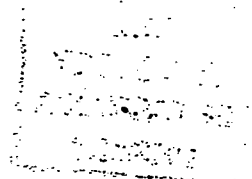# Vascular Involvement in Skeletal Muscle Metabolism

by

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Submitted in fulfilment of the requirements

for the degree of Doctor of Philosophy

Division of Biochemistry

University of Tasmania (September, 1996)

DECLARATION

This thesis contains material which has not been accepted for the award of any other degree or diploma, except where due acknowledgment is given. 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.

23.12.96

T.P.D. Eldershaw

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PREFACE AND ACKNOWLEDGMENTS

The majority of the experimental work outlined in this thesis was performed part-time between January 1992 and December 1995 during my employment as a Graduate Research Assistant/Junior Research Fellow in the Division of Biochemistry, University of Tasmania. Consequently data from a recently published study (June 1996, see publication 10 below), of which I am a co-author, using the vanilloid antagonists capsazepine and ruthenium red are not included in the body of the thesis.

I am fortunate to have been under the supervision of Associate Professor Eric Colquhoun throughout the study. His astute advice and broad knowledge combined with an affable nature and quick wit made him a supervisor without peer. I owe Eric a debt of gratitude for his patience and friendship.

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The synthesis and molecular modelling of vanilloid analogue molecules (Chapter 4) was performed by Honours students in the Department of Chemistry in consultation with myself, and under the direct supervision of Dr Adrian Blackman and Dr Brian Yates. I acknowledge the efforts made by Mr Andrew Clippingdale, Mr Bruce Reardon, and Mr Mark Mackey to model and synthesise target molecules. Testing of vanilloid compounds in whole rats was performed with the assistance of Dr Steve Edwards.

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Experimental work involving lean and obese Zucker rats (Chapter 6) was performed during July and August 1993 in the Diabetes Unit, Department of Vascular Biology, SmithKline Beecham Pharmaceuticals, United Kingdom. The support and assistance of Dr Bob Poyser, Dr Robin Buckingham, Dr Michael Cawthorne, Dr

The bulk of the data contained in this thesis have been published and/or presented at scientific meetings. These publications and presentations are listed below.

Publications arising from data presented in this thesis:

1. **Eldershaw, T.P.D.**, Colquhoun, E.Q., Dora, K.A., Peng, Z.-C., and Clark, M.G. (1992). Pungent principles of ginger (*Zingiber officinale*) are thermogenic in the perfused rat hindlimb. *Int. J. Obesity* **16**, 755-763.

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3. Clark, M.G., Colquhoun, E.Q., Dora, K.A., Rattigan, S., **Eldershaw, T.P.D.**, Hall, J.L., Matthias A., and Ye J.-M. (1994). Resting muscle: a source of thermogenesis controlled by vasomodulators. In: Milton, A.S. (Ed.) *Temperature regulation: recent physiological and pharmacological advances.* Karger: Basel, pp. 315-320.

4. **Eldershaw, T.P.D.,** Rattigan, S., Dora, K.A., Cawthorne, M.A., Buckingham, R.E., Colquhoun, E.Q., and Clark, M.G. (1994). Potential defect in the vascular control of nonshivering thermogenesis in the obese Zucker hind-limb. *Can. J. Physiol. Pharmacol.* **72**, 1567-1573.

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9. **Eldershaw, T.P.D.,** Duchamp, C., Ye, J.-M., Clark, M.G., and Colquhoun. E.Q. Nonshivering thermogenesis in perfused chicken *(Gallus domesticus)* muscle. *Comp. Biochem. Physiol.* (in press).

Related publications:

10. Griffiths, C.D., **Eldershaw, T.P.D.,** Geraghty, D.P., Hall, J.L., and Colquhoun E.Q. (1996). Capsaicin-induced biphasic oxygen uptake in rat

muscle: Antagonism by capsazepine and ruthenium red provides further evidence for peripheral vanilloid receptor subtypes (VN$_1$/VN$_2$). *Life Sci.* **59**, 105-117.

11. Marmonier, F., Duchamp, C., Cohen-Adad, F., **Eldershaw T.P.D.**, and Barré H. Hormonal control of muscle nonshivering thermogenesis in muscovy ducklings. *Am. J. Physiol.* (accepted subject to revision).

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1. Dora, K.A., **Eldershaw, T.P.D.**, Clark, M.G., and Colquhoun E.Q. A vallinoid receptor on vascular smooth muscle? *Proc. Aust. Biochem. Soc.* (1991) **23**, 34.

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7.   Colquhoun, E.Q., Bennett, K.L., Eldershaw, T.P.D., Hall, J.L., Dora, K.A., and Clark, M.G. Two different vanilloid (VN_1 and VN_2) receptors stimulate or inhibit oxygen consumption in perfused rat hindlimb. *Proc. Aust. Soc. Biochem. Mol. Biol.* (1993), **25**, 93.

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CONTENTS

ABBREVIATIONS

Å	Ångström units
ADP	adenosine-5'-diphosphate
ADR	adrenaline
AMP	adenosine-5'-monophosphate
ANOVA	analysis of variance
ATP	adenosine-5'-triphosphate
A-V	arteriovenous
BAT	brown adipose tissue
BSA	bovine serum albumin
CAP	capsaicin
CGRP	calcitonin gene-related peptide
Ci	Curie
CNS	central nervous system
Cr	creatine
CrP	creatine phosphate
d.p.m.	disintegrations per minute
GC-MS	gas chromatography-mass spectroscopy
GTN	glyceryl trinitrate
HPLC	high performance liquid chromatography
IMGU	insulin-mediated glucose uptake
i.p.	*intraperitoneal*
IU	international units
i.v.	*intravenous*
$\dot{M}O_2$	muscle oxygen consumption
NIDDM	non-insulin dependent diabetes mellitus
NMR	nuclear magnetic resonance (spectroscopy)
NST	nonshivering thermogenesis
NOR	noradrenaline
P	probability
PO_2	oxygen partial pressure

P_aO_2	arterial oxygen partial pressure
P_vO_2	venous oxygen partial pressure
PPAHV	phorbol 12-phenylacetate 13-acetate 20-homovanillate
R'g	2-deoxy-D-[1-^{3}H]glucose uptake in muscle
RTX	resiniferatoxin
SE	standard error
SNS	sympathetic nervous system
TLC	thin-layer chromatography
TTX	tetrodotoxin
VN_1/VN_2	putative vanilloid receptor subtypes in rat hindlimb muscle
$\dot{V}O_2$	oxygen consumption
2DG	2-deoxy-D-[1-^{3}H]glucose
5-HT	5-hydroxytryptamine (serotonin)
Δ	change in

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ABSTRACT

Perfused hindlimb preparations have been used to investigate vasoconstrictor-mediated control of skeletal muscle metabolism, with particular emphasis on the regulation of oxygen consumption ($\dot{V}O_2$) as an index of muscle nonshivering thermogenesis (NST). The ability of a group of molecules known as vanilloids to modulate muscle $\dot{V}O_2$ was investigated using hindlimb preparations of hooded Wistar rats. Both naturally-occurring and synthetic vanilloids were examined. Infused vanilloids gave dose-dependent $\dot{V}O_2$ changes in association with increased perfusion pressure (PP). Vanilloid $\dot{V}O_2$ concentration-response curves were biphasic, lower concentrations stimulating and higher concentrations inhibiting $\dot{V}O_2$.

Nitrovasodilators demonstrated an association between the $\dot{V}O_2$ changes and vasoconstriction, whilst α- and β-adrenergic antagonists showed that neither adrenergic receptors nor secondary catecholamine release were responsible for the increased $\dot{V}O_2$. The observed effects may have been due to specialised vanilloid receptors. The data in fact supported two vanilloid receptor subtypes; the putative higher affinity (VN$_1$) receptor mediated increased $\dot{V}O_2$ and vasoconstriction, and was dependent on the presence of oxygen and external Ca^{2+}. The putative lower affinity (VN$_2$) receptor mediated an inhibition of $\dot{V}O_2$ with vasoconstriction, but the vasoconstriction was independent of external Ca^{2+} or O_2 presence.

A range of vanilloid structural analogues were synthesised and used to construct a structure-activity profile for hindlimb thermogenic action. A distinct set of structural features required for thermogenic activity (a pharmacophore) was defined. However, there was no clear distinction between the pharmacophore for thermogenesis and the structural features deduced by others to be necessary for antinociceptive action in sensory neurone studies. Complete separation of the responses attributed to the putative dual vanilloid receptors was not observed, although there was some evidence of partial selectivity.

The concept of vascular metabolic control in muscle was further examined in a series of comparative perfusion studies. The first study established a viable technique for perfusing bird lower limbs. Since birds are reported to be devoid of brown adipose tissue, the perfused chicken lower limb was an appropriate model for examining the

potential of skeletal muscle, via vascular metabolic control, as a major contributor to NST. Infused catecholamines increased PP and gave biphasic $\dot{V}O_2$ concentration-response curves. Low dose $\dot{V}O_2$ stimulation was blocked by prazosin and nitrovasodilation, but was unaffected by propranolol. The demonstration of potential muscle NST in another taxon raised the possibility of vascular thermogenic control being a widespread and perhaps a fundamental NST mechanism.

In a further comparative study, genetically obese *(fa/fa)* Zucker rats were used primarily to examine the hypothesis that the obesity was related to a defect in vascular metabolic control. Differences in basal and noradrenaline-mediated $\dot{V}O_2$ related to lower muscle content and higher fat content in the obese hindlimb. 5-HT-mediated $\dot{V}O_2$ inhibition was significantly greater in age-matched lean *(Fa/?)* hindlimbs, even when the data were expressed in terms of muscle mass. This may indicate a reduced potential for vascular metabolic control with possible implications for the whole-body energy balance of the obese phenotype. In a separate series of experiments measuring glucose uptake, perfused obese hindlimbs were found to be markedly insulin-resistant relative to lean counterparts. The possibility of a link between the impaired insulin effectiveness and altered haemodynamic function is discussed.

The studies undertaken underline the potential significance of altering regional nutrient and hormone access in regulating skeletal muscle metabolism, and in particular support a critical role for the vasculature in the control of skeletal muscle thermogenesis.

Chapter 1

General Introduction

1.1 Experimental Techniques Used in Muscle Metabolism Studies

The currently favoured techniques for studying muscle metabolism are those in which muscle preparations are perfused or incubated. Both approaches allow strict control of experimental variables. However, the methods fundamentally differ in terms of the manner of nutrient delivery. The advantages, problems, and viability of muscle perfusion and incubation methods have recently been reviewed by Bonen *et al.,* (1994).

1.1.1 Incubated or perifused (superfused) muscle preparations

Incubated muscle techniques are generally popular, one of the main advantages being the relative lack of associated technical difficulty. On the other hand, a major source of concern regarding incubated or perifused preparations is that nutrient delivery relies entirely on diffusion from the outer extremities of the tissue. Thus under some circumstances, adequate distribution of nutrients to the inner zones of the tissue may be questionable (Bonen *et al.,* 1994). Efforts to avoid this problem have led to consideration of parameters such as the 'critical radius' of tissue (Segal and Faulkner, 1985) and the careful selection of experimental muscles with regard to size and shape.

