et al.*, 1994). Thus future studies need to examine the concentrations of these vasoconstrictor in the circumstances where thermogenesis occurs.

To understand the role of a vasoconstrictor on muscle thermogenesis, a better knowledge of its effect on cardiac output is also essential. Moreover, the ultimate effect of a vasoconstrictor on thermogenesis needs to judged on a whole body basis. Comparative studies between animals with and without BAT and between endotherms and ectotherms would still be a powerful approach.

Vascular thermogenesis is a new hypothesis to explain the vasoconstrictor-mediated increase in $\dot{V}O_2$ in the perfused tissues, especially in skeletal muscle. One of the problems in addressing this issue is the extreme difficulty of direct measurement of $\dot{V}O_2$ or heat efflux at the microvessel (ie. small artery and arterioles) level. The results of manipulating vascular contraction or relaxation from the perfused tissues so far, are generally but not completely supportive. However, they can be clouded by changes in the parenchymal tissues, such as the difference between the effects of norepinephrine in the perfused intestine and mesenteric artery (Chapter 5). The data available in the literature are mostly obtained from larger blood vessels (Paul 1980). An evaluation of vascular thermogenesis using these values would probably lead to substantial discount of the true values of the microvasculature. For instance, the values obtained from the perfused mesenteric artery (Chapter 5) and bovine cerebral microvessels are much higher than those reviewed by Paul (1980) for the larger vessels. If a relationship between the size of the artery and its $\dot{V}O_2$ can be established, a better estimate of microvessels $\dot{V}O_2$ may be more representative. Other indicators for the capability of $\dot{V}O_2$, such as mitochondria content, cytochrome oxidase are also important. It would be interesting to compare vascular $\dot{V}O_2$ between warm-adapted and cold-adapted animals. Most importantly, vascular thermogenesis needs to be further assessed according to vascular $\dot{V}O_2$ and its total mass of tissue within the body.

The notion that vasoconstrictors stimulate muscle $\dot{V}O_2$ by diverting flow to specific nutritive areas or cells needs to be further investigated in the following key areas: 1) whether the proposed nutritive and nonnutritive capillaries are morphologically discernible; 2) whether there are site-specific receptors on the arterioles associated with nutritive and nonnutritive capillaries; 3) the energy dissipating mechanism at the cellular level; and finally 4) the linkage between vasoconstrictor-controlled flow to the "switch" of the energy dissipating process. From a broader perspective, there seems to be a need to find out whether any such

energy dissipating mechanism in muscle is present in other tissues as well, since vasoconstrictor-mediated changes $\dot{V}O_2$ also occurs in other tissues.

Oxidative phosphorylation uncoupling or decoupling has been considered as a possible cellular mechanism for skeletal muscle thermogenesis (Barré, *et al.*, 1987b; Herpin & Lefaucheur, 1992). Recent studies using isolated skeletal muscle mitochondria in this laboratory appear to support this proposal (Matthias *et al.*, 1993). A key issue to evaluate this mechanism would be the measurement of the changes in ATP turnover rate during vasoconstrictor-mediated increases in $\dot{V}O_2$ in the perfused skeletal muscle preparation. A comparison of $\dot{V}O_2$ between the stoichiometrical value of the changed ATP turnover rate and the directly measured value would provide further insight into this hypothesis.

9.5 Conclusion

In conclusion, no matter what the mechanism it is, the results of this thesis reveal the important potential role of vascular control of thermogenesis in muscle and also in other tissues. These findings suggest that vasoconstrictor-mediated control of $\dot{V}O_2$ plays an important role in regulating whole body thermogenesis.

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APPENDIX

Publications originating from the thesis

1. YE, J.M, E.Q. COLQUHOUN, M. HETTIARACHCHI, AND M.G. CLARK. Flow-induced oxygen uptake by the perfuse rat hindlimb is inhibited by vasodilators and augmented by norepinephrine: a possible role for the microvasculsature in hindlimb thermogenesis. *Can. J Physiol. Pharmacol.* **68:** 119-125, 1990.

2. YE, J.M, E.Q. COLQUHOUN, AND M.G. CLARK. A comparison of vasopressin and norepinephrine on oxygen uptake by perfused rat hindlimb, kidney, intestine, and mesenteric arcade suggests that it is in part due to contractile work by blood vessels. *Gen. Pharmacol.* **21:** 805-810, 1990.

3. YE, J.M, S.J. EDARDS, R. W. ROSE, S. RATTIGAN, M.G. CLARK, AND E.Q. COLQUHOUN. Vasoconstrictors alter oxygen, lactate and glycerol metabolism in the perfused hindlimb of a rat kangaroo. *Am. J. Phyisol.* **268** (*Regulatory, Integrative and Comp. Physiol.* 37): R1217-1223, 1995.

4. YE, J.M. M.G. CLARK. AND E.Q. COLQUHOUN. Creatine phosphate as the preferred early indicator of ischemia in muscular tissues. *J. Surg. Res.* **(in press** 1995)

5. YE, J.M., M.G. CLARK, AND E.Q. COLQUHOUN. Constant-pressure perfusion of the rat hindlimb shows α- as well as β-adrenergic stimulation of oxygen consumption. *Am. J. Physiol.* **(in press)** 1995.

6. YE, J.M, S.J. EDARDS, R. W. ROSE, S. RATTIGAN, J.T. STEEN, M.G. CLARK, AND E.Q. COLQUHOUN. Alpha-adrenergic stimulation of thermogenesis in a rat kangaroo (Marsupialia: *Bettongia gaimardi*). Submitted to *Am. J. Phyisol.* in 1995.

Contributions to related papers

1. COLQUHOUN, E.Q., M. HETTIARACHCHI, J.M. YE, E.A. RICHTER, A.J. KNIAT, S. RATTIGAN, AND M.G. CLARK. Vasopressin and angiotensin II stimulate oxygen uptake in the perfused rat hindlimb. *Life Sciences.* **43:** 1747-1754, 1988.

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1. YE, J.M, S.J. EDWARDS, R.W. ROSE, M.G. CLARK, AND E.Q. COLQUHOUN. Effect of Vasoconstrictors on oxygen uptake, and lactate and glycerol production in a perfused marsupial (*Bettong*) muscle. *Proceeding of the 37th Annual Conference of Australian Society for Biochemistry and Molecular Biology.* Adelaide. **25:** 98, 1993

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3. DORA. K.A., S. RATTIGAN, J.M. YE, T.P.D. ELDERSHAW, J.L. HALL, E.Q. COLQUHOUN, AND M.G. CLARK. Vascular control of rat hindlimb nutrient delivery, uptake and release. **In:** *Abstracts of XXXII Congress of the International Union of Physiological Sciences.* Glassgow, UK. p 47, 1993.

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6. MATTHIAS, A., K.A. DORA, S. RATTIGAN, J.M. YE, E.Q. COLQUHOUN, AND M.G. CLARK. Comparison of thermogenesis in perfused skeletal muscle and brown adipose tissue. *6th Biothermokinetics Conference.* **In:** *What is controlling life?* Edited by GNARGER, E., F.N. GELLERICH, AND M. WYSS. Innsbruck University Press, Innsbruck-Schröken, Austria. p 249, 1994.

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8. TONG, A. C.Y., K.A. DOAR, J.T. STEEN, J.M. YE, S. RATTIGAN, E.Q. COLQUHOUN, AND M.G. CLARK. Sub-populations of vascular α-adrenoceptors mediate different metabolic responses in the perfused rat hindlimb. *33th National Scientific Conference of the Australian Society for Medical Research.* Melbourne. Oral 13-7, 1994.

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A COMPARISON OF VASOPRESSIN AND NORADRENALINE ON OXYGEN UPTAKE BY PERFUSED RAT HINDLIMB, KIDNEY, INTESTINE AND MESENTERIC ARCADE SUGGESTS THAT IT IS IN PART DUE TO CONTRACTILE WORK BY BLOOD VESSELS

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Abstract—1. The rat hindlimb, kidney and intestine were each perfused in a nonrecirculating mode at 25°C using an artificial perfusate (initial pressure 85 ± 5 mmHg) and the effects of vasopressin and noradrenaline on oxygen uptake and perfusion pressure determined.

2. Both vasopressin ($K_{0.5}$ = 0.1 nM) and noradrenaline ($K_{0.5}$ = 2 nM) increased oxygen uptake as well as perfusion pressure by the perfused hindlimb; changes in oxygen uptake were closely matched by changes in pressure. The maximum increase in oxygen uptake was approx. 9 μmol/hr per g wet wt of hindlimb.

3. The perfused kidney also responded to vasopressin and noradrenaline with parallel increases in oxygen uptake and perfusion pressure for each agent. The largest increase in oxygen uptake was approx. 30 μmol/hr per g wet wt but this was not maximal.

4. Vasopressin increased oxygen uptake and pressure by the perfused intestine over the range 0.01–2 nM, but the changes in pressure only became significant at doses >0.1 nM.

5. Noradrenaline inhibited oxygen uptake and increased perfusion pressure in a dose-dependent manner at pharmacological concentrations (>30 nM) when shunting of perfusate may have contributed to unperfused regions.

6. A network of mesenteric blood vessels estimated to contain approx. 6% vascular tissue by weight, with the remainder white fat cells, lymphatics and connective tissue, was also perfused.

7. Vasopressin ($K_{0.5}$ = 0.3 nM) and noradrenaline ($K_{0.5}$ = 30 nM) each increased oxygen uptake and perfusion pressure in a dose-dependent manner.

8. Calculations, based on the vascular tissue content and the overall oxygen uptake in the mesenteric artery preparation suggest that perfused blood vessels consume oxygen at very high rates (115 μmol/hr per g wet wt at 25°C) and that this consumption is further increased by 75% when the smooth muscle constricts.

9. It is concluded that agonist-mediated constriction of blood vessels is energetically expensive and that the consumption of oxygen for this process has significant implications for whole body thermogenesis.

INTRODUCTION

Our previous experiments using the perfused rat hindlimb (Colquhoun *et al.*, 1988, 1989; Ye *et al.*, 1990) have highlighted the central role of the vascular system in the control of hindlimb oxygen uptake and raised the novel possibility that a considerable proportion of oxygen consumed by the hindlimb during vasoconstriction is taken up by working vascular tissue itself (i.e. the "hot pipe" hypothesis).

The evidence supporting the centrol role of the vasculature includes observations where each of the vasoconstrictors, noradrenaline, adrenaline, vasopressin and angiotensin II increase oxygen consumption by the hindlimb in conjunction with an increase in perfusion pressure (Colquhoun *et al.*, 1988, 1989) and where the vasodilators, nitroprusside, nifedipine and isoproterenol, which each act by different mechanisms, oppose vasoconstrictor-mediator increases in both oxygen uptake and perfusion pressure (Colquhoun *et al.*, 1989).

The notion that the vascular tissue itself consumes oxygen during constriction, although consistent with the observations referred to above, is indirect and based on data where oxygen uptake due to skeletal muscle contraction appears to be additional to that produced by vasoconstrictors (Colquhoun *et al.*, 1989) and on experiments where near maximal flow-induced oxygen uptake by the hindlimb is further increased by noradrenaline and inhibited by nitroprusside (Ye *et al.*, 1990). If the vascular tissue is largely responsible for the increase in oxygen uptake during vasoconstriction, rates of oxygen uptake would approach that of vigorously contracting skeletal or cardiac muscle. This would assign a much higher respiratory rate to vascular tissue than has previously been estimated from fragments of large vessels, whether incubated or cannulated (Paul, 1980). In the absence of direct information on oxygen uptake by isolated perfused vascular tissue in the presence of vasoconstrictors, the present experiments were undertaken to assess whether the vasoconstrictor, vasopressin, increases oxygen uptake by perfused organs in general. For this study, hindlimb, kidney,

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intestine and a mesenteric artery network preparation were compared. Noradrenaline, the putative physiological thermogenic neurotransmitter/hormone was also included.

MATERIALS AND METHODS

Animals

Male hooded Wistar rats (180 ± 5 g body wt) were fed a commercial diet with water *ad libitum*.

Chemicals

Bovine serum albumin (Fraction V), was obtained from Boehringer Mannheim and was dialysed 5 times against distilled water and lyophilized before use. Arginine vasopressin and noradrenaline-HCl were obtained from Sigma U.S.A. All other chemicals were analytical grade.

Perfusion system for hindlimb, kidney, intestine and the mesenteric artery network

The perfusate consisted of a Krebs–Ringer bicarbonate buffer containing 1.27 nM $CaCl_2$, 2% bovine serum albumin and 8.3 mM glucose and was pumped from a reservoir kept at 4°C through a silastic tube oxygenator continuously equilibrated against a gas mixture of 95% O_2 and 5% CO_2. The temperature of the perfusate was brought to 25°C by passage through a heat exchange coil. Perfusion pressure was measured in the pump outflow line proximal to the arterial cannula. As indicated all perfusions were conducted at a constant flow rate; changes in perfusion pressure were assumed to reflect changes in vascular resistance. The venous effluent flowed through a 0.5 ml thermostatically controlled chamber (25°C) containing a Clark-type oxygen electrode. The effluent was discarded following measurement of its oxygen content. The oxygen electrode was calibrated before and after each experiment with gas mixtures of 100%, 20.9% and 0% oxygen and periodically checked with a Radiometer Model ABL300 blood gas analyzer. The PO_2 of the venous effluent and the arterial perfusion pressure were recorded continuously. Experiments were started when these parameters reached steady-state, usually within 40 min. Agonists were freshly prepared in isotonic saline containing 0.1% ascorbic acid. They were infused continuously into the perfusion line prior to the bubble trap at a rate not greater than 1% of the rate of perfusion. Perfusions were conducted in a temperature-controlled chamber at 25 ± 0.2°C.