1.1.2 Perfused muscle preparations

Perfused preparations (unlike their incubated counterparts) have the advantage of being supplied with nutriment via their own vascular networks. As a result, metabolic effects partially or wholly governed by characteristics of presumably complex vascular networks are likely to be more appropriately modelled by perfusion preparations.

Perfusion media (reviewed by Bonen *et al.,* 1994) vary, and may or may not contain red cells. The ability to directly measure venous PO_2 represents a distinct advantage of cell-free media, given that the venous PO_2 is linearly related to total oxygen content under such circumstances. However, in the absence of red cells the adequacy of oxygen delivery becomes an issue which is best resolved by determining the perfusate

lactate/pyruvate ratio and measuring the concentrations of high energy phosphate compounds within the tissue (Bonen *et al.*, 1994).

Since the early use of perfused rat hindlimb preparations described by Ruderman *et al.* (1971), the technique has become widespread. The preparation has been used to investigate a wide range of physiological phenomena: from the metabolism of glucose (Ploug *et al.*, 1987), to mechanisms of capillary exchange (Paaske and Sejrsen, 1989), physiological pharmacokinetics of solutes (Wu *et al.*, 1993), and exercise physiology (Côté *et al.*, 1985). There are a number of variations of the hindlimb perfusion technique, and the flow distribution may vary markedly depending on the particular method chosen (Gorski *et al.*, 1986). As a consequence, the comparison of results between research groups generally requires caution as the data may quantitatively differ.

There is a growing body of compelling evidence that the vasculature of skeletal muscle plays a critical role in controlling its metabolic behaviour (reviewed by Clark *et al.*, 1995). Such evidence suggests that the use of incubation and perifusion techniques may be inappropriate under many circumstances and points to an increasing use of perfusion to address questions relating to the metabolism of skeletal muscle.

Indeed, three constant-flow perfused skeletal muscle models were chosen for the present studies. Previous research in this laboratory has identified a number of vasoconstrictors capable of influencing perfused - but not incubated - skeletal muscle metabolism, both in terms of stimulation and inhibition (reviewed by Clark *et al.*, 1995). This work has given rise to the proposal that the skeletal muscle vasculature in fact plays a key role in the controlling the metabolism of the organ as a whole. This concept of vascular control was investigated using a comparative approach in the present work. The primary metabolic parameter examined was muscle oxygen consumption ($\dot{V}O_2$), an indirect index of muscle heat production. However, other parameters such as lactate release and glucose uptake were also examined at various stages.

One agent originally identified as being able to alter perfused skeletal muscle $\dot{V}O_2$ was capsaicin (Cameron-Smith *et al.*, 1990), the pungent principle of hot peppers (discussed in section 1.7). The present work involved an extended study of the capsaicin-like (vanilloid) molecules as a unique group of agents capable of enforcing vascular metabolic control. Metabolic (particularly $\dot{V}O_2$) effects, mechanisms of action, and structure-activity relationships of both naturally-occurring and synthetic molecules were

studied in perfused skeletal muscle. Preliminary studies of vanilloid thermogenic actions in intact rats were also undertaken.

1.2 Skeletal Muscle Contribution to Nonshivering Thermogenesis

1.2.1 Thermogenesis

This study examined skeletal muscle metabolism primarily from a thermogenesis viewpoint. The ability to modulate the facultative nonshivering thermogenesis of skeletal muscle emerges as a recurrent theme. Nonshivering thermogenesis (NST) may be briefly defined as heat production by processes not involving the contraction of skeletal muscles. Facultative NST refers to heat production over and above the obligatory component required to maintain the metabolic integrity of the animal at thermoneutrality. More expansive thermogenesis definitions are given in Appendix 1.

1.2.1.2 Measurement of thermogenesis

Heat production is measured by calorimetry. In thermal physiology, calorimetry measures the heat transfer between a tissue, organ, or an organism and its environment (Bligh and Johnson, 1973). Direct calorimetry physically measures heat whilst indirect calorimetry measures the rate of transfer of a material involved in the transformation of chemical energy into heat. The technique involves using an empirically established relation between the material transfer and the heat transfer to calculate the actual heat transfer (Bligh and Johnson, 1973).

The most common method of indirect calorimetry is to measure the uptake of oxygen and/or the elimination of carbon dioxide. In the present study, the measurement of oxygen uptake was used as an index of heat production.

1.2.2 Importance of brown adipose tissue in mammalian facultative NST

Brown adipose tissue (BAT) is a specialised tissue found in eutherian mammals. Richly vascularised and highly innervated by sympathetic fibres, the metabolic processes of BAT are centred on the production of heat. The tissue possesses large mitochondria of high cristae density. BAT is generally found in immediate contact with major blood vessels (Nechad, 1986).

BAT appears to be widely distributed within, but restricted to, eutherian mammals (Trayhurn, 1989). Within this taxon, the tissue is particularly prevalent in hibernators, cold-adapted or overfed rodents, and in the newborn and young of a number of species. (Trayhurn and Nicholls, 1986).

Whilst it is clear that BAT makes a large thermogenic contribution in some species (around 60% of total thermogenesis in rats, Foster and Frydman, 1978), a number of workers using various species conclude that BAT is unable to account for the entire cold-induced thermogenic response. These species (reviewed by Janský, 1995) include adult rats, gerbils, mice (40% contribution to cold-induced thermogenesis), Djungarian hamsters (20-45%) and hamsters (28-61%). The lack of congruence between BAT presence and observed NST applies especially to larger species such as adult humans (Astrup, 1986). Indeed, a growing body of evidence suggests that substantial facultative NST is possible in the complete absence of BAT. Marsupials and monotremes (Haywood and Lisson, 1992), pigs (Jamieson *et al.*, 1984), and birds (Saarela *et al.*, 1989) represent large groups of endotherms in which BAT is most likely absent. However, facultative NST has been demonstrated to exist in both young pigs (Dauncey and Ingram, 1979; Heath and Ingram, 1983; Jamieson *et al.*, 1984;) and birds (Duchamp *et al.*, 1993a), and may well comprise part of the facultative response in marsupials (Ye *et al.*, 1995).

1.2.3 Skeletal muscle

Skeletal muscle constitutes a substantial proportion of whole body mass in mammals (e.g. around 40% in adult male humans, Snyder *et al.*, 1975) and therefore has the potential, even with relatively low metabolic increases per gram of tissue, to make a substantial contribution to whole body thermogenesis. The facultative heat produced by skeletal muscle may be derived from exercise, shivering, or nonshivering processes. Whilst the heat produced during muscular contractions is well understood, that due to nonshivering processes is not as well defined - and is in fact disputed in some species. Although consensus has not yet been reached regarding the mechanisms responsible for facultative heat generation in the absence of contraction, compelling evidence for muscle NST has been outlined for fish, birds, and mammals (reviewed by Block, 1994).

A number of studies involving endotherms suggest that skeletal muscle may make an important contribution to overall NST under varying circumstances (Table 1.1). Amongst the ectotherms, swordfish and other related billfish species have been found to possess a specialised thermogenic organ derived from extraocular skeletal muscles beneath the brain (Carey, 1982). The cells of this organ are characterised by numerous mitochondria, extensive smooth endoplasmic reticula, and the absence of contractile elements and thermogenin (Block, 1987). Block (1994) has reviewed the literature dealing specifically with the thermogenic mechanisms associated with the billfish heater organ, and with muscle NST in other animal groups and concludes that ATP-cycling of Ca^{2+} between the sarcoplasmic reticulum and the cytosol is emerging as a common key pathway for muscular thermogenesis.

1.3 Other Possible Sources of Facultative NST

1.3.1 Liver

The literature contains conflicting accounts regarding the possible contribution of the liver to facultative heat production. Depocas (1958, 1960) reported that functionally eviscerated rats responded no differently to cold exposure or infused noradrenaline than intact animals. In addition, noradrenaline infusions did not increase the metabolic rate of the isolated rat liver (Janský *et al.*, 1964). Later studies, on the other hand, recorded increases in liver thermogenesis both in cold-exposed dogs (Bacconier *et al.*, 1979) and rats (Stoner, 1973).

Table 1.1 Quantitative estimates of skeletal muscle contribution to NST *in vivo* in various endotherms.

species	NST stimulus	acclimation status	estimated NST skeletal muscle contribution	experimental method	reference
rat	noradrenaline	CA	48% of NOR response	perfused muscle $\dot{V}O_2$	Grubb & Folk (1977)
rat	cold exposure	CA	up to 50% of NST response	cytochrome oxidase activity and muscle blood flow	Jansky (1963; 1971), +(1978)
rat	increased PO_2	TN	25% of NST response	perfused gracilis muscle $\dot{V}O_2$ and heat production	Chinet & Mejsnar (1989)
chicken	cold exposure	CA	155% increase in total metabolic rate, no shivering	EMG and indirect calorimetry	El-Halawani *et al.* (1971)
duckling	cold exposure	CA	71% of cold-induced thermogenesis	muscle blood flow and blood oxygen content	Duchamp & Barré (1993)
duckling	glucagon induced	CA	53% of thermogenesis increase	muscle blood flow and blood oxygen content	Duchamp *et al.* (1993a)
duckling	glucagon induced	TN	28% of thermogenesis increase	muscle blood flow and blood oxygen content	Duchamp *et al.* (1993a)

CA, cold-acclimated; WA, warm-acclimated; TN, thermoneutral acclimation; EMG, electromyography

1.3.2 Kidney, heart, intestine, and brain

In this laboratory, perfused rat kidney and intestine preparations were capable of increasing their oxygen uptake by 45% and 15% respectively in response to vasopressin stimulation. Noradrenaline induced similar $\dot{V}O_2$ stimulation in perfused kidney preparations. These increases were accompanied by elevated perfusion pressure (Ye *et al.*, 1990a; see section 1.4.5.1). The remaining literature contains relatively little support for these organs being significant contributors to facultative NST (reviewed by Janský, 1995), although the metabolic rate of the brain (MacKenzie *et al.*, 1976) and the gastrointestinal heat production of dogs (Durotoye *et al.*, 1976) have been observed to increase after noradrenaline and cold exposure respectively.