Surgical preparations

Rats were anaesthetized with pentobarbital sodium (5 mg/100 g body wt, i.p.). Surgery for hindlimb perfusion was essentially as described by Ruderman *et al.* (1971) and Richter *et al.* (1982). Additional details were as given previously (Colquhoun *et al.*, 1988, 1989). The hindlimb was perfused at a constant flow rate of 18.6 ± 0.2 ml/leg per min to give a basal pressure of 80–90 mmHg.

Details for kidney perfusion were essentially as described by Little and Cohen (1974). Ligatures of size 000 silk (Ethicon Inc.) were placed around the superior mesenteric artery, left renal vein, inferior vena cava below the left renal vein, inferior vena cava above the left renal vein, inferior vena cava above the right renal vein and right renal artery. The right renal artery was cannulated, and a cannula was placed in the inferior vena cava between the two ties adjacent to the entry point for the left renal vein. The cannulae were tied in place and the perfusion commenced. Ligatures around the superior mesenteric artery, left renal vein, inferior vena cava below the left renal vein, vena cava above the right renal vein were tied off. Renal arterial perfusion was not interrupted at any time. The kidney was perfused *in situ* at a constant flow rate of 4.7 ± 0.4 ml/min per kidney to give a basal pressure of 80–90 mmHg.

For perfusion of the intestine, the abdomen was opened along the midline and the large intestine, duodenum, and pancreas were surgically removed. The remainder of the small intestine was covered with a saline-soaked gauze and wrapped with plastic wrap to minimize evaporation and tissue dehydration. An intravenous injection of heparin (200 IU·100 g^{-1}) was made immediately before cannulation of the superior mesenteric artery and portal vein. The intestine was perfused at a constant flow rate of 15.2 ± 0.2 ml/min intestine to give a basal pressure of 80–90 mmHg.

Details for perfusion of the mesenteric artery network were essentially as given above for the intestine except that only the superior mesenteric artery was cannulated. In addition the mesenteric network (Fig. 1) was cut free from the intestine and transferred to a perfusion apparatus where it was hung from the cannula inside a sealed glass vessel. Perfusate draining from the cut ends of the arterial network collected at the bottom of the vessel and flowed into the oxygen electrode chamber before being discarded. The flow rate was 2 ml/g wet wt of mesentery per min.

Determination of vascular content

This was attempted only for the mesenteric artery network. Evans Blue (0.5 g·100 ml^{-1}) in perfusion medium was introduced for sufficient time to fill all of the blood vessels. A representative segment of the preparation was blotted and weighed. Extraneous tissue was dissected away to leave only the dye filled blood vessels which were then weighed.

Calculations

Oxygen contents and uptake rates were calculated from arteriovenous oxygen differences and measured steady-state flow rates. Data are shown as the mean ± SE.

RESULTS

Oxygen uptake and pressure development by the perfused hindlimb

We have previously reported that the perfused rat hindlimb responds to noradrenaline and vasopressin with increases in both oxygen uptake and pressure development (Colquhoun *et al.*, 1988, 1989). In those studies flow rate was constant at 4.0 ± 0.1 ml/min and basal perfusion pressure 29 ± 1 mmHg. In the present study perfusate flow was also constant but set so

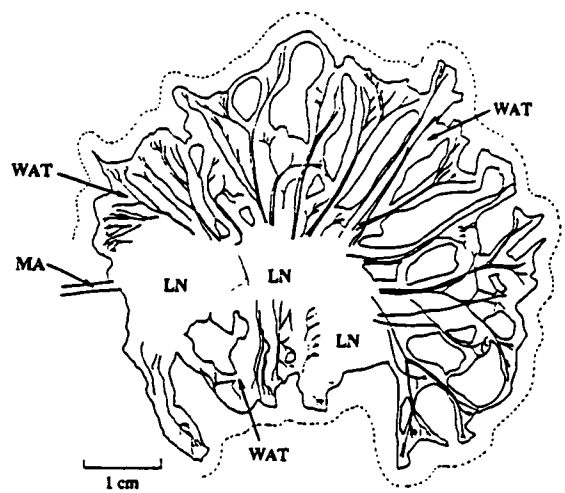

Fig. 1. Rat intestinal mesenteric artery network showing white adipose tissue (WAT), lymph node (LN), and the cannula positioned in the superior mesenteric artery (MA). The broken line shows the original position of the intestine.

that pressures approximating *in vivo* condition were attained. Thus the flow rate was 18.6 ± 0.2 ml/min and basal pressure 90 ± 2 mmHg. Under these conditions (25°C) the rate of oxygen uptake was 14.5 ± 0.4 μmol/h per g hindlimb. This is a little more than twice the value at the lower flow rate (4.0 ml/min) of 6.4 ± 0.2 μmol/h per g wet wt (Colquhoun *et al.*, 1989).

Figure 2 shows dose–response curves for noradrenaline and vasopressin-mediated increases in oxygen uptake and perfusion pressure development. The increase in oxygen uptake from both agonists reached maximal values and concentrations giving half-maximal effects were 0.1 nM vasopressin and 3 nM noradrenaline. The increases in pressure did not appear to have reached maximal values even though there were parallel trends between pressure and oxygen changes for each agonist (Fig. 1). Thus threshold doses for the changes in oxygen uptake and pressure development were similar. The maximum rate of oxygen uptake was 23 μmol/h per g hindlimb, representing an increase of 59% over basal.

Oxygen uptake and pressure development by the perfused kidney

As for the perfused hindlimb, a perfusion flow rate was set for the kidney to maintain a physiological perfusion pressure of approx. 90 mmHg at 25°C. This was achieved at 4.7 ± 0.4 ml/min per kidney and under these conditions oxygen uptake (basal) was 70.3 ± 3.4 μmol/h per g kidney. This compares reasonably well with published values of 171–425 μmol/h per g kidney (Cohen *et al.*, 1980; Franke *et al.*, 1976) obtained at 37°C and at higher

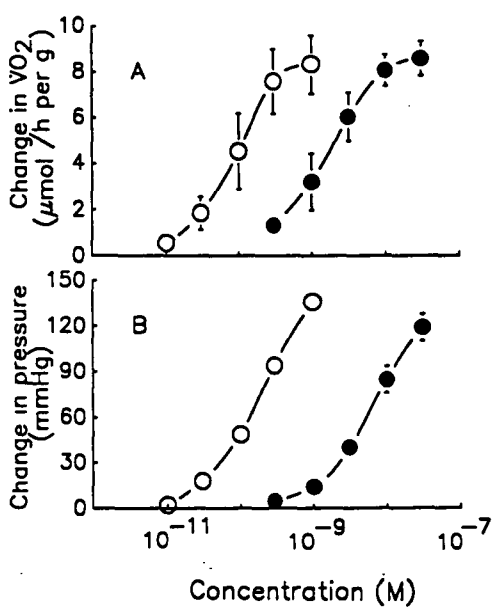

Fig. 2. Effect of vasopressin (○) and noradrenaline (●) on oxygen uptake (A) and perfusion pressure (B) in perfused rat hindlimb. The flow rate was maintained constant at 18.6 ± 0.2 ml/min per hindlimb. Basal values ($n = 6$) were 90 ± 2 mmHg and 14.5 ± 0.4 μmol/hr per g hindlimb for pressure and oxygen uptake, respectively. The weight of hindlimb perfused was 15 ± 2 g. Means ± SE are shown for a total of 6 perfusions involving three with vasopressin and three with noradrenaline. When not visible the error bar is within the symbol.

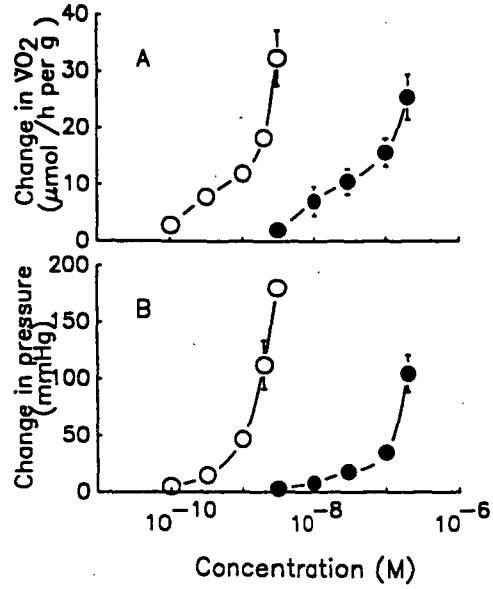

Fig. 3. Effect of vasopressin (○) and noradrenaline (●) on oxygen uptake (A) and perfusion pressure (B) in perfused rat kidney. The flow rate was maintained constant at 4.7 ± 0.4 ml/min per kidney. Basal values ($n = 6$) were 89 ± 5 mmHg and 70.3 ± 3.4 μmol/hr per g kidney for pressure and oxygen uptake, respectively. The weight of kidney perfused was 0.9 ± 0.1 g. Means ± SE are shown for a total of six perfusions. When not visible the error bar is within the symbol.

flow rates (approx. 25 ml/min per g kidney) and therefore higher arterial pressure (112–120 mmHg). The temperature difference could be expected to at least double the rate and the pressure difference between 80 and 112 mmHg could be expected to increase oxygen uptake by a further 23% (Franke *et al.*, 1976).

Figure 3 shows dose-response curves for noradrenaline and vasopressin-mediated increases in oxygen uptake and perfusion pressure development. Although dose-dependent, the increases in oxygen uptake and pressure did not reach maximal values for either agonist. However, it was apparent that changes in pressure and oxygen uptake followed each other closely; the shapes of the dose–response curves were similar for a given agonist. The highest rate of oxygen uptake attained was 102 μmol/h per g kidney, representing an increase of 45% over basal.

Comparisons between Figs 2 and 3 suggest that the hindlimb was at least one order of magnitude more sensitive to both agonists than the kidney.

Oxygen uptake and presssure development by the perfused intestine

Perfusion conditions for the intestine were also chosen to allow maintenance of a pressure approaching physiological. Thus to achieve an arterial perfusion pressure of 82 ± 2.4 mmHg, the flow rate was maintained constant at 15.2 ± 0.1 ml/min per intestine. Under these conditions (at 25°C) the basal rate of oxygen uptake was 33.3 ± 1.0 μmol/h per g intestine. This value compares with values of 98.5 and 150.4 μmol/h per g for fasted and fed rat intestine *in vivo*, respectively (Stevenson and Weiss, 1988) and 60 μmol/hr per g for autoperfused dog intestine

(Granger *et al.*, 1982). As argued above (for kidney) the lower rate observed for intestine in the present study would largely be the result of the experiments being conducted at 25°C rather than 37–39°C.

Figure 4 shows dose–response curves for noradrenaline and vasopressin-mediated increases in oxygen uptake and pressure development. Noradrenaline infusion results in a dose-dependent increase in pressure (+134%) which was accompanied by a parallel decrease in oxygen uptake (−24%). As discussed below the decrease in oxygen uptake within a scenario of increased pressure (vasoconstriction) may result from shunting of perfusate away from regions of respiring tissue.

Vasopressin is regarded as a pure intestinal vasoconstrictor (Richardson, 1984) and in Fig. 4 dose-dependent increases in both oxygen uptake and pressure are shown. However, it is important to note that oxygen uptake increased uniformly over the dose range of 10 pM–3 nM vasopressin whilst pressure changes were relatively small at the lower doses and larger at the top end of the concentration range. The highest rate of oxygen uptake attained was 39 μmol/h per g, representing an increase of 18% over basal.

Oxygen uptake and pressure development by the perfused mesenteric artery network

The mesenteric artery network was perfused *via* the superior mesenteric artery as shown in Fig. 1. Major arteries of this network were severed closely proximal to the intestine and the entire network perfused in a sealed chamber so the effluent could be collected for PO_2 measurement. Because of the nature of the preparation it is likely that most capillaries and veins were not perfused. Consequently the perfusion pressure was quite low at the relatively high flow rate of 2 ml/min per g artery network. Under these conditions the basal rate of oxygen uptake was 6.9 ± 1.5 μmol/hr per g wet wt.

Figure 5 shows dose–response curves for noradrenaline and vasopressin-mediated increases in oxygen uptake and pressure development for the mesenteric preparation. Noradrenaline infusion resulted in a dose-dependent increase in oxygen uptake with a half-maximal effect occurring at 0.3 μM. Pressure also increased in a dose-dependent manner but did not reach a maximal value and was still increasing at the highest dose used of 3 μM noradrenaline. Vasopressin also resulted in a dose-dependent increase in oxygen uptake and pressure development. Half maximal responses occurred for both at approximately 0.3 nM vasopressin. Vasopressin tended to have the greatest effect on oxygen uptake and at maximum dose the rate of oxygen uptake was 12.4 μmol/hr per g artery network, representing an increase of 80% over basal.

Comparison of the four tissues indicated that the hindlimb was the most sensitive to vasopressin and noradrenaline for both oxygen uptake and pressure development. However, since the dose–response curves were incomplete in some instances quantitative comparisons could not be made.