1.4 Role of the Vasculature in Skeletal Muscle Metabolism

1.4.1 Perfusion heterogeneity in skeletal muscle

It has long been recognised that the relationship between bulk muscle blood flow and the distribution of capillary flow within the muscle is complex; it is possible for bulk flow to increase whilst local perfusion of some regions simultaneously decreases (Duling, 1983). Vetterlein and Schmidt (1975) observed that vasodilation was able to increase bulk muscle blood flow whilst simultaneously reducing capillary red cell velocity in some vessels. Schroeder and Rathscheck (1973) noted that acetylcholine increased bulk flow but decreased tissue PO_2. Raising arterial PO_2 led to increased PO_2 variability yet the overall rise in tissue PO_2 was insignificant (Lund *et al.* 1980). These studies illustrate the complex nature of microcirculatory perfusion. The use of radioactive microspheres (Iversen and Nicolaysen, 1995 and references therein) have revealed flow heterogeneity within single skeletal muscles of a number of species, both in conscious and anaesthetised animals. These studies conclude that such uneven perfusion patterns represent genuine physiological phenomena.

1.4.2 Actions of vasoconstrictors in perfused skeletal muscle

1.4.2.1 Stimulation of basal metabolism

A number of perfused rat hindlimb studies, both in this laboratory (Colquhoun *et al.*, 1990; Dora *et al.*, 1992a) and elsewhere (Grubb and Folk, 1977; Richter *et al.*, 1982a; Côté *et al.*, 1985), have demonstrated the ability of catecholamines to induce increased $\dot{V}O_2$ in conjunction with vasoconstriction via an α-adrenergic receptor-mediated mechanism. A range of other agents examined in this laboratory (reviewed by Clark *et al.*, 1995) have been observed to increase hindlimb $\dot{V}O_2$ in association with vasoconstriction. In addition to increasing $\dot{V}O_2$, vasoconstrictors in this category (termed 'type A' vasoconstrictors, Table 1.2) appear capable of stimulating metabolism in general; various members of the group have been shown to accelerate the efflux of urate and uracil (Clark *et al.*, 1990), lactate (Hettiarachchi *et al.*, 1992), and glycerol (Clark *et al.*, 1994), as well as to elevate glucose uptake (Richter *et al.*, 1982b).

The metabolic and vascular effects of type A vasoconstrictors would appear to be closely associated. Neither the use of various types of vasodilator (e.g. nitrovasodilators, Ye *et al.*, 1990b, Colquhoun *et al.*, 1990; Ca^{2+} channel blockers and β-adrenergic agonists, Colquhoun *et al.*, 1990) nor metabolic poisons (Dora *et al.*, 1992a; Richards *et al.*, 1992) have succeeded in breaking the apparent link between the two. Furthermore, the results obtained when skeletal muscle preparations do not receive nutrients and hormones via the vasculature fail to correspond with those outlined above. That is, when incubated or perifused with noradrenaline, isolated skeletal muscle shows no increase in $\dot{V}O_2$, heat flux nor lactate efflux (Dubois-Ferrière and Chinet, 1981; Hettiarachchi *et al.*, 1992). In contrast, incubated *in vitro* preparations of BAT (Girardier and Stock, 1983) and liver (Binet and Claret, 1983) show increased thermogenesis in response to noradrenaline, although the effect in the latter tissue is attributed to increases in a diverse range of metabolic processes. Taken together, these observations form the nucleus of the argument supporting a critical role of the vasculature in controlling skeletal muscle metabolism.

Table 1.2. Type A and type B vasoconstrictor stimuli in the perfused rat hindlimb (modified from Clark *et al.*, 1995).

Agonist	Change From Control at Maximum Dose of Agonist, %		Reference
	Perfusion Pressure	Oxygen Uptake	

Type A

Agonist	Perfusion Pressure	Oxygen Uptake	Reference
Noradrenaline	52	46	Colquhoun *et al.*, 1988
	130	52	Côté *et al.*, 1985
	NR	77	Grubb & Folk, 1977
Adrenaline	67	25	Richter *et al.*, 1982a
	NR	67	Grubb & Folk, 1977
Phenylephrine	160[#]	50[#]	Shiota & Masumi, 1988
	NR	57	Grubb & Folk, 1977
Methoxamine	167	25	Hettiarachchi *et al.*, 1992
Amidephrine	24	28	Clark *et al.*, 1994*
Ephedrine	70	10	Clark *et al.*, 1994*
Norephedrine	57	18	Hettiarachchi *et al.*, 1991
Angiotensin II	133	64	Colquhoun *et al.*, 1988
Vasopressin	121	66	Colquhoun *et al.*, 1988
Capsaicin	54.5	22	Cameron-Smith *et al.*, 1990
Dihydrocapsaicin	49	18	Cameron-Smith *et al.*, 1990
Low frequency sympathetic nerve stimulation (0.5-4 Hz)	6	6	Hall *et al.*, 1996

Type B

Agonist	Perfusion Pressure	Oxygen Uptake	Reference
Noradrenaline	190	-11	T.P.D. Eldershaw, unpublished
(≥1 μM)	206	-3.0	Dora *et al.*, 1994
	200	-15	Côté *et al.*, 1985
	380	-10	Grubb & Folk, 1976
Serotonin	76	-30.3	Dora *et al.*, 1994
Capsaicin (>1 μM)	110	-35	Chapter 3
Dihydrocapsaicin (>1 μM)	+	-	Clark *et al.*, 1994
High-frequency sympathetic nerve stimulation (>4 Hz)	37	-16	Hall *et al.*, 1996

Hindlimbs were perfused at 25°C with constant flow. NR, not reported. [#]Cold-acclimated rats. *Actual values appear in M. Hettiarachchi PhD Thesis, University of Tasmania 1991. + and -, indicate an increase or decrease, respectively, when compared with control (vehicle only) perfusions.

It is important to address the possibility of type A metabolic effects being artefacts of the hindlimb perfusion technique. Many of the observations described above have been made using cell-free perfusates under fully dilated conditions, the resultant low vascular resistance raising the possibility of artefactual heterogeneity within the preparation. In basal preparations perfused for three hours, measurements of high energy phosphate concentrations and the ratio of creatine phosphate to creatine (a sensitive index of muscle hypoxia; Ye et al., 1996), were similar to measurements made using freshly sampled muscle. Comparable increases in $\dot{V}O_2$ have been observed using noradrenaline and vasopressin in preparations at higher flow rates with basal perfusion pressures approaching in vivo values (Ye et al., 1990b). In addition, experiments conducted at 37°C with red blood cell perfusates and concomitant basal perfusion pressures of 90 mm Hg demonstrated marked increases in $\dot{V}O_2$ in response to angiotensin II (Rattigan et al., 1996). The fact that several groups (Grubb and Folk, 1977; Richter et al., 1982a; Côté et al., 1985; Colquhoun et al., 1988) have reported catecholamine-induced increases in perfused rat hindlimb $\dot{V}O_2$ under a variety of conditions (i.e. with or without red blood cells, differing arterial PO_2, temperatures of 25, 32 or 37°C) implies that the results represent a genuine biological effect. Interestingly, studies in this laboratory suggest that the phenomenon is not confined to skeletal muscle, but may also be present in perfused rat kidney, intestine, and mesenteric arcade (Ye et al., 1990a).

1.4.2.2 Inhibition of basal metabolism

A second group of stimuli that vasoconstrict but inhibit basal metabolism have been identified ('type B' vasoconstrictors, Table 1.2). In addition to depressing basal $\dot{V}O_2$, the metabolic effects induced by type B agents in perfused rat hindlimb preparations are generally the opposite of type A agents. Thus such agents act to decrease lactate, glycerol, urate and uracil efflux (Clark et al., 1994), as well as inhibiting insulin-mediated glucose uptake (Rattigan et al., 1993, 1995). Serotonin (5-HT) acts as a type B agent at all effective concentrations (Dora et al., 1991, 1992a). Others agents such as noradrenaline (Dora et al., 1992a) and vanilloids (Chapters 2a and 2b) display bell-shaped $\dot{V}O_2$ dose-response curves, acting as type A agents at lower concentrations and type B agents at higher concentrations. Perfusion pressure,

on the other hand, shows dose-dependent increases over the full concentration range. As noted for type A vasoconstrictors, vasodilation opposes both the vasoconstricting and metabolic actions of type B agents (Rattigan *et al.*, 1993).

Interestingly, the effects of noradrenaline at concentrations thought to occur at sympathetic nerve synapses on vascular smooth muscle (i.e. $\geq$ 1 μM, Esler *et al.*, 1990) are negatively thermogenic (Table 1.2). Actual sympathetic stimulation of the hindlimb preparation results in qualitatively similar type B responses at high frequencies (Table 1.2), whilst lower frequencies towards the left of the bell-shaped dose response curve (Hall *et al.*, 1996) produce type A $\dot{V}O_2$ effects. Analogous frequency-dependent changes in oxygen extraction have been reported during sympathetic nerve stimulation of the auto-perfused dog hindlimb (Pappenheimer *et al.*, 1941; Durán and Renkin, 1976).

The actions of type B agents on glucose metabolism in the perfused rat hindlimb have been of particular interest (refer to section 1.5.2). 5-HT (Rattigan *et al.*, 1993), high-dose noradrenaline (Rattigan *et al.*, 1995) and high doses of vanilloids (T.P.D. Eldershaw, unpublished preliminary data from this laboratory) are all capable of markedly inhibiting insulin-mediated glucose uptake. Once again, it is noteworthy that these metabolic alterations only manifest when the hindlimb nutrients are supplied via the vascular route. In the studies with both 5-HT and high-dose noradrenaline, isolated incubated muscles gave no indication of similar metabolic depression when exposed to matching agonist concentrations (Rattigan *et al.*, 1993, 1995).

1.4.3 Nutritive and non-nutritive flow patterns

The concept of two circulatory patterns being present in skeletal muscle is not new. Indeed, the notion of circulatory systems within skeletal muscle being either nutritive or non-nutritive has been discussed at length in a number of communications (Barlow *et al.*, 1961; Grant and Payling Wright, 1970; Lindbom and Arfors, 1984; Saltzman *et al.*, 1992). These investigations have reported no evidence of thoroughfare channels (shunts) within the fibre-containing tissue which might act to short circuit the respiring tissue. Nevertheless, a general consensus was that the vessels within the associated connective tissue may serve a non-nutritive function. In addition to the proposal that the circulation in cat inter-muscular septa and tendons

was essentially non-nutritive, Barlow *et al.* (1961) noted that the two circulatory patterns responded differently to *i.v.* noradrenaline. Lindbom and Arfors (1984) observed that transverse arterioles fed circulatory systems in rabbit tenuissimus muscle connective tissue after branching into the muscle fibres. The two systems were thought to be controlled by their relative contributions to vascular resistance; it was hypothesised that sensitivity differences to stimuli may be a functional basis for the control of blood supply and capillary perfusion within the muscle. The same research group subsequently described the connective tissue microcirculation as representing a significant functional red-cell shunt (Ley *et al.*, 1988).

Although the work of Hammersen (1970) found no evidence for anatomical arteriovenous anastamoses in skeletal muscle, studies in this laboratory have postulated the presence of functional vascular shunting to explain the observed type B metabolic effects (Dora *et al.*, 1991, 1992a; Rattigan *et al.*, 1993). More recent experiments using 8-16 µm microspheres (Newman *et al.*, 1996) in perfused rat hindlimb preparations have resulted in low microsphere clearance and an unchanged distribution, indicating that any shunting (if occurring at all) must be within the microcirculation.

A recent study has successfully demonstrated differing vascular flow routes in perfused rat hindlimbs subjected to type A or type B stimulation (Newman *et al.*, 1996). The measurement of post-equilibration red blood cell efflux and the entrapment of fluorescent dextran, as well as corrosion casting of the microvasculature revealed that type A stimulation recruited a new vascular space. This space was not recruited by type B stimulation, despite higher perfusion pressure. Type B stimulation in fact restricted perfusate distribution, producing corrosion casts of lower mass than control. Casts for type A stimulation revealed a greater number of filled vessels, although the cast mass did not differ from control.

Taken together, the contemporary evidence for flow redistribution together with the longer standing suggestions of nutritive and non-nutritive circulation systems within skeletal muscle comprise a strengthening argument in support of vascular involvement in skeletal muscle metabolic control.

1.4.4 Window of vascular metabolic control

Given that vasoconstricting stimuli have the ability to both enhance and reduce muscle metabolism *in vitro*, the capacity for such vascular control of metabolism may be represented as the combined maximal changes due to type A and type B stimuli (Fig. 1.1). The overall size and location of such a vascular metabolic control window may have important implications for disease states such as obesity and diabetes. The size of this window in obese animal models, for example, may be related to the phenotypic expression of obesity. Furthermore the presence and range of this window in other animal taxa - particularly those lacking BAT such as birds and marsupials - may have implications regarding the potential importance of skeletal muscle as a NST effector in these groups.
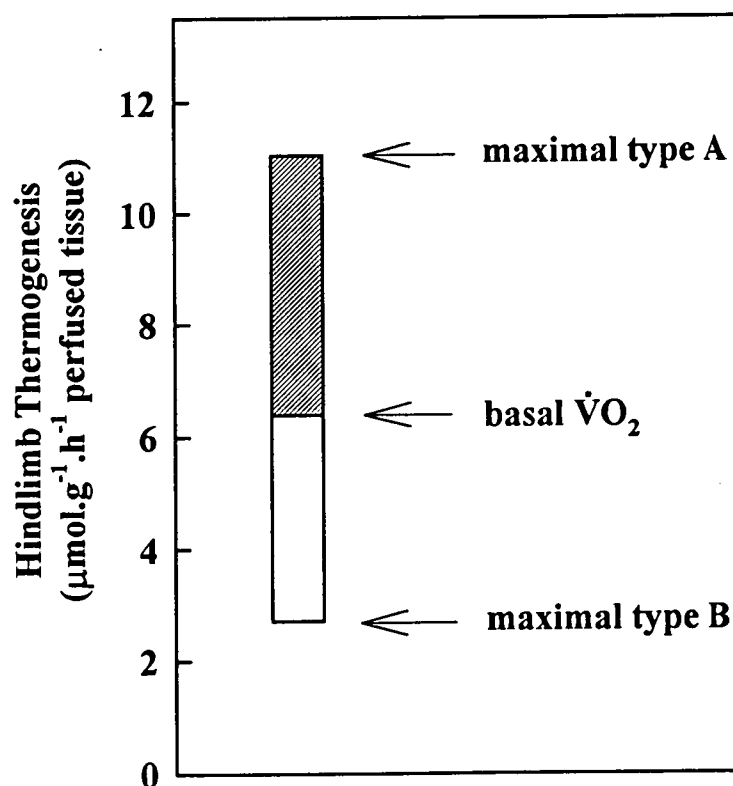
Fig. 1.1. Proposed vasoconstrictor-controlled metabolic window in perfused hindlimbs of lean Zucker rats (data from Chapter 6). The shaded bar represents increased metabolism (in this case $\dot{V}O_2$) due to type A (noradrenaline) stimulation. The unshaded bar represents decreased metabolism due to type B (serotonin) stimulation. The basal state is represented by the boundary. The combined bars therefore represent the capacity of the vasculature to control skeletal muscle metabolism.

1.4.5 Possible mechanisms to account for increased $\dot{V}O_2$

1.4.5.1 Working vascular smooth muscle

There is no doubt that the process of vasoconstriction involves a thermogenic component, given that vascular smooth muscle (VSM) contraction by definition involves an energy conversion. However, the proposal that working VSM of the resistance vessels may significantly contribute to facultative NST ('vascular thermogenesis'; Colquhoun and Clark, 1991) represents a new and interesting addition to the group of proposed NST effector tissues. The wide distribution of VSM throughout the body, and the close proximity of this putative thermogenic tissue to the blood are certainly amongst the more attractive features associated with this concept. Furthermore, much of the vasculature is highly innervated by sympathetic nerve fibres (Burnstock, 1975), a property shared with BAT.

The evidence in support of VSM being a potential thermogenic effector is centred on the observations that a range of vasoactive agents were able to stimulate oxygen uptake ($\dot{V}O_2$) in close association with vasoconstriction in perfused rat hindlimb preparations (Colquhoun *et al.*, 1988, 1990). This relationship also applied in other perfused systems such as the rat kidney and intestine (Ye *et al.*, 1990a), as well as the mesenteric arcade (Ye *et al.*, 1990a; Dora *et al.*, 1991). The vasoconstrictor-induced $\dot{V}O_2$ in hindlimb preparations was additive to that induced by muscle contraction (Colquhoun *et al.*, 1990). Furthermore, $\dot{V}O_2$ increases induced by higher flow rates were enhanced by vasoconstriction and reduced by vasodilation (Ye *et al.*, 1990b). Studies with isolated small arteries (approximately 500 µm) from the rat indicate that the presence of mitochondria and cytochrome c oxidase activity in the VSM are consistent with a potential for a significant thermogenic contribution (Z.-C. Peng, unpublished data from this laboratory). A series of calculations based on these data, and the estimated 3.4% presence of VSM in the human whole body (Paul, 1980) indicated that this potential VSM thermogenic contribution was around 30% of basal metabolism (Z.-C. Peng, *opp. cit.*).

However, the proposed high economy of VSM tension maintenance (e.g. the 'latch' hypothesis of Murphy *et al.*, 1988) presents some problems for this theory. Nevertheless, knowledge of the energetics of VSM remains incomplete (reviewed by

Paul *et al.*, 1989). The possibility of the energetic tension costs of the microvasculature being higher than previously estimated, particularly if components of the microcirculation maintain active tension (Colquhoun and Clark, 1991) should not be dismissed. Similarly, the estimated 3.4% presence VSM (Paul, 1980) may not be reliable, since it is an indirect measurement based on calculations involving projections of the metabolic cost of maintaining the tension required for vascular regulation of circulation, and the fraction of total metabolism estimated to be attributable to the maintenance of tone.

Given the current technical hurdles associated with isolating resistance vessels, direct evidence for this hypothesis is difficult to obtain. Whilst a substantial thermogenic contribution by VSM remains possible, further supporting evidence is required for this notion to be regarded as being of physiological importance. Nevertheless, the vasculature is increasingly being viewed as exerting a major influence on tissue metabolism, regardless of the magnitude of the direct thermogenic contribution of vascular smooth muscle.

1.4.5.2 Proposed paracrine signalling

The observed increases in perfused muscle $\dot{V}O_2$ are unlikely to be the result of increasing the hormone and nutrient access within skeletal muscle *per se*. It has recently been postulated that the release of a paracrine (or endocrine) signaling agent may activate the calorigenic mechanism (Clark *et al.*, 1995). Speculation regarding such an agent has centred on the hypothesis that site-specific vasoconstriction and the resultant flow increase to the nutritive circulation causes a shear-stress activated release of an endothelial autacoid substance which subsequently acts either in a paracrine role to increase muscle fibre metabolism, or an autacoid role to relax vessels distal to the impending flow.

1.4.5.3 Supply limitation

In the absence of endocrine/paracrine signals or specialised effector mechanisms for increasing metabolism within skeletal muscle, the concept of localised supply limitation (Duling, 1983) may account for modest increases in metabolism. Indeed, it has been demonstrated that oxygen supply can be limiting if the flow rate or

arterial PO_2 is sufficiently reduced relative to the muscle metabolic rate (Horstman *et al.*, 1976). If heterogeneity of muscle nutrient supply exists, as outlined in previous sections, it is reasonable to postulate that localised zones within the muscle may have sufficiently low PO_2 to inhibit oxidative phosphorylation. Type A actions to reduce such heterogeneity would result in the removal of the supply limitation and a concomitant $\dot{V}O_2$ increase.

Use of the Krogh equation (Krogh, 1919) to predict oxygen supply to tissue assumes relative homogeneity of spatial oxygen distribution. Heterogeneity, be it due to vessel geometry, flow pattern, red cell distribution, or tissue type (e.g. fibre type distribution) influences the overall level of tissue oxygenation (Duling, 1983) and creates potential for supply limitation independent of bulk tissue flow. A number of more contemporary physiological models now recognise the roles of diffusion limitation and heterogeneity of blood flow (reviewed by Piiper, 1994). In the perfused rat hindlimb model, physiological pharmacokinetics of solutes under varied perfusion conditions have been described by Wu *et al.* (1993).

The notion of supply limitation within skeletal muscle is, on the other hand, not supported by the findings of Gayeski, Honig, and colleagues concerning the role of myoglobin in facilitating oxygen diffusion within myocytes (Gayeski *et al.*, 1985; Honig and Gayeski, 1993). Indeed these authors conclude that the facilitation effect of myoglobin is such that the principal resistance to oxygen diffusion in fact lies outside the myocyte (Honig and Gayeski, 1993). Furthermore, myoglobin is reported to buffer myocyte intracellular PO_2 above the tension found to limit cytochrome turnover (Gayeski *et al.*, 1985). This oxygen buffering proposal was supported by the apparent lack of anoxic regions (using cryomicrospectroscopy) in working dog gracilis muscle - even at high $\dot{V}O_2$ (Gayeski *et al.*, 1988).

In light of the magnitude of some type A-induced thermogenic increases (e.g. 77% increase with noradrenaline, Table 1.2), it is difficult to rationalise that such a large amount of oxygen could be coupled to ATP synthesis in resting skeletal muscle preparations. It therefore becomes necessary to consider specialised energy-dissipating mechanisms or uncoupled respiration.

1.4.5.4 Substrate cycling

Shiota and Masumi (1988) reported that ouabain was capable of blocking NOR-induced $\dot{V}O_2$ in perfused rat hindlimb preparations, suggesting that active transport of sodium and potassium ions across the plasma membrane was the primary energy-dissipating mechanism. Recent data from this laboratory (A.C.Y. Tong, S. Rattigan, K.A. Dora, and M.G. Clark, submitted) support this interpretation. On the other hand, other studies (Clausen *et al.*, 1991) have dismissed Na^+-K^+ transport as a major contributor to NST.