DISCUSSION

The present study was undertaken to address the possibility that vasoconstrictor-mediated oxygen uptake by perfused tissue was a general phenomenon. Two agonists were used and four tissues compared. In all cases except one both vasopressin and

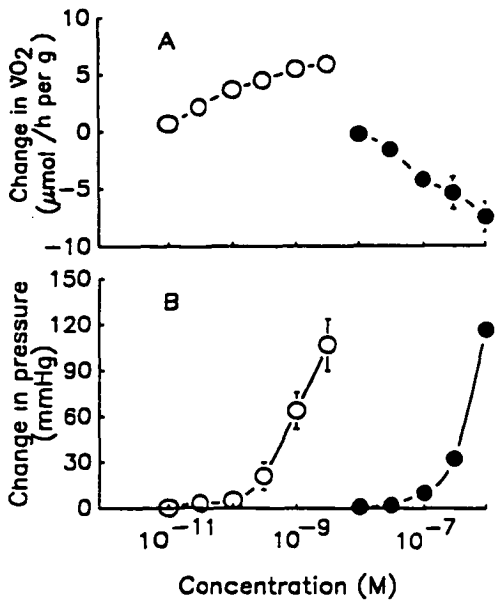

Fig. 4. Effect of vasopressin (○) and noradrenaline (●) on oxygen uptake (A) and perfusion pressure (B) in perfused intestine. The flow rate was maintained constant at 15.2 ± 0.1 ml/min per intestine. Basal values (n = 7) were 82 ± 0.1 and 33.3 ± 1.0 μmol/hr per g intestine for pressure and oxygen uptake, respectively. The weight of intestine perfused was 11.6 ± 0.1 g. Means ± SE are shown for a total of 7 perfusions (four perfusions with noradrenaline). When not visible the error bar is within the symbol.

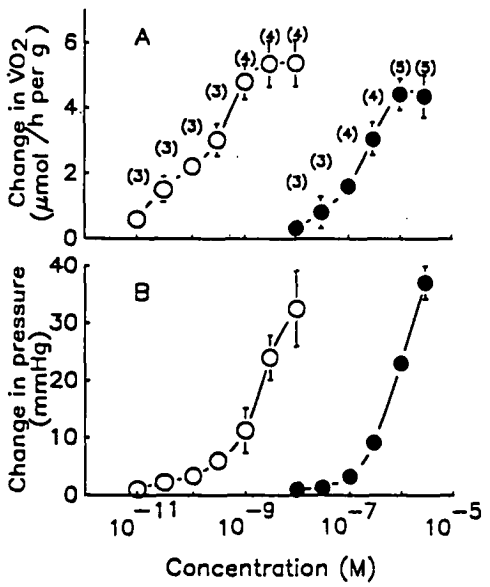

Fig. 5. Effect of vasopressin (○) and noradrenaline (●) on oxygen uptake (A) and perfusion pressure (B) in perfused rat mesenteric artery network. The flow rate was maintained constant at 2 ml/min per g artery network. Basal values (n = 6) were (14 ± 1 mmHg and 6.9 ± 1.5 μmol/hr per g wet wt, for pressure and oxygen uptake, respectively. The weight of perfused mesenteric artery network as shown in Fig. 1 was 1.0 ± 0.1 g. Means ± SE are shown with the number of determinations shown in parentheses. When not visible the error bar is within the symbol.

noradrenaline increased oxygen uptake in association with an increase in perfusion pressure.

Our previous studies using the perfused rat hindlimb have shown that noradrenaline, vasopressin and angiotensin II each stimulate vasoconstriction and oxygen uptake. Both effects were blocked by vasodilators, e.g. nitroprusside, nifedipine or isoproterenol (Colquhoun *et al.*, 1988, 1989). In addition, oxygen consumption by electrically stimulated skeletal muscle was not inhibited by nitroprusside and was additive to vasoconstrictor-induced oxygen uptake (Colquhoun *et al.*, 1989). Furthermore, when flow was increased (and therefore perfusion pressure) oxygen uptake also increased and the addition of noradrenaline or nitroprusside increased or decreased, respectively, the oxygen uptake at all flow rates (Ye *et al.*, 1990). Together these findings led to the conclusion that the vascular system plays a key role in the regulation of resting muscle thermogenesis and we have considered several possible explanations for the data, all of which should be reconsidered in relation to the findings of the present study. These are (a) heterogenous perfusion of the tissue (in the case of the hindlimb this would be the skeletal muscle) that is altered by perfusion pressure; (b) redirection of perfusate to specialized heat-producing cells in the skeletal muscle bed; (c) the presence of receptors on the skeletal muscle for the vasoconstrictors that activate thermogenic mechanisms; and (d) the vascular smooth muscle during constriction consumes the oxygen.

We believe the argument in support of heterogenous perfusion is nullified by the use of flow rates sufficiently high to exceed the threshold pressure (50 mmHg) at which there is a demonstrable decrease in the number of capillaries perfused (Lindbom and Arfors, 1985). Accordingly the hindlimb, kidney and intestine have all been perfused, under basal conditions, at 80–90 mmHg. In addition the argument for heterogenous perfusion of the mesenteric artery network is weak. If the artery network is tied off rather than cut at the points proximal to the intestine there is almost no flow at all even though the pressure may exceed 200 mmHg (Ye, Colquhoun and Clark, unpublished observations). These latter observations suggest that there is a minimal potential for arteriovenous shunting in the mesenteric artery network. However, the perfused intestine shows both an increase in oxygen consumption with perfusion pressure (below 60 mmHg) (Anzueto *et al.*, 1984) and the capacity to decrease oxygen uptake with those vasoconstrictors which decrease blood flow under constant flow conditions (Kvietys and Granger, 1982). This latter effect may be due to a decrease in capillary exchange capacity (Kvietys and Granger, 1982) in turn a product of shunting of perfusate.

The presence of specialized heat producing cells have recently been described in a modified eye muscle of the billfish (Block, 1987). They are proposed to heat blood going to the brain and eye. The cells are characterized by numerous mitochondria, extensive smooth endoplasmic reticulum, and the absence of contractile elements as well as thermogenin. This specialized muscle also has a very extensive vascular counter-current heat-exchanging vascular rate. Block (1987) proposes that heat production in these cells originates from Ca^{2+} cycling by the sarcoplasmic reticulum and that this phenomenon may also be of thermogenic importance in skeletal muscle generally. As yet there is no evidence for this tissue in mammalian skeletal muscle and its presence in intestine, kidney and the mesenteric artery network would seem unlikely.

A direct receptor-mediated metabolic effect of vasoconstrictor agonists on oxidative metabolism is often discussed. However, for rat hindlimb muscle there appears to be few if any α-adrenergic receptors (Rattigan *et al.*, 1986) and the status of vasopressin and angiotensin receptors in this tissue is unknown. Regardless of the presence or otherwise of receptors, the agonist-mediated increase in oxygen uptake would have to be blocked by nitroprusside, nifedipine and isoprenaline (Colquhoun *et al.*, 1989). In addition the flow (pressure)-induced increase in oxygen uptake (Ye *et al.*,1990) could be difficult to explain on the basis of a receptor-mediated effect on oxidative metabolism although stretch-mediated stimulation of smooth muscle contraction is well known (Somlyo and Somlyo, 1960 and references therein). For intestine, vasoactive agents are considered to have either direct or indirect effects on oxidative metabolism (Kvietys and Granger, 1982) although the mechanisms are not clear. For kidney a stimulatory effect of noradrenaline and vasopressin on oxygen uptake due to their effects on metabolism (e.g. Roobol and Alleyne, 1973) is highly likely. However, a metabolic effect of either agonist on oxidative metabolism of tissue other than the smooth muscle/endothelial cells of the mesenteric arteries would seem unlikely, although there is some evidence that the lymph nodes can consume oxygen at high rates (Altman and Dittmer, 1968) and it is possible this rate may be stimulated by noradrenaline and vasopressin.

Of the four possibilities we believe the present data is consistent with the notion that the vascular tissue itself consumes the oxygen as it constricts. In support of this notion calculations based on the vascular content of the hindlimb and the mesenteric artery network yield similar results for the estimated rates of oxygen uptake. Thus for the hindlimb, and assuming a content of 3.4% vascular tissue (Paul, 1980) the basal rate of oxygen uptake is 2.65 μmol/g vascular tissue per min at 37°C increasing to 7.50 and 9.40, respectively with noradrenaline or vasopressin. From our estimates of perfused vascular tissue content of the mesenteric artery network of 6%, the basal rate of oxygen uptake is calculated to be 4.8 μmol/g vascular tissue per min at 37°C. This increases to 8.0 and 8.7 μmol/g per min at 37°C with noradrenaline or vasopressin, respectively.

Conceptually the notion of vascular tissue consuming oxygen in proportion to work done during and whilst maintaining constriction is attractive. The association between striated muscle work and energy consumption is well established (e.g. see Gibbs, 1978 and references therein) and thus an analogy for the vascular smooth muscle/endothelium exists. However, there are properties of the vascular smooth muscle itself as well as the vascular tree of a complex organ such as the hindlimb, intestine or kidney which make a simple relationship between perfusion pressure (an index of work load) and putative oxygen

uptake by the vascular tissue inappropriate. Firstly, vascular smooth muscle is considered to have the potential for "latch bridge" formation. These are noncycling or very slowly cycling cross bridges formed when myosin is dephosphorylated (Murphy *et al.*, 1983). Such "latch bridges" are envisaged as providing an internal resistance to shortening and thereby allow conservation of energy. Secondly vascular tissue is considered to undergo vasomotion (Wiedman, 1984) characterized by oscillatory constriction and dilation. Consequently, the mean arterial pressure of a vascular bed (which may be static) may not necessarily reflect the dynamic work being performed by the vasculature.

At the organ level it seems likely that thoroughfare channels exist and that opening of these may be agonist-specific. For example, serotonin, which in the perfused rat hindlimb increases perfusion pressure also inhibits oxygen uptake. Estimation of perfused tissue volume by dye infusion and washout kinetics (Dora, Colquhoun and Clark, unpublished observations) supports the view that serotonin inhibits oxygen uptake of the rat hindlimb by closing major arterioles causing a pressure rise which in turn opens thoroughfare channels. Thus most of the vascular tree is not perfused due to the closing of a relative few major branch points. Effects of this kind may explain the noradrenaline-mediated inhibition of oxygen uptake by the perfused intestine (Fig. 4) and the attainment of maximal values for oxygen uptake in all four tissues even though perfusion pressures were continuing to rise (Figs 2–5).

In conclusion the present data extend our previous observations (Colquhoun *et al.*, 1988, 1989; Ye *et al.*, 1990) by demonstrating that the vasoconstrictor agents, noradrenaline and vasopressin, not only increase pressure and oxygen uptake in perfused hindlimb but also in kidney, intestine and the mesenteric artery network. Therefore this now appears to be a general phenomenon and exceptions, when they do occur, may be explained by shunting of perfusate (opening of thoroughfare channels). Of the possibilities that can account for the increase in oxygen uptake in conjunction with vasoconstriction in these four preparations we favour the notion that it is the vascular tissue itself (i.e. thermogenesis by "hot pipes"). Further, experiments with pressure-loaded isolated perfused arteries to directly test this proposal are currently in progress.

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Flow-induced oxygen uptake by the perfused rat hindlimb is inhibited by vasodilators and augmented by norepinephrine: a possible role for the microvasculature in hindlimb thermogenesis

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Oxygen uptake in the perfused rat hindlimb was studied at 25°C using an artificial perfusate, and the effects of perfusate flow rate, norepinephrine, and vasodilators were compared. Hindlimb oxygen uptake and perfusion pressure each increased as the flow rate was increased stepwise from 2 to 18.5 mL/min per hindlimb. At each flow rate, the rate of oxygen uptake was inhibited by the vasodilator nitroprusside (0.5 mM) and increased by norepinephrine (5 nM). A corresponding change in perfusion pressure also occurred, with norepinephrine leading to a marked increase and nitroprusside leading to a decrease; however, changes in oxygen uptake and pressure were not linearly related. The lactate/pyruvate ratio of the perfusate was used as an index of tissue perfusion and was determined at each flow rate. Lactate and pyruvate efflux increased as the flow rate was increased stepwise from 2 to 18.5 mL/min per hindlimb. At 2 mL/min per hindlimb, the lactate/pyruvate ratio was 15; at flow rates equal or greater than 4 mL/min per hindlimb, the ratio was constant at 9. Nitroprusside had no significant effect on the ratio at any flow rate even though a marked inhibitory effect on oxygen uptake was evident. Muscle content of high energy phosphates at 8 mL/min per hindlimb did not differ before and after treatment with vasodilators. In addition, the vasodilators had no apparent effect on skeletal muscle oxygen uptake or force development during electrical stimulation. The findings indicate that oxygen uptake by the hindlimb is not limited by inadequate perfusion and that oxygen uptake can be further increased by norepinephrine. The close association of the vasoconstrictive action of norepinephrine with the increase in oxygen uptake and the finding that the increase in oxygen uptake is minimal when nitroprusside is present suggest a central role of the vasculature in the control of muscle thermogenesis. The novel possibility that a significant proportion of oxygen uptake by the resting hindlimb is a consequence of the work done by cells contributing to vasoconstriction (i.e., thermogenesis from "hot pipes") is discussed.

Key words: hindlimb oxygen uptake, oxygen uptake and vasoconstriction, norepinephrine, pressure-induced oxygen uptake, nitroprusside.

YE, J-M., COLQUHOUN, E. Q., HETTIARACHCHI, M., et CLARK, M. G. 1990. Flow-induced oxygen uptake by the perfused rat hindlimb is inhibited by vasodilators and augmented by norepinephrine: a possible role for the microvasculature in hindlimb thermogenesis. Can. J. Physiol. Pharmacol. **68** : 119–125.