Calcium ion cycling between the cytoplasm and the sarcoplasmic reticulum (SR) has been proposed as the primary energy-dissipating mechanism in billfish heater organs (Block, 1994). Central to the cycling process is an increase in the permeability of the SR membrane. However, it appears likely that this mechanism is relatively specialised, and indeed makes no contribution to resting $\dot{V}O_2$ in perfused hindlimb preparations (Chinet and Mejsnar, 1989).

1.4.5.5 Uncoupling of oxidative phosphorylation

Uncoupling of oxidative phosphorylation in brown adipose tissue is widely recognised as a specialised thermogenic mechanism (Trayhurn, 1994). BAT uncoupling protein (UCP) acts as a proton conductance pathway across the inner mitochondrial membrane. Thus protons generated from substrate oxidation through the respiratory chain are able to return through the inner membrane allowing the bypass of ATP synthesis. The energy of substrate oxidation is therefore expressed as heat.

It is conceivable that the increased $\dot{V}O_2$ observed during type A stimulation is associated with specialised uncoupling subsarcolemmal mitochondria situated within the capillary recruitment areas. Such "loose-coupled" mitochondria have been reported to be present in the skeletal muscle of birds (Barré *et al.*, 1989) where the glucagon-mediated liberation of free fatty acids may be implicated in the uncoupling process (Barré *et al.*, 1986).

Recent studies have suggested the presence of atypical β-adrenoreceptors (presumed to be β₃-adrenoreceptors, the receptors responsible for activation of BAT thermogenesis) in rat skeletal muscle (Roberts *et al.*, 1993; Abe *et al.*, 1993; Liu and

Stock, 1995). Accordingly, β_3-adrenoreceptor messenger RNA (mRNA) has recently been detected in rat skeletal muscles (Summers *et al.*, 1995; Evans *et al.*, 1996). However, the substantial adipsin mRNA relative to the low levels of β_3-adrenoreceptor mRNA suggested that these receptors may only have been present in associated white fat cells and not associated with UCP. Nevertheless, Evans *et al.* (1996) did report a weak signal for UCP mRNA in a single rat gastrocnemius muscle preparation.

1.4.5.6 Passive proton leakage

Studies in the United Kingdom (Brand *et al.*, 1994; Rolfe and Brand 1996a, 1996b) have suggested that a substantial proportion (up to 52%) of the perfused rat hindlimb $\dot{V}O_2$ may be associated with maintaining the mitochondrial proton motive force in the presence of passive proton leak across the inner mitochondrial membrane (as opposed to facilitated proton leak associated with uncoupling mechanisms). This mechanism has therefore been postulated as a major contributor to whole body thermogenesis. In addition, small changes in membrane potential can markedly affect the rate of proton leakage and therefore cellular thermogenesis (Brand *et al.*, 1994).

In this laboratory, Steen *et al.* (1996) have duplicated the data of Brand *et al.* (1994), using oligomycin (ATP synthase inhibitor) in the perfused rat hindlimb to provide preliminary support to the notion that 50% of basal respiration is unlikely to be coupled to ATP synthesis, and may be attributable to the aforementioned inner mitochondrial membrane proton leak. Furthermore, the use of type A and type B vasoconstrictors in the presence of oligomycin results in significant alterations of the remaining respiratory component (Steen *et al.*, 1996) in the established pattern (Table 1.2).

It seems feasible that the initial alteration in perfusate access induced by vasoconstrictor-associated flow redistribution may act to modulate the inner mitochondrial membrane potential which in turn changes the rate of proton leak.

The involvement of an endothelium-derived paracrine signal substance to initiate a change in membrane potential of muscle mitochondria, or simple supply-limitation of oxygen to the location of specialised thermogenic (proton leaking) mitochondria are both possibilities which at present cannot be excluded. However,

the concept of passive proton leak being responsible for marked changes in muscle $\dot{V}O_2$ remains just one of a number of possible mechanisms.

1.5 Possible Physiological Correlates of Vascular Metabolic Control

1.5.1 Control of muscle contractility

Type A and type B vasoconstriction have been demonstrated to have opposite effects on the contractile performance of constant-flow perfused muscle (reviewed by Clark et al., 1995), in similar fashion to the opposing effects noted for the metabolic parameters discussed thus far. Again, matching treatments of non-perfused preparations had little or no effect on contractile performance. The type B vasoconstrictors serotonin (Dora et al., 1994) and high doses of noradrenaline (Clark et al., 1995) act to decrease the aerobic phase of contractile performance, reinforcing the argument for reduced nutritive flow under such circumstances. Indeed the contractile performance of skeletal muscle is dependent on oxygen supply (Walker et al., 1982) and similar reductions in tetanic tension can be achieved by imposing conditions of anoxia, ischaemia, or anaemia on skeletal muscle (Dodd et al., 1993; Walker et al., 1982). Vasodilators represent another group of agents capable of reducing aerobic contractility, as demonstrated by experiments in autoperfused (i.e. variable flow) muscle preparations in cats (Hirvonen et al., 1964). Significantly, vasodilation has been shown to induce microcirculatory perfusion heterogeneity in variable flow models (Vetterlein and Schmidt, 1975), although bulk tissue perfusion actually increases.

The type A vasoconstrictors adrenaline, low dose noradrenaline, and angiotensin II all improve skeletal muscle tension development (Richter et al., 1982a; Clark et al., 1995; and Rattigan et al., 1996 respectively). In the most recent study, infusion of angiotensin II improved aerobic tetanic tension by 80%; the improvement was attributed to flow redistribution, either to nutritive circulation or from type I to type II contracting muscles (Rattigan et al., 1996).

1.5.2 Control of insulin-mediated glucose uptake

Skeletal muscle is the major site of insulin-mediated glucose uptake (IMGU, Baron et al., 1994). Data indicate that the degree of skeletal muscle perfusion can be

an important determinant of IMGU (reviewed by Baron, 1994). Parallel (but separate) research in this laboratory has centred on the vasoconstrictor-induced haemodynamic responses of perfused skeletal muscle as they relate to IMGU.

Consistent with the hypothesis of vasoconstrictor-induced flow redistribution, perfusion experiments have confirmed a role for vasoconstrictors in the control of IMGU. Such control was not apparent in parallel incubation studies.

Serotonin infusion was capable of inducing an acute state of insulin resistance in perfused preparations, with IMGU being inhibited by 30%. Furthermore, the uptake of 2-deoxy-D-[1-^{3}H]glucose in individual muscles was inhibited by 32%-80% (Rattigan *et al.*, 1993). The infusion of type B doses of noradrenaline (10 μM) identified an α-adrenergic inhibition of IMGU (Rattigan *et al.*, 1995). This concentration of noradrenaline is similar to those thought to occur at sympathetic synapses (Esler *et al.*, 1990).

Type A vasoconstrictor-induced stimulation of IMGU in the perfused rat hindlimb preparation was demonstrated using 1 μM noradrenaline which markedly increased IMGU in perfusion preparations at 37°C (Clark *et al.*, 1996).

The experiments outlined above provide compelling evidence for a haemodynamic basis of skeletal muscle insulin resistance. Indeed, insulin itself has been recently reported to include a haemodynamic component in its spectrum of pharmacological activity (reviewed by Baron, 1994). The dilatory actions of insulin in human skeletal muscle vasculature, apparently via nitric oxide release, are reportedly impaired in insulin-resistant disease states. The dilatory behaviour is therefore increasingly being recognised as an important part of the overall action of insulin (Baron, 1994).

1.6 Possible Link Between Impaired Flow Distribution and Disease States

Based on the observation that the disease states of hypertension, hyperlipidaemia, and diabetes mellitus often cluster in the same individuals, a metabolic syndrome ("Syndrome X") has been proposed (Reaven, 1988). It has been suggested that increased sympathetic nerve activity (Lind *et al.*, 1988) and a decrease

in peripheral blood flow (reviewed by Lind and Lithell, 1993) may have pathogenic importance for the development of the syndrome.

Certainly the data obtained using the perfused rat hindlimb preparation suggest that increased sympathetic nerve activity and the resultant increase in noradrenaline concentrations may manifest in restricted perfusate flow within skeletal muscle (J.L. Hall, unpublished data from this laboratory). Such impaired flow distribution *in vivo* could ultimately lead to vascular rarefaction (Zeman *et al.*, 1988) and therefore a permanent state of reduced nutritive flow within the skeletal muscle, potentially presenting an individual with an impaired ability to clear plasma glucose and lipids, as well as a diminished capacity for regulatory skeletal muscle thermogenesis (Fig. 1.2).

Perfusion studies with obese and insulin-resistant animal models (e.g. obese Zucker rats) may provide insights regarding the relationship of skeletal muscle vascular control mechanisms to the expression of the obesity and insulin-resistance. Evidence of impaired vascular control would strengthen the case for a causal relationship between skeletal muscle dysfunction and the development of hypertension, obesity, and diabetes.

Increased Sympathetic Nerve Activity

|

Non-Nutritive Flow Predominates

|

Vessel Rarefaction and Hypertrophy

|

Decreased Peripheral Blood Flow

Hyperinsulinaemia   Hypertension   Hyperlipidaemia
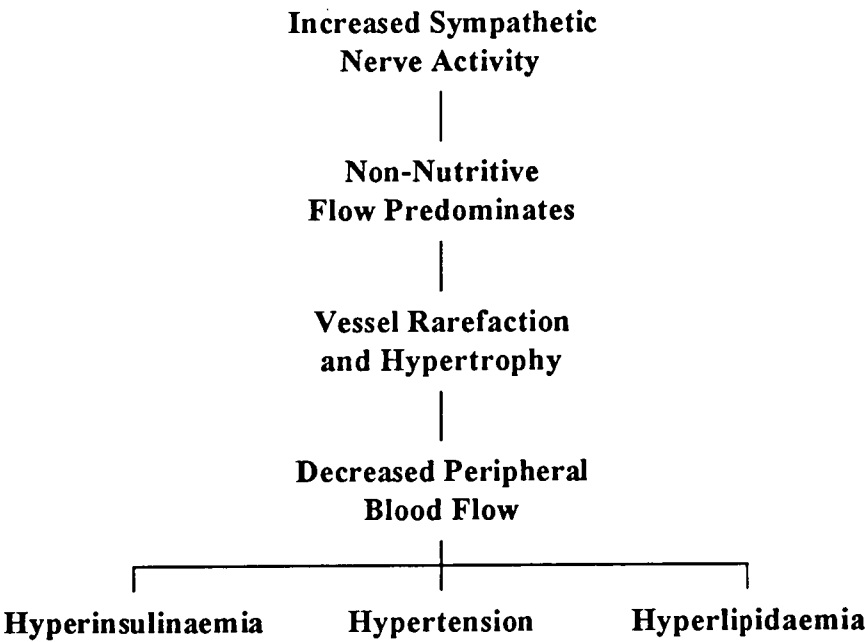
Fig. 1.2. Possible chain of events leading to the development of metabolic "Syndrome X" (redrawn and modified from Lind and Lithell, 1993).