On a examiné la consommation d'oxygène par le membre postérieur perfusé du rat à 25°C en utilisant un perfusat artificiel, et comparé les effets de vasodilatateurs, de la norépinéphrine et du débit dans le perfusat. Tant la consommation d'oxygène par le membre postérieur que la pression de perfusion ont augmenté quand le débit a été accru en échelons de 2 à 18,5 mL/min/membre postérieur. À tous les échelons utilisés, le taux de consommation d'oxygène a été inhibé par le vasodilatateur nitroprusside (0,5 mM) et augmenté par la norépinéphrine (5 nM). Des variations correspondantes ont aussi été observées pour la pression de perfusion, qui a été augmentée de façon marquée par la norépinéphrine et diminuée par le nitroprusside; toutefois, les variations de consommation d'oxygène et de pression n'étaient linéairement reliées. La relation lactate/pyruvate dans le perfusat a été utilisée comme indice de perfusion tissulaire et déterminée à chaque débit. L'efflux de pyruvate et de lactate a augmenté quand le débit a été accru en échelons de 2 à 18,5 mL/min par membre postérieur. À 2 mL/min par membre postérieur, le quotient lactate/pyruvate a été de 15; à des débits égaux ou supérieurs à 4 mL/min par membre postérieur, il s'est maintenu à 9. Le nitroprusside n'y a pas eu d'effet significatif à aucun débit, malgré un effet marqué sur la consommation d'oxygène. La concentration de phosphates riches en énergie dans le muscle à 8 mL/min par membre postérieur n'a pas différé avant, ni après le traitement avec les vasodilatateurs. En outre, les vasodilatateurs n'ont pas eu d'effet manifeste sur la consommation d'oxygène ou le développement de force dans le muscle squelettique durant une stimulation électrique. Les résultats indiquent que la consommation d'oxygène dans le membre postérieur n'est pas limitée par une perfusion inadéquate et qu'elle pourrait être augmentée davantage par la norépinéphrine. L'étroite association entre l'action vasoconstrictive de la norépinéphrine et l'augmentation de la consommation d'oxygène, ainsi que la constatation que l'augmentation de la consommation d'oxygène est minime en présence de nitroprusside suggèrent un rôle clé pour le système vasculaire dans le contrôle de la thermogenèse musculaire. On discute de la possibilité qu'une proportion significative de la consommation d'oxygène par le membre postérieur au repos soit le résultat du travail effectué par les cellules participant à la vasoconstriction (c.-à-d. thermogenèse par « conduits de chaleur »).

[Traduit par la revue]

Introduction

Thermogenesis in resting skeletal muscle has been long considered as a significant contributor to whole body heat production (e.g., Jansky 1971; Mejsnar et al. 1980). Indeed data of Foster and Frydman (1978), which focused on the role of brown adipose tissue, were also indicative of a substantial role for skeletal muscle. Their findings showed that for warm-adapted and normal rats norepinephrine administration resulted in a 30% increase in skeletal muscle blood flow and a 60% increase in oxygen consumption. Such data suggested that muscle could produce an equal amount of heat to brown fat (Foster and Frydman 1979), even though oxygen consumption was very much less per gram of tissue. Attempts to address the

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issue more directly by using the perfused hindlimb have supported the above conclusions. The perfused rat hindlimb has been found to respond consistently to norepinephrine administration with a marked increase in the rate of oxygen uptake ($\dot{V}O_2$) (Mejsnar and Jansky 1973; Grubb and Folk 1976; Richter et al. 1982*a*; Côté et al. 1985). This response occurred at rest when the hindlimb was perfused with erythrocyte-containing medium at 37°C (Grubb and Folk 1976; Richter et al. 1982*b*) or without erythrocytes at 25°C (Côté et al. 1985) and at high flow rate at 37°C during muscle contraction with erythrocyte-containing perfusate (Richter et al. 1982*a*). A surprising finding has been that the norepinephrine-mediated increase in rat hindlimb $\dot{V}O_2$ was mediated by an α-adrenergic receptor mechanism (Grubb and Folk 1977) that was invariably associated with an increase in perfusion pressure (Richter et al. 1982*a*, 1982*b*). Recently we have reported that both vasopressin and angiotensin II stimulated $\dot{V}O_2$ and perfusion pressure in the perfused rat hindlimb with both effects blocked by nitroprusside (Colquhoun et al. 1988), thereby supporting the possibility that perfusion pressure (and possibly flow) controls oxygen consumption. Thus in the present study, perfusion flow and pressure have been varied over a wide range and the effects of norepinephrine and vasodilators compared during varied flow.

Since the effects of norepinephrine to increase $\dot{V}O_2$ and of vasodilators to decrease $\dot{V}O_2$ were not diminished as the flow approached maximum, it appeared unlikely that changes in perfusate distribution were responsible. Other possibilities are discussed.

Methods

Hindlimb perfusion

Male hooded Wistar rats (180 ± 5 g body wt.) were fed a commercial diet with water *ad libitum* and anaesthetized with pentobarbital sodium (5 mg/100 g body wt., intraperitoneal). Surgery and perfusion details were as described by others (Ruderman et al. 1971; Richter et al. 1982*a*; Côté et al. 1985). Only one limb was perfused and a tight ligature was placed around the common iliac artery supplying the contralateral hindlimb. To restrict spillover blood flow to the lower back muscles with any rise in perfusion pressure, an addition ligature was placed around the abdomen of the rat approximately at the level of the L3, L4 vertebrae. A ligature was also placed around the tarsus of the perfused limb. Perfusions were conducted in a temperature-controlled chamber at 25 ± 0.2°C (Côté et al. 1985; Colquhoun et al. 1988). The perfusate consisted of a Krebs–Ringer bicarbonate buffer containing 1.27 mM $CaCl_2$, 4% bovine serum albumin, and 8.3 mM glucose.

For perfusions where pressure was varied stepwise, the perfusate was allowed to flow under gravity from a reservoir kept at 4°C through a silastic tube oxygenator continuously equilibrated against a gas mixture of 95% O_2 and 5% CO_2. The temperature of the perfusate was brought to 25°C by passage through a heat exchange coil. Perfusion pressure was measured in the pump outflow line proximal to the aorta. The venous effluent flowed through a 0.5-mL thermostatically controlled chamber (25°C) containing a Clark-type oxygen electrode and the effluent was discarded following measurement of its oxyen content. The pressure was adjusted by relocating the perfusate reservoir to predetermined positions above the hindlimb. Flow was allowed to vary and was monitored by collection into a fraction collector set on time mode. $\dot{V}O_2$ was calculated from the arteriovenous difference in Po_2 and flow rate after allowing for the delay in venous Po_2 measurement due to electrode characteristics. Pressure readings were corrected for the pressure contributed by the aortic cannula (determined without the animal).

For perfusions where flow was varied, the hindlimb was initially equilibrated at 4 mL/min (15 g fresh weight of muscle) for 40 min. Flow was then adjusted to 2.0 mL/min using a calibrated pump and maintained at that rate for 30 min. The flow was then sequentially readjusted to 4, 6, 8, and 18.5 mL/min maintaining constant flow until steady-state pressure and venous Po_2 were achieved. Venous Po_2 and perfusion pressure were monitored continuously. Other details were as described previously (Colquhoun et al. 1988).

Electrical stimulation

The gastrocnemius–plantaris–soleus muscle group was stimulated electrically via the sciatic nerve (Richter et al. 1982*a*). Twitch tension was recorded during electrical stimulation (200-ms trains of 100 Hz applied every 2 s; each impulse in the train being of a duration of 0.1 ms) adjusted (15–20 V) to attain full fibre recruitment. Muscles were stimulated for three periods of 2 min each with rest periods of 30 min between consecutive runs. Venous Po_2 was monitored continuously and maximum $\Delta\dot{V}O_2$ were noted; these occured 1–2 min after commencement of stimulation.

Muscle metabolites

Skin was carefully removed from the hindlimb and the gastrocnemius–plantaris–soleus muscle group exposed. The group was freeze-clamped from anaesthetized rats (denoted "*in vivo*") or from constant-flow perfused hindlimbs following perfusion at 25°C for 120 min with medium at 8 mL/min containing 4% bovine serum albumin and the additions indicated.

Perfusate lactate and pyruvate

Perfusate samples were taken for analyses when steady state was reached as judged by pressure and $\dot{V}O_2$. Lactate and pyruvate were determined on neutralized perchlorate extracts (Bergmeyer 1974).

Statistics

Significance of difference was assessed using the unpaired Student's *t*-test.

Results

Pressure-induced changes in flow and oxygen uptake

These experiments were conducted to assess the effect of changes in pressure on the rates of flow and oxygen uptake. The conditions chosen (erythrocyte-free perfusate at 25°C) were considered likely to minimize autoregulation of flow and the related control of capillary density (e.g., see Chinet and Mejsnar 1989). Figure 1 shows the effect of periodically varying the perfusion pressure from high to low while allowing flow to self-regulate. Initially the rat hindlimb was perfused at a pressure of 30 mmHg (1 mmHg = 133.3 Pa) for 30 min to allow complete equilibration; a constant flow rate of 5.4 mL/min was attained and the $\dot{V}O_2$ was 7.0 $\mu mol \cdot h^{-1} \cdot g^{-1}$ muscle. The perfusate reservoir was then raised to give a new pressure of 57 mmHg. Perfusate flow immediately increased to 13 mL/min then, over the next 30 min, gradually decreased to 11 mL/min. $\dot{V}O_2$, calculated from the arteriovenous difference in Po_2 and flow rate, peaked at 19.5 μmol/h per g muscle immediately following the increase in pressure, then decreased gradually over the next 30 min to 8 $\mu mol \cdot h^{-1} \cdot g^{-1}$ muscle. When the pressure was decreased from 59 to 20.5 mmHg by lowering the perfusate reservoir, the flow decreased to 1.5 mL/min and then increased marginally to 2.0 mL/min (Fig. 1). $\dot{V}O_2$ decreased immediately following the fall in pressure and flow to 0.5 $\mu mol \cdot h^{-1} \cdot g^{-1}$ muscle but then gradually increased over the next 30 min to 3 $\mu mol \cdot h^{-1} \cdot g^{-1}$ muscle. A similar relationship between pressure, flow, and $\dot{V}O_2$ as described above was again evident when the pressure was reset to 32 mmHg (Fig. 1).

Overall the extent of flow regulation in the present system was slow when compared with blood perfused systems where an increase in perfusion pressure (or flow) is followed by a

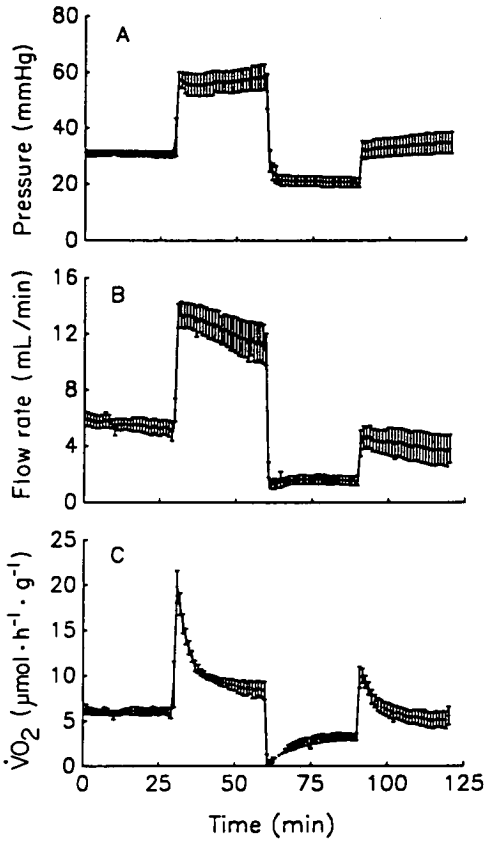

FIG. 1. Effect of perfusion pressure (A) on flow (B) and $\dot{V}o_2$ (C) of the perfused rat hindlimb. Data are means ± SEM from four perfusions.

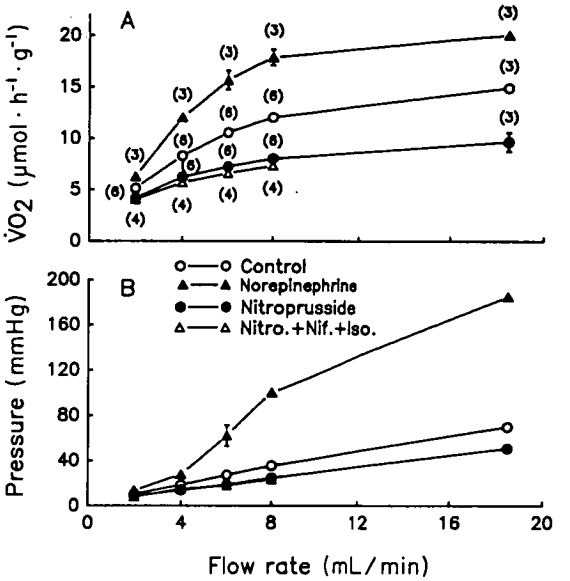

FIG. 2. Effect of perfusate flow, norepinephrine, and vasodilators on $\dot{V}o_2$ (A) and pressure development (B). Additions to the perfusate were none (○), 5 mM norepinephrine (▲), 0.5 mM nitroprusside (●), and a combination of 0.5 mM nitroprusside, 1 μM nifedipine, and 1 μM isoprenaline (△). The number of perfusions are given in parentheses. Data are means ± SEM; bars not visible are within the symbols.

rapid increase (2 min) in resistance to blood flow, and a decrease in perfusion pressure (or flow) is followed by a reduction in resistance to flow (Stainsby and Renkin 1961). The slow responses shown in Fig. 1, sometimes taking 20 to 30 min to reach steady state, may be those of drift in a passive vascular bed but are in the autoregulatory direction. The lack of rapid autoregulation may be attributable to the use of artificial perfusate as well as to the lower temperature (25°C).

Flow-induced changes in oxygen uptake and pressure

The relationship between flow and steady-state oxygen consumption and pressure development, as well as the effect of vasodilators and norepinephrine on these latter parameters is shown in Fig. 2. Perfusate flow rate was set at 2, 4, 6, 8, or 18.5 mL/min for at least 15 min and sometimes 40 min during which time pressure and oxygen uptake were recorded. Figure 2A shows that $\dot{V}o_2$ increased as the flow rate was increased and that this increase was further augmented by 5 nM norepinephrine.

Vasodilators and flow-induced changes

Three types of vasodilators were chosen for this study. Nitroprusside (acting via cyclic GMP), nifedipine (a Ca^{2+} channel blocker), and isoproterenol (acting via cyclic AMP) were used. Figure 2A shows that the increase due to flow was inhibited by either 0.5 mM nitroprusside or a combination of 0.5 mM nitroprusside + 1 μM nifedipine + 1 μM isoproterenol. The flow-dependent rise in pressure was also inhibited by the vasodilators (Fig. 2B). However, there was no significant difference between the effects of nitroprusside alone or the three vasodilators together.

Vasodilators and working skeletal muscle

Since oxygen uptake by the hindlimb at all flow rates was markedly inhibited by the vasodilators, it was considered relevant to assess the effects of these agents on working skeletal muscle $\dot{V}o_2$. Table 1 shows that neither nitroprusside alone nor the combination of nitroprusside, nifedipine, and isoproterenol had any significant effect on the increase in $\dot{V}o_2$ by the perfused contracting hindlimb at constant flow (4 mL/min). More importantly, the twitch tension force development was also unaffected by the vasodilators (Table 1).

Muscle metabolites

The experiments of Table 2 were conducted to assess whether nitroprusside-induced inhibition of hindlimb O_2 uptake was associated with a decrease in muscle content of high energy phosphates. Table 2 shows metabolite concentrations for the gastrocnemius–plantaris–soleus muscle group from hindlimbs freeze-clamped before surgery (*in vivo*) and following perfusion for 120 min with no additions, 0.5 mM nitroprusside, and a combination of the vasodilators nitroprusside (0.5 mM), nifedipine (1 μM), and isoprenaline (1 μM). None of the differences between muscles perfused with or without vasodilators or between muscles perfused and muscles *in vivo* was significant.

Perfusate lactate/pyruvate ratio

The lactate/pyruvate ratio is considered a reliable indicator of muscle oxygenation (Bylund-Fellenius et al. 1981; Shiota and Sugano 1986) and was thus used in the present study.

TABLE 1. Effect of vasodilators on twitch tension force development and $\dot{V}O_2$ of the hindlimbs

Addition	Twitch tension (g) 0 min	Twitch tension (g) 2 min	$\Delta\dot{V}O_2$ ($\mu mol \cdot h^{-1} \cdot g^{-1}$)
None	603±132	220±49	3.90±0.38
0.5 mM nitroprusside	641±75 ns	200±64 ns	4.50±0.34 ns
0.5 mM nitroprusside +1 μM nifedipine + 1 μM isoproterenol	547±151 ns	217±47 ns	3.86±0.42 ns

NOTE: Data are mean ± SEM; three hindlimbs per group and perfusions were conducted at 4.0 mL/min; ns, not significantly different from "none."

TABLE 2. Metabolite concentrations in the gastrocnemius–plantaris–soleus muscle group of rat hindlimb

Muscle origin	n	Wet/dry wt.	Metabolite concentration (μmol/g dry wt.) ATP	CrP	Lactate	Pyruvate	Lactate/pyruvate
In vivo	3	4.4±0.1	27.3±1.1	71.9±4.1	10.5±1.6	0.7±0.1	16.8±5.3
Perfused 120 min							
No additions	5	4.5±0.2	29.7±0.9	72.5±1.9	9.2±1.0	1.0±0.1	9.2±1.1
Nitroprusside	5	5.3±0.3	27.2±1.7	70.1±2.32	9.7±1.2	1.1±0.1	8.9±1.3
NNI	4	4.9±0.2	26.0±0.9	63.3±0.9	9.0±0.9	0.9±0.2	10.3±2.0

NOTE: Data are mean ± SEM; perfusions were conducted at 8.0 mL/min. NNI, combination of nitroprusside, nifedipine, and isoprenaline.

Figure 3A shows lactate and pyruvate release at each flow rate ±0.5 mM nitroprusside. Lactate release increased as the perfusate flow rate was changed from 2 to 18.5 mL/min. Pyruvate release also increased over this range of flow rates (Fig. 3B). The vasodilator, nitroprusside, had no significant effect on either lactate release or pyruvate release. Thus the net effects of changing flow rate from 2 to 18.5 mL/min was to lower the perfusate lactate/pyruvate ratio from approximately 15 at 2 mL/min to a constant value of approximately 9 at 4 mL/min and beyond. In addition, and as shown in Fig. 3C, the ratio was not affected by the presence of nitroprusside. Overall these data suggest that inadequate oxygen supply to the resting perfused rat hindlimb at 25°C occurs only at flow rates less than 4 mL/min.

Discussion

In the present study, the effects of perfusate flow rate, norepinephrine, and vasodilators on oxygen uptake have been assessed using a system where rapid autoregulation has been largely avoided (Fig. 1). A principal finding was that oxygen uptake by the perfused rat hindlimb increased as flow was increased to approach a plateau at 18.5 mL/min or 1.3 mL · $min^{-1} \cdot g^{-1}$ (Fig. 2). At flow rates less than 8 mL/min, the hindlimb data illustrate a widely noted property of perfused noncontracting skeletal muscle preparations where $\dot{V}O_2$ increases with an increase in perfusion pressure or flow. This relationship has been reported for rat gracilis muscle (Honig et al. 1971), dog gastrocnemius–plantaris muscle group at low but not high blood flow levels (Stainsby and Otis 1964), dog hindlimb with (Coburn and Pendleton 1979) or without (Pappenheimer 1941) norepinephrine infusion, isolated autoperfused preparations of the cat soleus (Whalen et al. 1973) and gracilis (Whalen et al. 1973) muscles, resting and isolated gracilis muscles of dogs (Beer and Yonce 1972), and hindlimbs of lemmings (Grubb and Folk 1978) and rats (Grubb and Folk 1978; Shiota and Sugano 1986). The relationship appears to be readily observed in rat muscle preparations without exception (Honig et al. 1971; Grubb and Folk 1978; Shiota and Sugano 1986) but, for as yet unknown reasons, is not always apparent in dogs (Duran and Renkin 1974, 1976; Honig et al. 1971; Chapler et al. 1980).

To date, the dependence of $\dot{V}O_2$ on blood flow has been interpreted as a physiological limitation of O_2 consumption in resting muscle by O_2 transport, owing to the inhomogenity of a capillary function (Lindbom and Arfors 1985) and uneven distribution of O_2 within the organ (Honig et al. 1971; Shiota and Sugano 1986). To address this issue we have used a range extending from approximately half to four times the normal hindlimb blood flow rate of the conscious rat (Armstrong and Laughlin 1985). Under these conditions it was noted that nitroprusside reduced pressure and inhibited oxygen uptake at all flows and particularly at the highest flow where tissue perfusion could be expected to be approaching the maximum. Furthermore, the effect of norepinephrine to increase $\dot{V}O_2$ and pressure was greater at high than low flow rates. It could be argued that if areas of the hindlimb were inadequately perfused, any shunting effect of norepinephrine would be greater at the lowest flow and pressure. This reasoning is based on the notion that the increase in pressure due to norepinephrine results in increased perfusate O_2 delivery to previously underperfused regions of skeletal muscle and that these regions are more extensive at low pressures.

The question of tissue underperfusion was also addressed by assessing the biochemical indices of hypoxia. Poorly perfused muscle has a lower creatine phosphate concentration (Ruderman et al. 1971; Challiss et al. 1986) and increased production of lactate (Ruderman et al. 1971). Thus, to assess the possibility that nitroprusside-induced inhibition of O_2 uptake resulted from tissue underperfusion, a representative sample of muscle (the gastrocnemius–plantaris–soleus group) was freeze-clamped and assayed for the content of ATP, creatine

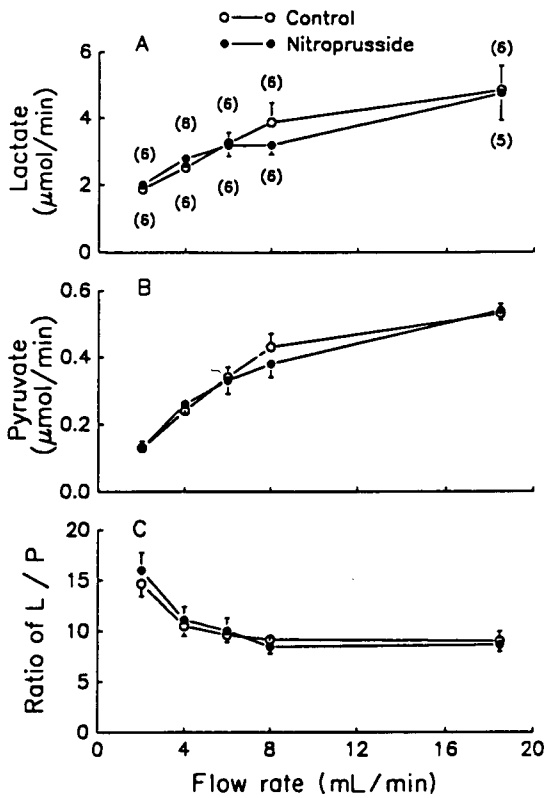

FIG. 3. Effect of perfusate flow and nitroprusside on lactate (A) and pyruvate (B) release as well as the perfusate lactate/pyruvate ratio (C). Additions to the perfusate were none (○) or 0.5 mM nitroprusside (●). The numbers of perfusions are given in parentheses. Values in A and B are the product of concentration (μmol/mL) × flow rate (mL/min). Data are means ± SEM; bars not visible are within the symbols.

phosphate, lactate, and pyruvate. If the vasodilators decreased $\dot{V}O_2$ by decreasing the area of skeletal muscle actually perfused, then it could be expected that creatine phosphate and ATP levels would be lower following this treatment. Despite the fact that the vasodilators had resulted in a 40% inhibition of O_2 uptake under the conditions chosen, there was no significant difference between vasodilator-treated and untreated muscle with respect to metabolite concentration.

The lactate/pyruvate ratio is a sensitive index of hypoxia (Bylund-Fellenius et al. 1981) and was also used in the present study to assess whether the increase in $\dot{V}O_2$ by the hindlimb induced by the increase in perfusate flow rate was the result of increasing O_2 delivery to previously underperfused regions. Since the ratio was higher at 2 mL/min per hindlimb than at all other flow rates and was constant at rates equal to or greater than 4 mL/min per hindlimb, it appeared unlikely that hindlimb tissue was underperfused at flow rates other than 2 mL/min per hindlimb. It is also worthy of note that 0.5 mM nitroprusside which inhibited $\dot{V}O_2$ at 18.5 mL/min per hindlimb to a value similar to that of the untreated hindlimb perfused at 5 mL/min had no effect on the lactate/pyruvate ratio at the high flow rate. These data taken together imply that flow- and agonist-induced $\dot{V}O_2$ results from processes other than changes in O_2 delivery to skeletal muscle fibres.

Another finding made in this study was that the vasodilator, nitroprusside, did not inhibit oxygen uptake or twitch-tension development by working skeletal muscle. This contrasts with its marked effect to inhibit oxygen uptake induced by vasoconstrictors (Colquhoun et al. 1988) or pressure (Fig. 2) where dilation of thoroughfare channels might have occurred. However, the opening of thoroughfare channels would be expected to compromise oxygen availability to working skeletal muscle and thereby reduce working skeletal muscle oxygen uptake and twitch-tension development. Clearly this was not evident from the present experiments (Table 1) and thus further investigations are required.

A direct effect of norepinephrine on skeletal muscle to increase oxygen uptake would be consistent with the observations made in the present study. However, it must be kept in mind that the effect of norepinephrine is mediated by α-adrenergic receptors (Grubb and Folk 1977; Richter et al. 1982*a*) and that oxygen uptake by the hindlimb is also increased by vasopressin or angiotensin II (Colquhoun et al. 1988). There are relatively few, if any, α-adrenergic receptors on striated muscle cells (Rattigan et al. 1986) and a biochemical mechanism to account for the large increase in oxygen consumption is yet to be defined. Furthermore, the failure to observe stimulatory effects of norepinephrine on oxygen uptake by isolated (unperfused) muscles *in vitro* (Hannon et al. 1963; Eaton 1964) or homogenates (Hannon et al. 1963) comparable to those reported for the perfused hindlimb (Mejsnar and Jansky 1973; Grubb and Folk 1976; Richter et al. 1982*a*; Côté et al. 1985; Colquhoun et al. 1988) suggests a complex mechanism, possibly dependent upon a functioning vascular system.