1.7 Potential Role for Vanilloid Agents in Controlling Muscle Metabolism

1.7.1 Vanilloid molecules

Vanilloids are a family of molecules bearing a structural resemblance to capsaicin (8-methyl-N-vanillyl-6-nonanamide, Chapter 2a, Fig. 2a.1), a pungent ingredient of hot peppers and chillies from the genus *Capsicum* (family Solanaceae). The group is distinguished by a homovanillyl structural moiety (Chapter 2a, Fig. 2a.1), and naturally-occurring members include the pungent compounds dihydrocapsaicin (reduced form of capsaicin), resiniferatoxin (RTX, an ultrapotent vanilloid present in the latex of some members of the genus *Euphorbia*), the gingerol and shogaol homologues (ginger), and piperine (black pepper).

1.7.2 Capsaicin actions in the perfused rat hindlimb

Cameron-Smith *et al.* (1990) identified capsaicin and dihydrocapsaicin as vasoconstrictors capable of modulating the $\dot{V}O_2$ of perfused rat hindlimb preparations. Both agents induced type A $\dot{V}O_2$ effects at concentrations less than 1 μM (20%-23% above basal). At higher concentrations, type B $\dot{V}O_2$ effects were apparent. The stimulatory $\dot{V}O_2$ actions were not mediated by α_1 nor β_1/β_2 adrenergic receptors, thereby identifying the vanilloids as a potential novel group of vasoconstrictors capable of influencing perfused muscle metabolism.

1.7.3 Vanilloid neuropharmacological activity

Since the early work by the Hungarian group of Janscó postulating the existence of a capsaicin-sensitive "pain receptor" (Janscó, 1968), vanilloids have attracted increasing interest (Bevan and Szolcsányi, 1990; Szallasi and Blumberg, 1990b; Dray, 1992; Craft and Porreca, 1992; Maggi, 1992), particularly with respect to their neuropharmacological activity (Bevan and Szolcsányi, 1990; Szallasi and Blumberg, 1990b; Dray, 1992). Much attention has centred on the well known excitatory, desensitising, and toxic effects of capsaicin on subsets of unmyelinated or thinly myelinated sensory nerves (Wood *et al.*, 1988). The role of vanilloids in the depolarisation of nerves and in the mediation of pain has been extensively reviewed (Wood, 1993). Vanilloid antinociceptive activity, the result of an ability to desensitise

a subset of primary afferent neurones (Buck and Burks, 1986), has led to the development of synthetic analogues as prototype analgesic agents (e.g. Park *et al.*, 1995).

1.7.4 Vanilloid actions in peripheral tissues

Whilst vanilloids generally are found to have a wide range of pharmacological activities, there are few accounts of vanilloid effects on oxygen consumption in individual tissues, although capsaicin and synthetic analogues have been found to interfere with mitochondrial enzymes (Satoh *et al.*, 1996). Reported actions on non-neuronal systems include cardiac muscle excitability and inhibition of visceral smooth muscle activity (Holzer, 1991 and references therein), as well as biological responses in the liver and uterus (Szallasi, 1994 and references therein). Many types of peripheral smooth muscle are documented as being vanilloid-sensitive, including rat urinary bladder (Szallasi *et al.*, 1993), colon (Goso *et al.*, 1993), urethra (Parlani *et al.*, 1993) as well as human and guinea pig airways (Szallasi *et al.*, 1995b).

In terms of vascular smooth muscle stimulation, capsaicin is reported to have both contractile and endothelium independent dilatory influences (Saito *et al.*, 1988; Duckles, 1986). It seems probable that both are components of acute *in vitro* treatment within a complex vascular system (Duckles, 1986).

1.7.5 Vanilloid receptors

The selectivity of capsaicin action and the defined set of structural requirements for capsaicin-like activity provided early evidence for a specific ligand-receptor interaction (reviewed by Szallasi and Blumberg, 1990b). Following indirect evidence obtained using capsaicin-like photoaffinity probes to inhibit the capsaicin response (James *et al.*, 1988), attempts to detect putative receptors with radiolabelled dihydrocapsaicin were thwarted by the highly lipophilic nature and relatively low potency of this agent (James *et al.*, 1988). However, the discovery that resiniferatoxin acted as an ultrapotent capsaicin analogue was significant since the use of [^{3}H]RTX has resulted in direct identification of a specific vanilloid receptor (Szallasi and Blumberg, 1990a, 1990b). Vanilloid receptors have subsequently been identified in a number of species, including humans (Acs *et al.*, 1994a). However, some species are

noteworthy due to their reported insensitivity to vanilloids. The chicken, for example, is reported to be practically insensitive to capsaicin (Pierau *et al.*, 1986) and accordingly [^{3}H]RTX does not bind to chicken dorsal root ganglia neurones (Szallasi and Blumberg, 1990b). Vanilloid actions on perfused chicken skeletal muscle have not been investigated.

The notion of a specific vanilloid recognition site intimately associated with the vanilloid-operated cation channel (James *et al.*, 1993) has been reinforced by the development of a competitive antagonist, capsazepine. This agent is selective for the actions of capsaicin on central nerve endings in the rat (Urban and Dray, 1991; Dickenson and Dray, 1991), in functional studies on capsaicin-mediated ion uptake in the same neurones (Bevan *et al.*, 1992), and on contraction of smooth muscle (Maggi *et al.*, 1993).

The presence of a vanilloid recognition site suggests the likely existence of endogenous ligands. However, this notion remains a topic of debate, and the current experimental evidence is inconclusive (reviewed by Szallasi, 1994). Nevertheless, recent studies have suggested that protons (i.e. low pH) may be the endogenous ligands (Szallasi *et al.*, 1995a), based on inhibited binding of [^{3}H]RTX to spinal cord receptors.

1.7.6 Vanilloid receptor subtypes

Following the discovery of the vanilloid receptor, evidence has emerged to support the existence of vanilloid receptor subtypes, as well as interspecies receptor heterogeneity (reviewed by Szallasi, 1994). In particular, differences between central and peripheral vanilloid receptors in terms of binding affinity and cooperativity have been noted, but there is also heterogeneity amongst peripheral receptors (Szallasi, 1994). However, receptors on differing parts of primary afferent neurones appear to be a uniform population (Acs *et al.*, 1994b).

The observed thermogenic effects of capsaicinoids in the perfused rat hindlimb system (Cameron-Smith *et al.*, 1990) are conceivably due to interactions with peripheral vanilloid receptors within skeletal muscle. Furthermore, the duel $\dot{V}O_2$ effects may be linked to the presence of receptor subtypes within the hindlimb preparation. If specific subtypes were responsible for the thermogenic perturbations,

then vanilloid agents - both natural and synthetic - warrant further investigation as lead compounds for a new class of thermogenic drug, particularly if selectivity for the peripheral activity within skeletal muscle, and more particularly the positive thermogenic actions, can be attained.

1.8 Objectives of the Present Study

In light of the evidence presented linking the vascular system to the metabolic control of skeletal muscle, and the potential of vanilloids as a new group of agents capable of enforcing such control, the aims of the present study were as follows:

1. To chemically isolate and subsequently assess the ability of naturally-occurring vanilloid compounds to modulate the oxygen consumption of perfused rat hindlimb preparations.

2. To investigate and characterise mechanisms of vanilloid action in the perfused rat hindlimb model, including the possibility of specific receptor interactions.

3. To use information obtained from experiments with natural vanilloids to develop synthetic vanilloid analogues in order to effectively study the structure-activity relationships of vanilloids in the perfused rat hindlimb model.

4. To develop a viable method for perfusing bird skeletal muscle in order to conduct further comparative studies addressing firstly the reported lack of vanilloid receptors in chickens, and secondly the potential of skeletal muscle as a source of NST in endothermic animals without detectable BAT.

5. To conduct comparative studies using an obese, insulin-resistant animal model (the genetically obese Zucker rat) in order to explore possible links between impaired skeletal muscle vascular control and the phenotypic expression of obesity and insulin-resistance.

6. To address the possibility, in view of the experimental results, of vasoconstrictor-controlled skeletal muscle thermogenesis being a general biological NST mechanism.

Chapter 2a

Ginger Vanilloid Principles: Direct-acting Thermogenic Agents in the Perfused Rat Hindlimb

2a.1 Introduction

Ginger, the rhizome of *Zingiber officinale* Roscoe, is extensively used as a flavouring additive in foods, beverages, and confectionery. A herbaceous perennial belonging to the family Zingiberaceae, it has medicinal qualities of importance in traditional Chinese medicine. Legendary Chinese herbalist Shen Nung (3000 B.C.) recommended ginger for colds, fever, chills, tetanus and leprosy (Castleman, 1991). The crude drug continues to be widely used for the treatment of a number of ailments: these include colds and influenza, motion sickness, digestive problems, and irregular menstruation (Castleman, 1991). Ginger is noted for its apparent ability to subjectively warm the body (Ou Ming, 1989).

This laboratory has previously demonstrated (Cameron-Smith *et al.*, 1990) that capsaicin and dihydrocapsaicin, the vanilloid spice principles present in hot chillies and capsicums, increase oxygen uptake in the isolated perfused rat hindlimb; the oxygen effects being associated with vasoconstriction. These findings may help to explain those of Henry and Emery (1986) who reported that human consumption of a meal containing chilli and mustard sauces resulted in a 25 per cent greater increase in diet-induced thermogenesis over a three hour period than a similar meal without spices. In addition, the response of the hindlimb to the capsaicinoids was consistent with the hypothesis that vascular smooth muscle directly consumed oxygen during sustained vasoconstriction (Colquhoun *et al.*, 1988, 1990; Ye *et al.*, 1990a, 1990b).

Diet-induced thermogenesis may contribute significantly to the regulation of body temperature and energy balance (Landsberg and Young, 1981; Rothwell and Stock, 1979). The magnitude of this phenomenon is influenced both by caloric intake (Rothwell and Stock, 1983) and dietary composition (Moore, 1987; Swick and Gribskov, 1983; Rothwell *et al.*, 1983).

Pungency, a feature of chillies and capsicums, is also an important characteristic of ginger. The pungent principles of ginger are present as two phenylalanine-derived (Denniff *et al.*, 1980) homologous series, the gingerols and shogaols (Connell and Sutherland, 1969). The shogaols are formed via an alkyl chain dehydration reaction from gingerols (Fig. 2a.1), hence they are usually present in dried rather than fresh rhizomes. Gingerols and shogaols primarily consist of the [6]-, [8]-, and [10]-homologues (Connell and McLachlan, 1972; Fig. 2a.1), although trace amounts of other homologues have been reported following gas chromatographic studies (Harvey, 1981; Chen *et al.*, 1986). Trace amounts of gingerols with methyl side chains have been described (Chen *et al.*, 1986). Zingerone, a pungent hydrolysis product of gingerols and shogaols (Connell and Sutherland, 1969) is present in many heat treated or roasted ginger preparations.