It is interesting that there has been no discussion by other researchers in this field of the possibility that work performed by the vascular system in holding or resisting the perfusion pressure could contribute to the $\dot{V}O_2$. This may be because the possibility has not been directly explored, or that $\dot{V}O_2$ by isolated vascular smooth muscle preparations so far tested appears to be considerably lower than that required to account for pressure- or agonist-induced changes in $\dot{V}O_2$. Literature values for $\dot{V}O_2$ obtained by Warburg techniques of various vascular smooth muscle fragments incubated *in vitro* at 37°C (see review by Paul 1980) range from 0.03 to 0.15 $\mu mol \cdot g^{-1}$ wet wt. $\cdot min^{-1}$. However, $\dot{V}O_2$ for cannulated canine femoral artery (0.446 $\mu mol \cdot g^{-1}$ wet wt. $\cdot min^{-1}$) is more than 10-fold greater than incubated segments (0.040–0.051 $\mu mol \cdot g^{-1}$ wet wt. $\cdot min^{-1}$ (Kosan and Burton 1966), which may reflect the effect of pressure loading of the vessel. Microvessels also have the potential for considerable rates of oxygen uptake. Isolated unstimulated, nonperfused brain microvessels have an endogenous rate of oxygen uptake of 10.9 $nmol \cdot min^{-1} \cdot mg^{-1}$ protein at 37°C (Brendel et al. 1974). At 150 mg protein/g wet wt. this corresponds to 1.6 $\mu mol \cdot g^{-1}$ wet wt. $\cdot min^{-1}$. Such vessels can further increase their oxygen uptake threefold when given succinate as a substrate (Sussman et al. 1988) and presumably would use even more oxygen when pressure loaded. Calculations based on the data of Fig. 2A indicate that the maximal norepinephrine-induced increase in $\dot{V}O_2$ of the hindlimb would be comparable to the above values. Thus the difference in oxygen uptake values between norepinephrine and nitroprusside at a flow rate of 18.5 mL/min of 10.4 $\mu mol \cdot h^{-1} \cdot g^{-1}$ muscle tissue at 25°C would equate to 5 $\mu mol \cdot min^{-1} \cdot g^{-1}$ vascular tissue if this latter tissue was 3.4% of the hindlimb muscle mass (Paul 1980). At 37°C, with a Q_{10} of 2.5 (Paul 1980) it would correspond to 12.7 $\mu mol \cdot min^{-1} \cdot g^{-1}$ vascular tissue. Working muscle is capable of high rates

of oxygen consumption and rates of approximately 6 μmol · $min^{-1} \cdot g^{-1}$ have been reported for contracting dog gastrocnemius muscle *in situ* (Stainsby et al. 1984) and the Langendorff perfused beating rat heart at 60 mmHg aortic pressure (Randle and Tubbs 1979). Even higher rates of oxygen uptake (approximately 14 μmol · $min^{-1} \cdot g^{-1}$) have been reported for the Langendorff perfused beating rat heart at 120 mmHg aortic pressure (Randle and Tubbs 1979). Thus the key issues are the actual hindlimb content of vascular smooth muscle and its inherent oxidative capacity during contractile activity. Histochemical properties of arteriolar smooth muscle are consistent with this tissue being capable of higher rates of oxidative metabolism than surrounding skeletal muscle (Granger et al. 1984).

Within the context of whole body thermogenesis, it is relevant to consider the hindlimb oxygen uptake data in relation to similar studies on brown adipose tissue. The difference in oxygen uptake values between norepinephrine and nitroprusside at the flow rate of 18.5 mL/min of 10.4 μmol · $h^{-1} \cdot g^{-1}$ muscle tissue at 25°C (Fig. 2A) can be used to calculate the maximum component of nonshivering thermogenesis that might be attributable to vascular control for the intact warm-acclimated rat. The increase in oxygen uptake of 10.4 μmol · $h^{-1} \cdot g^{-1}$ muscle corresponds to 1.32 W/kg muscle at 25°C, assuming a standard average energy value of 4.83 kcal/L O_2 at STP (1 kcal = 4.1855 kJ) (Brown and Brengelmann 1965). At 37°C, with a Q_{10} of 2.5 (Paul 1980) this would equate to 3.3 W/kg muscle, and for a 200-g rat with 43% of the body mass as muscle to 0.28 W. For comparison, brown adipose tissue content of the warm-acclimated rat of approximately the same body weight is 1.22 g (Smith and Horwitz 1969; Himms-Hagen 1970; Bukowiecki et al. 1980) and has a total capacity for catecholamine-stimulated thermogenesis of 1.5 mL O_2/min (Bukowiecki et al. 1980). This would correspond to 0.37 W. Thus the potential contributions from muscle tissue and brown adipose tissue are similar and together could account for 65% of the thermogenic response to catecholamines of adult warm-acclimated rats weighing 200–250 g of 3 mL O_2/min or 1 W (Bukowiecki et al. 1980).

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Vasoconstrictors alter oxygen, lactate, and glycerol metabolism in the perfused hindlimb of a rat kangaroo

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Ye, Ji-Ming, Stephen J. Edwards, Randy W. Rose, Stephen Rattigan, Michael G. Clark, and Eric Q. Colquhoun. Vasoconstrictors alter oxygen, lactate, and glycerol metabolism in the perfused hindlimb of a rat kangaroo. *Am. J. Physiol.* 268 (*Regulatory Integrative Comp. Physiol.* 37): R1217–R1223, 1995.—The Tasmanian bettong (*Bettongia gaimardi*) is a small marsupial rat kangaroo without detectable brown adipose tissue (BAT). The hindlimb was perfused with constant flow at 25°C after cannulation under anesthesia of the femoral artery and vein to one hindlimb. Norepinephrine (NE, 25 nM–2.5 μM) and vasopressin (VP, 10 nM–0.1 μM) each increased perfusion pressure, oxygen consumption ($\dot{V}O_2$), and lactate and glycerol efflux of the perfused hindlimb. NE-mediated increases in $\dot{V}O_2$ and the efflux of lactate and glycerol were unaffected by propranolol (10 μM) but were completely blocked by the further addition of phentolamine (10 μM). In contrast, serotonin (5-HT; 0.1–2.5 μM) inhibited $\dot{V}O_2$ and inhibited lactate efflux. The changes induced by NE, VP, and 5-HT were all rapidly reversed by nitroprusside. These results suggest that resting thermogenesis in bettong hindlimb can be differentially controlled by the vasculature, which may also contribute to the induced $\dot{V}O_2$. This vascular control of skeletal muscle $\dot{V}O_2$ appears widespread in homeotherm evolution.

marsupial; *Bettongia gaimardi;* vasoconstriction; energy metabolism; thermogenesis; skeletal muscle

MARSUPIALS ARE A DIVERSE GROUP of mammals with well-developed thermoregulation. The Tasmanian bettong (*Bettongia gaimardi*) is a rat kangaroo closely related to the larger kangaroos and wallabies. At birth, the bettong is ectothermic. The young becomes progressively endothermic before it finally vacates the pouch (25). Intramuscular injection of norepinephrine (NE) elicited a significant increment in oxygen consumption ($\dot{V}O_2$) in vivo in the potoroo *Potorous tridactylus* (Kerr), a close relative of the bettong, and this increase was blocked by propranolol but not by phenoxybenzamine (23). Hayward and Lisson (15) have presented strong anatomic and microscopic evidence that marsupials, including bettongs, do not possess brown adipose tissue (BAT), which is a major contributor to nonshivering thermogenesis in most newborn and small eutherian mammals. This characteristic makes the bettong a valuable animal for the study of nonshivering thermogenesis occurring in tissues other than BAT, such as skeletal muscle.

The perfused rat hindlimb preparation has proved to be a useful model in defining the controlling influences for thermogenesis originating from skeletal muscle, particularly under resting conditions. Thus in the perfused rat hindlimb, we have found that vasoconstrictors [excluding serotonin (5-HT)] such as NE, epinephrine (Epi), vasopressin (VP), and angiotensin (ANG) I, II, and III, increase $\dot{V}O_2$ within a limited dose range while causing vasoconstriction in a constant-flow preparation (8, 9, 32, 33). In addition, vasodilators, such as nitroprusside, nifedipine, and isoproterenol, blocked the vasoconstrictor-mediated $\dot{V}O_2$ (7–9). Increasing perfusion flow also increased $\dot{V}O_2$, which could be further augmented by NE but depressed by nitroprusside (33).

Thus the concept has emerged (7) that either increased flow to, or vasoconstriction within, the rat hindlimb led to enhanced thermogenesis from this vascular bed. However, the findings that 5-HT (11) and high, but not low, concentrations of NE decreased $\dot{V}O_2$ by the constant-flow perfused rat hindlimb have led to a modification of the above proposal (6) and suggested the possibility that preexisting vascular tone, particularly in vivo, might be under some circumstances inhibitory. If so, then vasodilators, such as β-adrenergic agonists, could also exert a positive thermogenic response by decreasing the preexisting inhibitory effect on $\dot{V}O_2$. Alternatively, β-activation, by increasing cardiac output and hence flow to muscle, may increase thermogenesis (33). These two explanations are not mutually exclusive and could coexist.

No study on $\dot{V}O_2$ in a perfused marsupial hindlimb has been found in the literature. As a result of a successful breeding program, a small number of bettongs, which are legally protected under state law, became available for experimentation. Accordingly, the present study attempted to examine whether the vasoconstrictive hormones, NE, VP, and 5-HT, have any effect on $\dot{V}O_2$ and on lactate and glycerol efflux from the perfused hindlimb of the bettong, as an example of marsupial muscle metabolism.

METHODS

Animals. Bettongs of either sex were obtained from the University of Tasmania breeding colony (by R. W. Rose). The experiments were approved by the Ethics Committee of the University of Tasmania under the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. The animals were housed in room-size cages (approximately 6 × 2 m) in an outdoor animal compound with free access to food and water. During the period of experiment, the seasonal temperature ranged between 18 and 28°C during the daytime and between 10 and 20°C at night. Diet was a choice of apples, bread, and Pal dog pellets (Uncle Ben's of Australia), containing 15% crude protein and 10% fat.

Hindlimb perfusion. The animals (mass 1.2 ± 0.11 kg) were anesthetized with an intraperitoneal injection of pentobarbital sodium at a dose of 60 mg/kg. Additional injections were given to maintain the anesthesia when required. An incision was made distal to the inguinal ligament. The femoral artery and

vein were exposed. Heparin (1,000 IU) was injected into the femoral vein at the level of the inguinal ligament 1 min before cannulation, and an arterial cannula and a venous cannula were then inserted into the respective vessels just below the branch points for the superficial iliac circumflex artery and vein. A tight ligature was placed around the circumference of the thigh ~1 cm above the two cannulas, and another tight ligature around the tarsus, so that only the muscle bed distal to the inguinal ligament of the limb was perfused. The mass of muscle perfused was determined for the first three animals by excising and weighing dye-containing muscle from the contralateral limb that had been infused with perfusion medium containing Evans blue (1% wt/vol) after the same surgical procedures and heparinization of the animal but before the perfusion commenced on the remaining hindlimb. A regression formula was then calculated [perfused muscle mass (g) = [31.87 × body wt (kg)] − 9.05 ($r = 0.9993$, $P < 0.05$)] and was applied to the following experimental animals, on the assumption that both hindlimbs were identical in muscle mass. A similar surgical strategy was adopted when muscle samples were collected from the contralateral hindlimb for the in vivo muscle lactate and phosphagen samples. After such sampling the femoral artery and vein were ligated together with a circumferential ligature around the thigh to prevent any hemorrhage before perfusion of the remaining hindlimb.

The perfusion method was essentially that previously described for the rat hindlimb by Colquhoun et al. (9). The perfusate consisted of Krebs-Ringer bicarbonate buffer containing 1.27 mM $CaCl_2$, 2% bovine serum albumin, and 8.3 mM glucose. The perfusate was pumped from a reservoir kept at 4°C through a Silastic tube oxygenator continuously equilibrated against a gas mixture of 95% O_2–5% CO_2 and a heat exchanger connected in series at 25°C. The $\dot{V}O_2$ was calculated from arteriovenous difference of oxygen partial pressure measured with a Clark-type oxygen electrode as follows

$$\dot{V}O_2(\mu mol \cdot g^{-1} \cdot h^{-1}) = 1.508 \times (Pa_{O_2} - Pv_{O_2})(mmHg)/[1{,}000\ (ml/l) \times \text{muscle mass (g)}] \times 60\ (min/h) \times \text{flow rate (ml/min)}$$

where 1.508 ($\mu mol \cdot l^{-1} \cdot mmHg^{-1}$) is the Bunsen coefficient for plasma at 25°C (5), and Pa_{O_2} and Pv_{O_2} are arterial and venous oxygen partial pressures, respectively.

The Pa_{O_2} was measured at the beginning and end of each perfusion by connection of arterial and venous lines without passing through the hindlimb, so that diffusional losses from the tubing would be the same during perfusion. Pv_{O_2} was continuously monitored throughout the perfusion by passing the effluent through a Clark-type oxygen electrode with a chamber volume of ~0.5 ml. The perfusion pressure was also continuously monitored through a sidearm connected to the perfusion line before the arterial cannula and recorded via a pressure transducer. To avoid the perturbation to metabolite assays by erythrocytes and to allow comparisons with results already obtained in our laboratory from other species (8, 9, 11, 16), the perfusion was conducted with an erythrocyte-free medium at 25°C. The perfusion flow rate per mass of hindlimb muscle (0.28 ± 0.02 $ml \cdot g^{-1} \cdot min^{-1}$) was adjusted to be as close as possible to the flow rate used previously for the perfused rat hindlimb (0.27 $ml \cdot g^{-1} \cdot min^{-1}$) (8, 9, 11, 16). For all perfusions the Pv_{O_2} remained >200 mmHg, indicating hindlimb flow was adequate.

Calorimetric measurement. The system was composed of a sealed respiratory chamber sitting in a water bath, a coil of copper piping placed in the water bath to act as a heat exchanger for the inlet air, a vacuum pump for controlling air flow through the chamber, a sampling pump, and a modified O_2–CO_2 gas analyzer (Datex, Labtech, Helsinki, Finland). The experiments were performed between 10 A.M. and 6 P.M., which is in the animal's normal sleep and quiescent phase.

Bettongs were placed in well-ventilated coarse-woven jute bags to reduce ambient light influences to a minimum and were then moved into the respiratory chamber maintained at 25°C. The flow rate of air through the chamber was adjusted to between 6.5 and 8.0 l/min to keep the expired CO_2 below . The expired air was continuously monitored with the analyzer, and the data were collected and stored in a computer for every 1-min interval during the period of experiment. The resting metabolic rate was taken as the minimum 5-min average when the animals stayed quiet. Normally, animals settled well within 15 min, but 30 min were allowed to elapse before data were collected. For comparison, metabolic rates were also determined on rats (male Wistar, 180–200 g) in the same system. Heat production was calculated from the oxygen consumed at rest under conscious condition with the method by Weir (30).