Recent studies have reported that both ginger and its isolated pungent principles exhibit a range of pharmacological effects. Suekawa *et al.* (1986a, 1986b) have found [6]-shogaol to have a triphasic effect on blood pressure in rats *in vivo*. It was suggested that this response was a complex phenomenon involving both CNS and peripheral activity. Other studies have found ginger principles to exhibit cardiac effects (Shoji *et al.*, 1982; Suekawa *et al.*, 1984), mutagenicity (Nakamura and Yamamoto, 1983; Nagabhushan *et al.*, 1987), gastro-intestinal and analgesic activity (Nagabhushan *et al.*, 1987), inhibition of human neutrophil 5-lipoxygenase activity (Flynn *et al.*, 1986), and inhibition of serotonin-induced hypothermia and diarrhoea (Huang *et al.*, 1990).

The gingerols and shogaols bear some similarities to the capsaicinoids in terms of both structure and function. All contain the 4-methoxy, 3-hydroxy phenyl (vanillyl) moiety, as well as a carbonyl-containing alkyl side chain. Each group of homologues is responsible for the pungent taste of the parent plant (Connell and Sutherland, 1969). As an on-going search for thermogenic dietary components, this study investigated the actions of ginger and its pungent principles in the isolated perfused rat hindlimb.

CAPSAICIN

n=4: [6]-gingerol
n=6: [8]-gingerol
n=8: [10]-gingerol

n=4: [6]-shogaol
n=6: [8]-shogaol
n=8: [10]-shogaol

PIPERINE

RESINIFERATOXIN
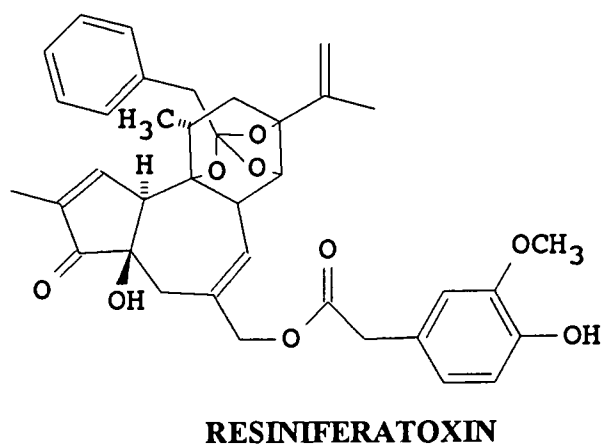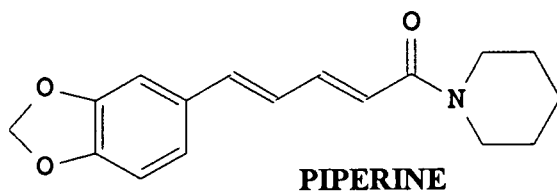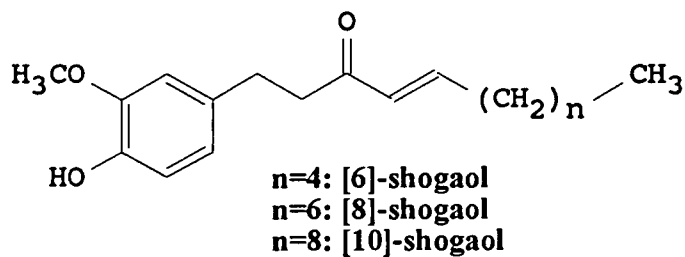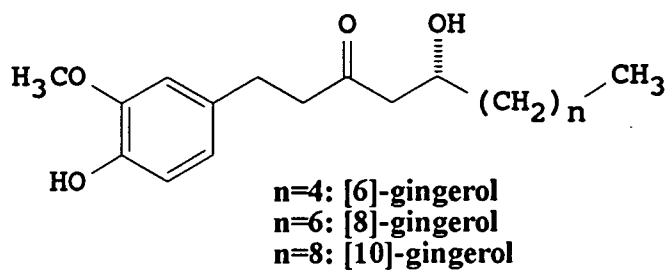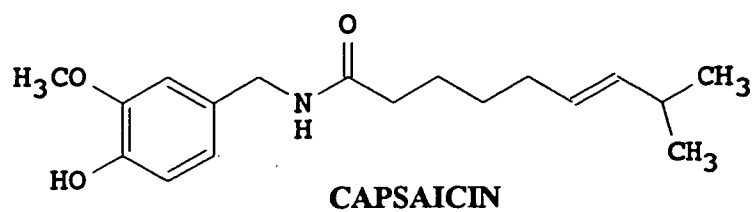
Fig. 2a.1. Structures of active vanillyl-containing compounds discussed in the text.

2a.2 Methods

2a.2.1 Animals

All procedures adopted and experiments undertaken were approved by the University of Tasmania Ethics Committee under the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (1990). Male hooded Wistar rats (180-200 g) were raised on a commercial diet containing 21.4 per cent protein, 4.6 per cent lipid, 68 per cent carbohydrate, and 6 per cent crude fibre with added vitamins and minerals (Gibsons, Hobart) together with water *ad libitum* at a constant temperature of 21 ± 1°C in a 12 h/12 h light/dark cycle.

2a.2.2 Materials and instrumentation

Bovine serum albumin (fraction V, Boehringer Mannheim, Germany) was dialysed 5 times against distilled water before use. NAD^+ (free acid) and lactate dehydrogenase were purchased from Boehringer Mannheim (Germany). Prazosin hydrochloride and DL-propranolol hydrochloride were obtained from Sigma (USA) whilst glyceryl trinitrate (GTN) was from G Pohl-Boskamp GmbH and Co. (Germany). Fresh ginger was purchased locally whilst ground dried ginger was supplied by Buderim Ginger (Australia) and Superior Rate Corporation (Taiwan).

Preparative TLC plates were prepared using a moving hopper slurry spreader over glass backing (Merck silica gel 60G, Germany). Short column chromatography was performed using Fluka (Switzerland) silica gel H for TLC (dry packed). A Chromatotron apparatus (Harrison Research, USA) was used for radial chromatography. Plates were prepared using Merck (Germany) silica gel 60 PF_{254} on a glass backing.

HPLC was performed using a Waters (Millipore, USA) system, incorporating 6000A pumps, a U6K injector, a model 440 UV absorbance detector, and a differential refractometer model R401. A Dynamax-60A C18 column (model no. 83-221C, 21.4 mm i.d. x 25 cm, Rainin Instruments, USA) was used for preparative separations whilst analytical work was carried out using a Waters Radial-Pak C18 column (8NVC184). The isocratic mobile phase was 80:20 methanol/water.

Solvents were all AR grade (Ajax Chemicals, Australia). Those used for HPLC were filtered (0.45 μm) and degassed prior to use.

2a.2.3 Surgical and perfusion procedures

The surgical and perfusion procedures were performed as described previously (Ruderman *et al.*, 1971; Colquhoun *et al.*, 1988). Briefly, following anaesthesia (sodium pentobarbital 60 mg·kg^{-1} *i.p.*) the tail, left tarsus, epigastric vessels and iliolumbar vessels were all ligated. An incision was made along the mid line of the abdomen. Ligatures were placed around the duodenum and the rectum, and the gut was excised. Heparin (2000 IU·kg^{-1} body weight) was injected into the vena cava prior to cannulation of the abdominal aorta and vena cava. The right common iliac artery and vein were ligated to ensure that flow was restricted to the left hindlimb. A further ligature was placed around the abdomen (L_3-L_4 vertebrae region) to prevent access of perfusate to the muscles of the back. Following commencement of perfusate flow, the rat was given a lethal cardiac injection of sodium pentobarbital.

Perfusion was performed at 25°C in a temperature-controlled cabinet with constant flow (4 ml·min^{-1}; 0.27 ml·min^{-1}·g muscle^{-1}) of an erythrocyte-free modified Krebs-Ringer bicarbonate buffer containing 2% dialysed bovine serum albumin, 8.3 mM glucose, and 1.27 mM calcium chloride (prepared according to Côté *et al.*, 1985). The buffer reservoir was kept on ice and continuously stirred, whilst the surface was gassed with 95% O_2 - 5% CO_2. Perfusate was pumped by a peristaltic pump (Masterflex, Cole Palmer, USA) to a 25°C heat exchanger prior to passing through a silastic lung, also gassed with 95% O_2 - 5% CO_2. Venous oxygen tension was continuously monitored with an in-line Clark-type electrode of 0.5 ml capacity. Water jackets maintained the heat exchanger and the oxygen electrode at 25°C. Perfusion pressure was monitored continuously at a bubble trap proximal to the arterial cannula using a gas-filled pressure transducer. The perfusion apparatus is shown in Fig. 2a.2.
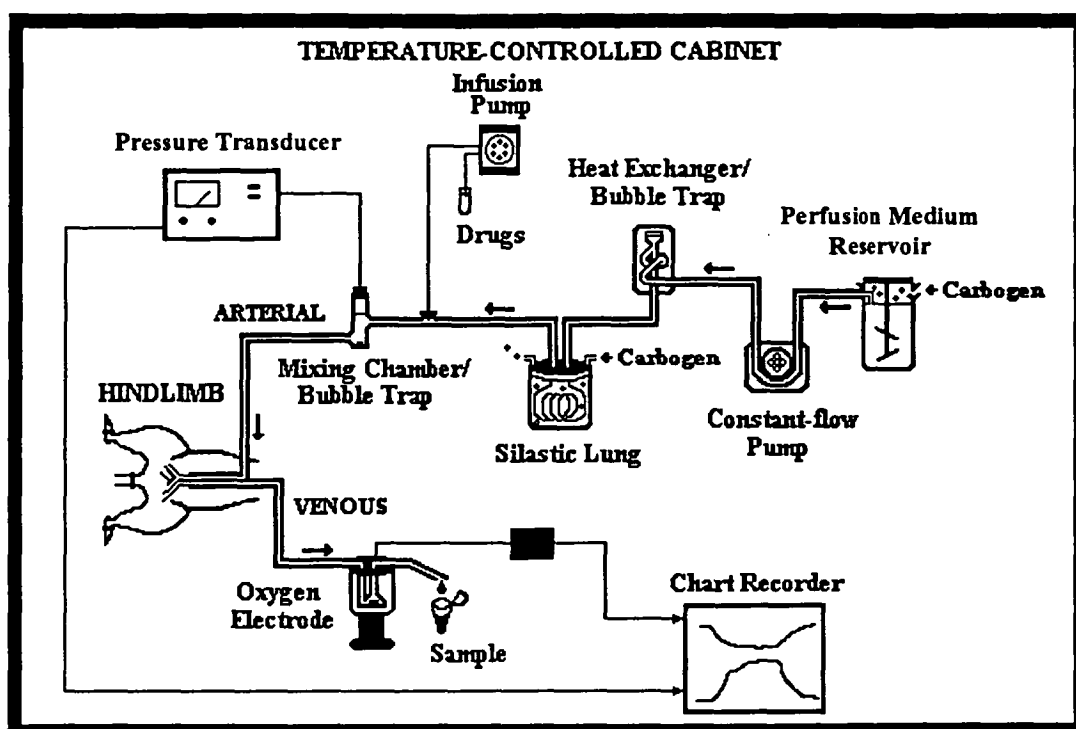
Fig. 2a.2. Constant-flow rat hindlimb perfusion apparatus.