Metabolite assay. For the measurement of muscle metabolites, the calf muscle group (gastrocnemius-plantaris-soleus group) was freeze-clamped with liquid N_2-precooled aluminium tongs. The in vivo samples were taken under anesthesia before perfusion from the contralateral leg. The perfused samples were taken at the end of the perfusion. Samples, after storage at −80°C, were processed by pulverizing under liquid N_2, and a portion (200 ± 20 mg) was homogenized in 2.0 ml of perchloric acid (0.42 M), which was then neutralized with dipotassium hydrogen phosphate (1.0 M) to pH 6.5. The neutralized perchloric acid extract was then used for the assay of muscle lactate (0.2 ml) and high-energy phosphate compounds (10–20 μl). For the measurement of lactate and glycerol release, 2.0 ml of venous effluent were collected either at each steady state or at nominated times for the time-response curves and were frozen (−20°C) until assay. The arterial perfusate was used as the blank in the assays. Lactate was assayed according to the method of Gutmann and Wahlfeld (14). Glycerol was measured enzymatically, essentially as described by Wieland (31), using the change in fluorescence of NADH measured with an Aminco-Bowman spectrophotofluorometer set at 340 nm (excitation) and 460 nm (emission). High-energy phosphate compounds were determined by high-performance liquid chromatography (26) with a Nova Pak C_{18}, 4-μm column (Waters Associates).

Chemicals. The enzymes, NAD, AMP, ADP, and ATP were purchased from Boehringer Mannheim (Germany). NE, VP, 5-HT, phentolamine, and propranolol were obtained from Sigma (St. Louis, MO). Bovine serum albumin (fraction V) was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN) and dialyzed five times against distilled water. Sodium nitroprusside was purchased from Merck. All other chemicals were analytic grade from Ajax Chemicals (Australia).

Statistics. Student's *t*-test was used for the statistics, and $P < 0.05$ was considered significantly different. All values are presented as means ± SE.

RESULTS

Validation of perfusion. Hindlimb perfusions were performed on eight bettongs (7 male, 1 female). Table 1 shows the mean values for body weight of these young animals aged 6–8 mo, which have left the pouch for some time. The mass of perfused skeletal muscle, as well as the Pa_{O_2}, perfusion pressure, hindlimb $\dot{V}O_2$, and rate of release of lactate and glycerol under basal conditions are also shown in Table 1. Perfusion conditions ap-

Table 1. *Basal measurements for perfused bettong muscle*

Body Mass, kg	Perfused Muscle Mass, g	Perfusate Flow Rate, ml·g^{-1}·min^{-1}	Perfusion Pressure, mmHg	Arterial Po_2, mmHg	$\dot{V}o_2$, μmol·g^{-1}·h^{-1}	Lactate Efflux, μmol·g^{-1}·h^{-1}	Glycerol Efflux, μmol·g^{-1}·h^{-1}
1.20 ± 0.11	25.0 ± 2.34	0.28 ± 0.02	32.0 ± 2.30	685.6 ± 10.3	4.18 ± 0.35	4.41 ± 0.39	0.28 ± 0.03

Values are means ± SE; n = 7–8 animals.

peared stable; $\dot{V}o_2$ as well as lactate and glycerol efflux remained constant for 3–4 h. Measurement of metabolites in the frozen gastrocnemius-plantaris-soleus muscle group at the end of the perfusion period showed that there were no significant statistical differences of adenine nucleotides between the muscle samples obtained in vivo and after perfusion. In fact, the concentration of lactate and creatine decreased ($P < 0.01$), and the phosphocreatine-to-ATP ratio was improved ($P < 0.05$) after the perfusion (Table 2). These results show an essentially normal metabolite profile of the perfused muscle without evidence of hypoxia.

Actions of vasoconstrictors. As shown in Fig. 1, NE, VP, and 5-HT each exerted a qualitatively similar dose-dependent vasoconstrictive effect in the perfused bettong hindlimb. Their other effects on $\dot{V}o_2$ and on lactate and glycerol efflux aggregated into two different sets of responses. Thus qualitatively, NE and VP induced increases in $\dot{V}o_2$ and in lactate and glycerol efflux, which appeared to be closely associated with the changes in perfusion pressure. NE-induced changes attained a maximal value at ~1.0 μM NE, with increases >100% over the basal in the three variables. Above this dose, values of $\dot{V}o_2$ tended to decline, although the perfusion pressure continued to rise. Similarly, VP stimulated, but to a lesser degree, the $\dot{V}o_2$ and the efflux of lactate and glycerol, with maximal values occurring at ~25 nM. Perfusion pressure continued to increase with dose. In contrast to NE and VP, 5-HT significantly inhibited $\dot{V}o_2$ ($P < 0.05$) in the concentration range 0.1–2.5 μM during vasoconstriction. However, the inhibition of lactate and glycerol efflux by 5-HT was not large in magnitude and reached statistical significance only for lactate efflux. After termination of infusion of each of the vasoconstrictors, values for Pv_{O_2} and perfusion pressure returned to basal values throughout the periods of perfusion, which lasted at least 3–4 h.

Effects of α- and β-adrenergic antagonists on NE-mediated effects. Changes in perfusion pressure, $\dot{V}o_2$, and lactate and glycerol release in response to NE were rapid in onset, sustained, and rapidly reversible (Fig. 2). In the presence of the β-adrenergic antagonist propranolol (10 μM DL-racemic), NE-induced perfusion pressure tended to be higher, but $\dot{V}o_2$ and lactate and glycerol release in the presence of NE and propranol were not statistically different from values with NE alone. However, the additional infusion of the α-adrenergic antagonist, phentolamine (10 μM), completely blocked the rise in perfusion pressure as well as the increases in $\dot{V}o_2$ and in lactate and glycerol release elicited by NE.

Effect of nitroprusside. The nitrodilator sodium nitroprusside (1.0 mM) completely blocked the rise in perfusion pressure and the changes in $\dot{V}o_2$ stimulated by NE, VP, and 5-HT (Fig. 3). Nitroprusside also blocked the increased release of lactate and glycerol elicited by NE and VP and reversed the inhibitory effects elicited by 5-HT on lactate and glycerol efflux (data not shown).

Metabolic rate. The bettongs studied by indirect calorimetry were slightly larger than those killed for perfusion studies. The weight (mean ± SE) of the animals was 1.7 ± 0.1 kg (n = 7). The $\dot{V}o_2$ was 23 ± 2 μmol·h^{-1}·g body wt^{-1}, which is equivalent to a resting heat production of 249 ± 21 kJ·kg^{-1}·day^{-1}. Under these conditions, the rat $\dot{V}o_2$ amounted to 60 ± 1 μmol·h^{-1}·g body wt^{-1} (i.e., 656 ± 10 kJ·kg^{-1}·day^{-1}).

DISCUSSION

In the present study, bettong hindlimbs were perfused at 25°C at flow rates chosen to give a constant flow per unit mass of muscle of 0.28 ml·min^{-1}·g^{-1} (Table 1). This constant flow rate was the same as that employed by us previously for rat hindlimb perfusions at the same temperature (8, 9, 11, 16). Perfusions were performed without red blood cells to prevent their contribution to lactate production (16). This necessitated a lower temperature of 25°C to allow delivery of sufficient oxygen to the preparation, which was stable throughout the experimental period. The characteristics of the perfused hindlimb preparation, as well as incubated muscle at 25°C for the study of muscle metabolism, have been recently reviewed, and the data were generally comparable to

Table 2. *Metabolites of bettong gastrocnemius-plantaris-soleus muscle group*

	μmol/g wet wt								EC	PCr/ATP
	Lactate	Cr	PCr	AMP	ADP	ATP	ΣAN	Cr + PCr		
In vivo	3.12 ± 0.55	71.98 ± 1.87	22.51 ± 0.44	0.036 ± 0.003	0.595 ± 0.023	5.22 ± 0.20	5.85 ± 0.21	94.48 ± 6.58	0.943 ± 0.002	4.33 ± 0.21
Perfused	1.21 ± 0.11*	59.91 ± 3.01*	23.41 ± 1.18	0.030 ± 0.004	0.476 ± 0.016	4.68 ± 0.23	5.18 ± 0.23	83.31 ± 14.03†	0.948 ± 0.002	5.03 ± 0.21†

Values are means ± SE; n = 5 samples/group. Muscle samples were freeze-clamped with liquid N_2-precooled aluminium tongs. The in vivo group was taken under anesthesia, whereas the perfused samples were taken at the end of the perfusion. Among the perfused group, 3 muscle samples were taken after the removal of vasoconstrictors, 1 in the presence of norepinephrine (0.1 μM) and the other in the presence of 5-hydroxytryptamine (1.0 μM). As no significant differences in the metabolites were found in the presence or absence of the vasoconstrictors, data were pooled in 1 group. ΣAN, total adenine nucleotides; Cr, creatine; PCr, phosphocreatine; EC, energy charge. EC = (½[ADP] + [ATP])/([AMP] + [ADP] + [ATP]), where [AMP], [ADP], and [ATP] refer to concentrations of AMP, ADP, and ATP, respectively. *$P < 0.01$, †$P < 0.05$ vs. in vivo.

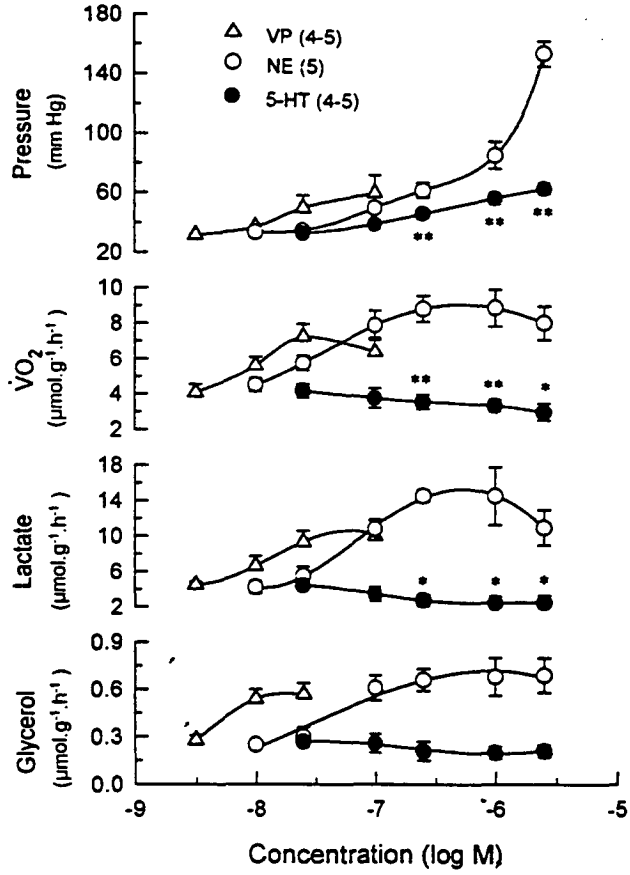

Fig. 1. Dose-response curves for vasoconstrictor-mediated changes in perfusion pressure, oxygen consumption ($\dot{V}O_2$), and lactate and glycerol efflux in the perfused bettong muscle. Basal values are shown in Table 1. Data are presented as means ± SE; nos. in parentheses are nos. of samples. Error bars when not visible are within symbols. VP, vasopressin; NE, norepinephrine; 5-HT, serotonin. Significance for 5-HT data is indicated: $^*P < 0.05$, $^{**}P < 0.01$.

those obtained at 37°C by applying a Q_{10} of 2.5 (2), where Q_{10} refers to the rate of change of muscle metabolism per 10°C increase in temperature.

While resting hindlimb perfusion pressures are at first sight low, arterial pressures will necessarily be lower than in vivo when a cell-free, low-viscosity perfusate is employed. In addition, there is neither resting sympathetic tone nor circulating vasoconstrictors in the perfused hindlimb. Thus the arterial tree is almost fully relaxed as shown by the fact that vasodilators (e.g., nitroprusside) do not lower the perfusion pressure (Fig. 3). The in vivo blood flow rates to the skeletal muscle of the bettong's larger relative, the red kangaroo (*Macropus rufus*), at rest under different environmental temperatures ranged from 0.010 to 0.053 $ml \cdot min^{-1} \cdot g^{-1}$ (22). Thus if similar flow rates apply to the bettong, the flow rate we employed is approximately 5 to 28 times greater than in vivo. Furthermore, because there is little diminution of pressure in the dilated arterial and arteriolar beds, the capillary exchange pressures will be sufficient. Adequate perfusion and oxygen delivery to the hindlimb preparation at rest and under the maximal vasoconstriction or $\dot{V}O_2$ are indicated by the normal values for intracellular lactate, high-energy phosphate compounds, and the calculated energy charge in perfused muscle (Table 2). This is further supported by values for $P\bar{v}_{O_2}$ that at no stage fell below 200 mmHg.

The basal $\dot{V}O_2$ of the perfused bettong hindlimb, which for calculation we have attributed to the mass of skeletal muscle present, was 4.18 ± 0.35 $\mu mol \cdot g^{-1} \cdot h^{-1}$, which is ~65% that of perfused rat hindlimb of 6.4 $\mu mol \cdot g^{-1} \cdot h^{-1}$ (8, 16). When the whole body resting metabolic rates were measured under the same conditions, the metabolic rate of 249 ± 21 $kJ \cdot kg^{-1} \cdot day^{-1}$ (n = 7) for the bettong was ~38% that of the rat at 656 ± 10 $kJ \cdot kg^{-1} \cdot day^{-1}$ (n = 7). These latter metabolic rates were similar to those obtained by others in a closely related species of bettong (29) and in the rat (20).

Although there are a number of reports on the effect of infused NE on $\dot{V}O_2$ in the perfused muscle in eutherian mammals such as the rat (8, 13, 32) and the dog (24), to the best of our knowledge this is the first report on the effects of vasoconstrictors (i.e., NE, VP, and 5-HT) on $\dot{V}O_2$ and on lactate and glycerol efflux in perfused marsupial hindlimb and hence in a noneutherian homeothermic species.

In the perfused bettong hindlimb, increases in $\dot{V}O_2$ and in both lactate and glycerol efflux were observed in response to NE and VP, whereas decreases in $\dot{V}O_2$ and in lactate and glycerol efflux were noted with 5-HT (Figs. and 2). The changes in $\dot{V}O_2$ and in lactate in response to these vasoconstrictors were qualitatively similar to those observed previously in the perfused rat hindlimb (8, 9, 11). A new observation for muscle perfusion was that changes in the glycerol efflux closely paralleled the changes in the hindlimb $\dot{V}O_2$ and lactate (Figs. 1 and 2). The production of lactate and glycerol will have thermogenic potential in vivo if they are subsequently taken up by other tissues such as the liver and resynthesized to carbohydrate or lipid, as these processes would require additional energy expenditure.

Analysis of the dose-response data (Fig. 1) for the perfused bettong hindlimb shows that the thermogenic response to NE started at 25 nM, which is almost 25 times higher than that required for the perfused rat hindlimb under the same conditions (9). The increase in $\dot{V}O_2$ stimulated by NE of ~100% above the basal value was greater than that of the perfused rat hindlimb, which increases by approximately 60–70% at the same flow rate (9, 16, 33). At present, no data on circulating NE are available for the bettong, and in general, data are sparse for all marsupials. However, a small marsupial, the sugar glider (27), and the platypus (21) show resting and stressed values of NE approximately 3- to 10-fold higher than those of the rat; thus the present observations may be relevant to field conditions.

Marsupials appear to have more skeletal muscle than eutherians. Red and gray kangaroos have 52% of body mass as skeletal muscle, which is 6–8% more than in the wildebeest or Thomson's gazelle and ~20% more than in domestic eutherian species (28). The blood flow to the marsupial skeletal muscle varies considerably in re-

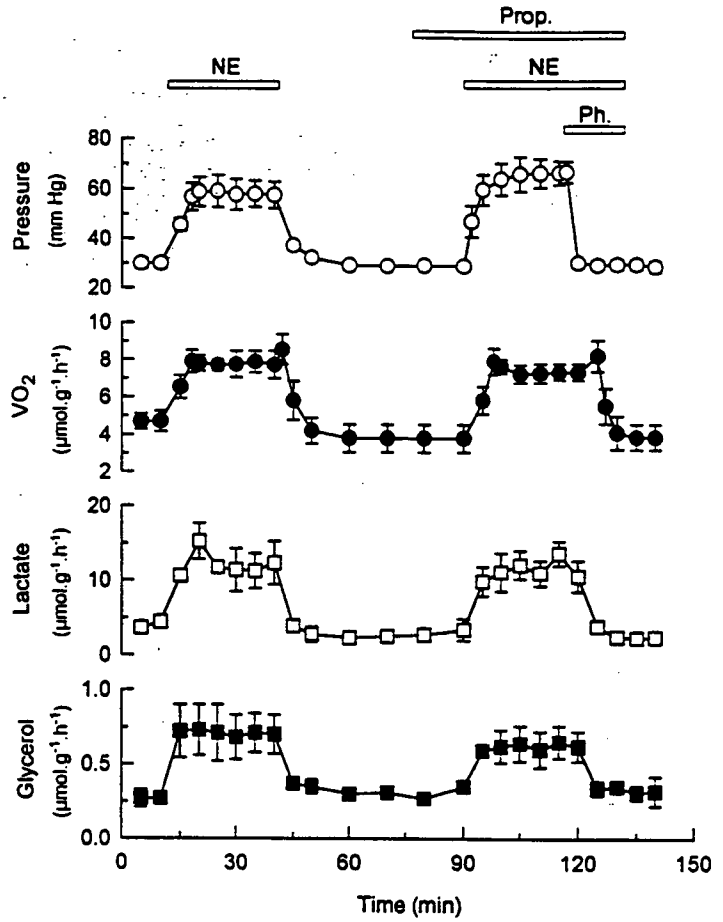

Fig. 2. Effects of α- and β-adrenergic antagonists on NE-mediated perfusion pressure, $\dot{V}O_2$, and lactate and glycerol efflux. Prop, propranolol (10 μM racemic); Ph, phentolamine (10 μM); NE (0.1 μM). Data are means ± SE from 3 experiments.

sponse to the environmental temperature. For example, distribution of cardiac output to skeletal muscle increased from 4% at 37°C to 23% at 7°C (22). When exposed to the cold, the maximal heat production of small marsupials can match or exceed that of rodents of similar size (10). Thus the present study, when taken in conjunction with these observations, suggests that marsupial skeletal muscle may contribute a larger proportion to whole body thermogenesis than the skeletal muscle in eutherian species. As the bettong and other marsupials have been shown not to possess histologically apparent BAT (15), the bigger relative increase in NE (or other vasoconstrictor)-induced energy metabolism and the greater proportion of skeletal muscle may compensate for the lack of BAT in these species.

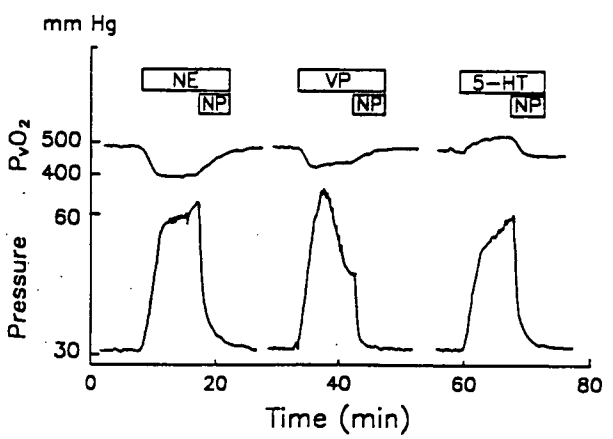

Fig. 3. Representative tracing of inhibitory effect (n = 2–3) of nitroprusside (NP, 1.0 mM) on partial pressure of venous oxygen (Pv_{O_2}; *top*) and perfusion pressure (*bottom*) mediated by NE (0.1 μM), 5-HT (2.5 μM), and VP (25 nM).

Unexpectedly, the NE-induced glycerol release was found to be mediated through α-adrenergic receptors as shown by its blockade by phentolamine plus propranolol but not by propranolol alone (Fig. 2). Activation of lipolysis by Epi has been reported in the perfused mouse hindlimb, and β-adrenergic receptors were thought to be responsible (3). We are unaware of any study so far that characterizes increased glycerol efflux mediated by α-adrenergic receptors, VP, or its inhibition by 5-HT. On the contrary, inhibition of adipose tissue lipolysis has been associated with activation of the α_2-adrenergic receptor (17). A recent autoradiographic study indicates that β-receptors were predominantly distributed on skeletal muscle cells while the α-receptors were much more

sparse and were 30 times higher on arterioles than in the surrounding skeletal muscle (19). Nevertheless, the NE-mediated $\dot{V}O_2$ and lactate and glycerol efflux in the present experiment is α-receptor dependent rather than β-receptor dependent (Fig. 2), suggesting blood vessels are somehow involved in the increased metabolism. The close parallel relationship between vasoconstrictor-mediated glycerol release and $\dot{V}O_2$ change implies that the vasoconstrictors may play a role in modulating lipid mobilization either directly by receptor activation or indirectly by association with smooth muscle contraction to adjust the fuel sources for skeletal muscle thermogenesis. We have recently made similar preliminary findings of NE-mediated increases in glycerol efflux in the perfused rat hindlimb in the presence of propranolol (6). Chin et al. (4) have shown that infused lactate can lead to increased glycerol production in the perfused rat hindlimb. Hence in the present study, which uses a once-through perfusion, another potential source of glycerol would be the conversion of a small proportion of lactate to glycerol downstream from the site of lactate production.

The lack of a β-adrenergic stimulation of $\dot{V}O_2$ in perfused bettong hindlimb (Fig. 2), which was also found in the perfused rat muscle (8), contrasts with the well-known stimulation of $\dot{V}O_2$ by β-adrenergic agonists in vivo in the rat and in another marsupial rat kangaroo, the potoroo (23). This discrepancy might in part be explained by noting that in general, β-adrenergic excitation is known to elevate cardiac output and relax blood vessels in vivo. These effects could result in increased blood flow to skeletal muscle. Increased flow to skeletal muscle has been shown to increase $\dot{V}O_2$ by perfused skeletal muscle in the rat (33). In addition, other thermogenic tissues, such as liver, might also contribute to β-adrenergic stimulation of the whole body metabolic rate (18). Alternatively, β-stimulation leading to vasodilation in vivo may oppose a preexisting pattern of vasoconstriction that inhibits $\dot{V}O_2$ as observed with 5-HT and high concentrations of NE in the perfused rat hindlimb (6).

The action of the nitric oxide generator, nitroprusside, to block both vasoconstriction and the changes in $\dot{V}O_2$, whether they be increased or decreased (Fig. 3), strongly suggests that vasoconstriction is tightly linked in some manner to these phenomena. Yet the mechanism for the vasoactive hormone-induced increase in $\dot{V}O_2$ is still controversial. A priori, to explain the increased $\dot{V}O_2$, there must either be an increased hydrolysis and subsequent synthesis of ATP or a stimulated mitochondrial uncoupling within the hindlimb analogous to that occurring in BAT. Four general possibilities are apparent for the bettong and other species that could explain the vasoconstrictor-mediated control of such processes in the perfused hindlimb.

First, the vasoconstrictors may act directly on skeletal muscle receptors to cause the above metabolic changes independent of the increases in perfusion pressure because some vasoconstrictor hormones have receptors on skeletal muscle. This possibility appears to be unlikely because for NE, β-receptors are predominantly distributed on skeletal muscle cells while α-receptors are much higher on arterioles than on surrounding skeletal muscle (19). Moreover, results from Fig. 2 show that rather than β-receptors are responsible for NE-induced metabolism in the perfused bettong hindlimb. Further evidence suggesting that the vasoconstrictor receptors coupled to $\dot{V}O_2$ appear not to be present on skeletal muscle comes from the observations that NE had little or no effect on $\dot{V}O_2$ from perifused mouse skeletal muscle (12), nor did NE or angiotensin stimulate $\dot{V}O_2$ or lactate production from perifused rat skeletal muscle (16).

The second possibility is that the rise in perfusion pressure associated with vasoconstriction may redistribute the perfusate within the hindlimb to previously underperfused zones and thus, by relieving regions of hypoxia, stimulate increased $\dot{V}O_2$. This is unlikely because, as discussed earlier, there is no evidence of regional hypoxia as the high-energy nucleotides are normal (Table 2). Furthermore, such a pressure-induced redistribution of flow to relieve hypoxia does not accord with the inhibition of $\dot{V}O_2$ associated with vasoconstriction induced by 5-HT (Figs. 1 and 3).

The third possibility is that arterial vasoconstriction is energetically expensive and uses the observed $\dot{V}O_2$. We have previously hypothesized vascular thermogenesis in the perfused rat hindlimb (7). The possibility of direct blood vessel smooth muscle $\dot{V}O_2$ during constriction is consistent with the close association of vasoconstriction and increases in $\dot{V}O_2$ and lactate and glycerol efflux and the block of all these changes by the vasodilator nitroprusside (Figs. 1–3). It is not readily apparent how vascular thermogenesis could explain the inhibition of $\dot{V}O_2$ and lactate efflux by 5-HT that accompanies a rise in vascular resistance in the bettong hindlimb (Fig. 1) or the rat hindlimb (11). However, when infused into the isolated perfused rat mesenteric artery, 5-HT (as well as NE and VP) caused vasoconstriction and increased $\dot{V}O_2$ (11, 32). These observations led to the idea that 5-HT initiates a state of functional shunting of perfusate within the muscle vascular bed away from actively metabolizing cells (11). Similarly, functional shunting may occur in the bettong hindlimb in response to 5-HT.

The fourth possibility is that vasoconstrictors by selective actions redirect flow to particular thermogenic skeletal muscle cells or other thermogenic cells in the hindlimb. This would provide more oxygen to those cells to allow activation of increased levels of uncoupling, ion pumping, or increased substrate cycles. So far, such specialized muscle cells have only been described in billfish, which heat blood going to the retina and brain by presumptive calcium ion cycling (1). However, data from the present experiments cannot discriminate between these two mechanisms of controlling or contributing for the involvement of blood vessels in the vasoconstrictor-altered $\dot{V}O_2$ and lactate and glycerol efflux in the perfused bettong hindlimb. They may not be necessarily mutually exclusive.

In summary, the present experiments show that vasoconstrictive hormones play an important role in controlling and/or contributing to $\dot{V}O_2$ and lactate and glycerol efflux in marsupial (bettong) muscle. There appears to be a greater contribution from skeletal muscle vascular bed to the whole body thermogenesis in

the bettong than in the eutherians both at rest and in response to NE, which may in vivo compensate for its lack of BAT. The responses, however, do not relate in a simple way to the degree of vasoconstriction because there are both increases and decreases in the metabolic parameters depending on the vasoconstrictor employed. The present findings on perfused bettong muscle when taken together with other studies on perfused rat muscle raise the possibility of vasoconstrictor control of $\dot{V}O_2$ and metabolism as an entrenched and widespread physiological mechanism in evolution.

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