2a.2.4 Agent infusions

Crude extracts, fractions, and pure ginger principles were infused as 25% ethanol (AR grade) solutions using a glass syringe with teflon tubing in a Sage Instruments Syringe pump (model 355). GTN was also infused using this apparatus, given its propensity for sorption by components of drug delivery systems (Roberts *et al.*, 1980). Other compounds were infused using LKB (Sweden) peristaltic pumps in water or saline solutions. Infusion rates were between 10 and 40 $\mu l \cdot min^{-1}$. Vehicle infusions (e.g. 25% ethanol/75% water, saline, and water) were shown not to perturb basal conditions. Solutions were infused into a bubble trap prior to the arterial cannula. The perfusate in this trap was subject to continual stirring. Oxygen uptakes and perfusion pressures were calculated from steady state values, usually attained within 5 minutes after applying the agent.

2a.2.5 Calculation of oxygen uptake

The oxygen electrode was calibrated before and after each experiment with pure oxygen and air. Arterial PO_2 (PaO_2) was determined by joining the arterial and

venous cannulae to bypass the perfused tissue. $\dot{V}O_2$ of perfused tissue was calculated from the difference between PaO_2 and venous PO_2 (PvO_2), the flow rate, and the perfused hindlimb muscle mass according to the following equation:

$$\dot{V}O_2 \ (\mu mol \cdot g^{-1} \cdot h^{-1}) = [1.508 \times (PaO_2 - PvO_2) \times \text{flow rate}]/[\text{perfused muscle mass}]$$

where 1.508 ($\mu mol \cdot L^{-1} \cdot mm \ Hg^{-1}$) is the Bunsen coefficient for oxygen solubility in human plasma at 25°C (Christoforides $et \ al.$, 1969); PaO_2 and PvO_2 are in mm Hg; flow rate is in $L \cdot hr^{-1}$; and perfused muscle mass is in g (assumed to be 1/12 of body mass in 180-200 g rats, Ruderman $et \ al.$, 1971).

2a.2.6 Lactate assay

The lactate assay using neutralised perchlorate samples was based on the method of Gutman and Wahlefeld (1974). Samples for lactate analysis were taken at times corresponding to steady state oxygen consumption and perfusion pressure (usually 5 minutes after applying the agent). Lactate release was calculated as follows:

$$\text{lactate efflux} \ (\mu mol \cdot g^{-1} \cdot h^{-1}) = \frac{\Delta E \times 1000 \times \text{cell volume} \times \text{total volume} \times \text{perfusion flow rate} \times 60}{6220 \times \text{neutralised volume} \times \text{perfusate volume} \times \text{muscle weight}}$$

where ΔE is the net change in absorbance due to lactate dehydrogenase; 6220 ($ml \cdot mmol^{-1} \cdot cm^{-1}$) is the extinction coefficient for NADH at 340 nm; perfusion flow rate is in $ml \cdot min^{-1}$; volumes are in ml; and muscle weight is in g.

2a.2.7 Statistics

The data are expressed as means ± standard errors. Curves were fitted using the Sigma-Plot program (Jandel Scientific). Significance of differences ($P \leq 0.05$) was assessed using Student's unpaired two-sided t test. In general, a minimum of five animals were used to determine a single data point.

2a.2.8 Preparation of extracts and isolation of ginger principles

Crude extracts of both fresh ginger and ground dried ginger were prepared by percolation (×4) in methanol (HPLC grade Ajax). Fresh ginger was chopped and blended with methanol to a slurry prior to extraction. Percolation periods were generally 8-12 hours after each fresh addition of methanol. Following extraction the methanol was removed under vacuum at temperatures no greater than 30°C. A typical fractionation procedure used to isolate principles from fresh ginger is shown in Fig. 2a.3.

Fresh Ginger (Bulk)

1. extraction (methanol)

Crude MeOH Extract

2. solvent removal

Oleoresin

3. extraction (ether/hexane 1:1)

Solute   **Residue**

4. short column chromatography (Si gel,2:1 ether/hexane)

n fractions
(monitored by TLC)

5. activity screening (hindlimb perfusions)

Active Fractions

6. preparative TLC (Si gel, 5:2 ether/hexane)
   multidevelopment (3X)

Discrete Bands (visible under UV)

7. recrystallisations
(n-hexane)

8. preparative HPLC

[6]-gingerol   **[8]-,[10]-gingerols**

Fig. 2a.3. Typical extraction, isolation and testing procedure for active principles of fresh ginger (gingerols). Dried ginger, the source of shogaols, was treated in a similar fashion except that an additional radial chromatography stage was used between stages 5 and 6 and step 7 was omitted.

The fractionation procedure for dry ginger was similar to that outlined in Fig. 2a.3, except that the active material following testing at stage 5 (Rf 0.65-0.8) was subjected to radial chromatography using silica gel of layer thickness 4 mm (5:2 ether/hexane). The combined active fractions were subjected to preparative TLC (Fig. 2a.3, step 6) and the resultant active band (Rf 0.75-0.80) was subjected to HPLC (Fig. 2a.3, step 8) to yield [6]- and [8]-shogaol.

The most abundant principle isolated from fresh ginger, [6]-gingerol, was identified by gas chromatography-mass spectroscopy (GC-MS) and proton nuclear magnetic resonance spectroscopy. Subsequent principles were identified by GC-MS. The purity of isolated principles was confirmed by TLC, HPLC, GC-MS and direct insertion mass spectroscopy.

2a.3 Results

The perfused hindlimb was initially allowed to reach steady state perfusion pressure and venous PO_2 (Colquhoun *et al.*, 1988). The mean arterial and venous PO_2 values were 688 ± 4 mm Hg ($n = 22$) and 413 ± 8 mm Hg ($n = 22$) respectively. The mean basal oxygen uptake was therefore 6.6 ± 0.2 $\mu mol \cdot g^{-1} \cdot h^{-1}$ ($n = 22$). The mean basal perfusion pressure was 26.0 ± 0.6 mm Hg, whilst the mean lactate efflux was 5.4 ± 0.3 $\mu mol \cdot g^{-1} \cdot h^{-1}$ ($n = 19$). These values are similar to those obtained during other studies from this laboratory (Cameron-Smith *et al.*, 1990; Colquhoun *et al.*, 1988, 1990; Ye *et al.*, 1990a, 1990b).

Exhaustive methanolic extracts of both fresh rhizomes and commercially peeled, dried and ground rhizomes were found to cause both an increase in oxygen uptake and perfusion pressure when infused into the perfused rat hindlimb. Hindlimb oxygen uptake was stimulated over final concentration ranges of 0.05-0.15 mg·ml⁻¹ (fresh ginger extract) and 0.005-0.01 mg·ml⁻¹ (dried ginger extract). Similar extracts of other spices including garlic, gin seng, horseradish and yellow mustard had no effect when infused at final concentrations of up to 25 mg·ml⁻¹.

Fractionation of the crude methanolic extracts from both fresh and dried ginger resulted in the isolation of the principles responsible for the observed

thermogenic activity (Fig. 2a.1, structures I-V). Fig. 2a.4 shows typical oxygen and pressure traces produced by a series of increasing [6]-shogaol doses. The order of dose infusion did not affect the observed dose responses. Fig. 2a.5 illustrates the increases in steady state oxygen uptake, perfusion pressure and corresponding lactate efflux of the perfused hindlimb as a function of [6]-, [8]- and [10]-gingerol, and [6]-shogaol concentration. Increasing alkyl chain length leads to decreased potency, although maximal stimulated oxygen consumption appears to increase. In addition, Fig. 2a.5 shows the effect of alkyl chain dehydration on the potency and maximal oxygen stimulation of [6]-gingerol, the major principle of fresh ginger, by comparison with responses obtained using [6]-shogaol, the major principle of dried ginger. Experimental results using [8]-shogaol (data not shown) found the half-maximal dose to be around 20 μM, indicating that the molar potency relationship between the shogaol homologues is similar to that existing between the gingerol homologues.
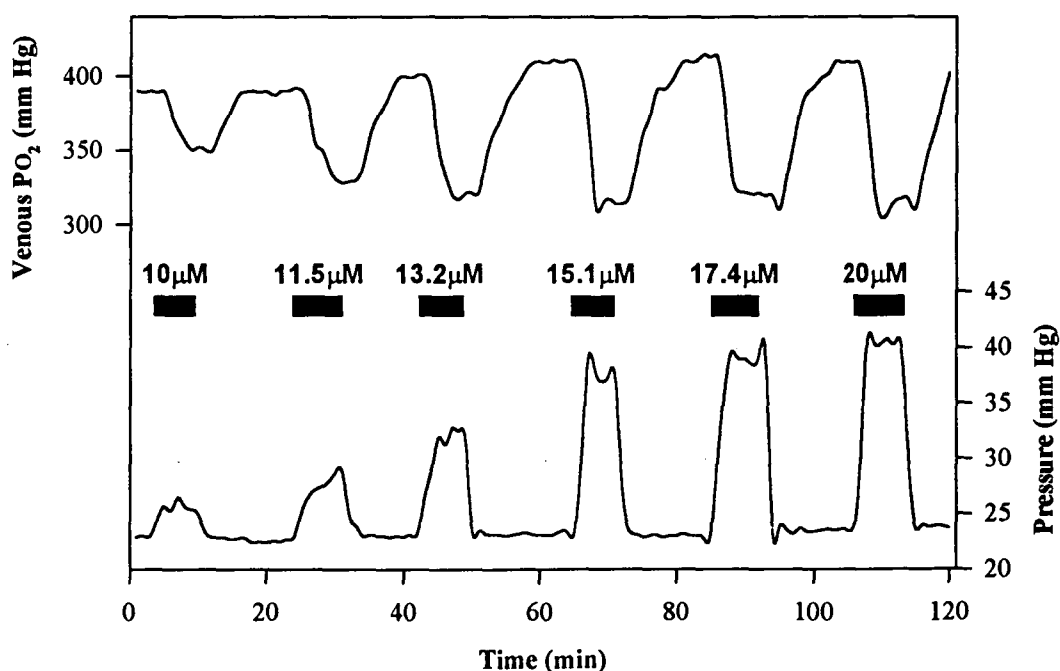
Fig. 2a.4. Typical dose response tracing of changes in venous PO_2 and perfusion pressure in perfused rat hindlimb preparations subjected to increasing concentrations of [6]-shogaol (10-20 μM, n = 5). Other ginger principles (discussed in the text) gave qualitatively similar response profiles. Randomising the order of addition did not alter the observed responses.
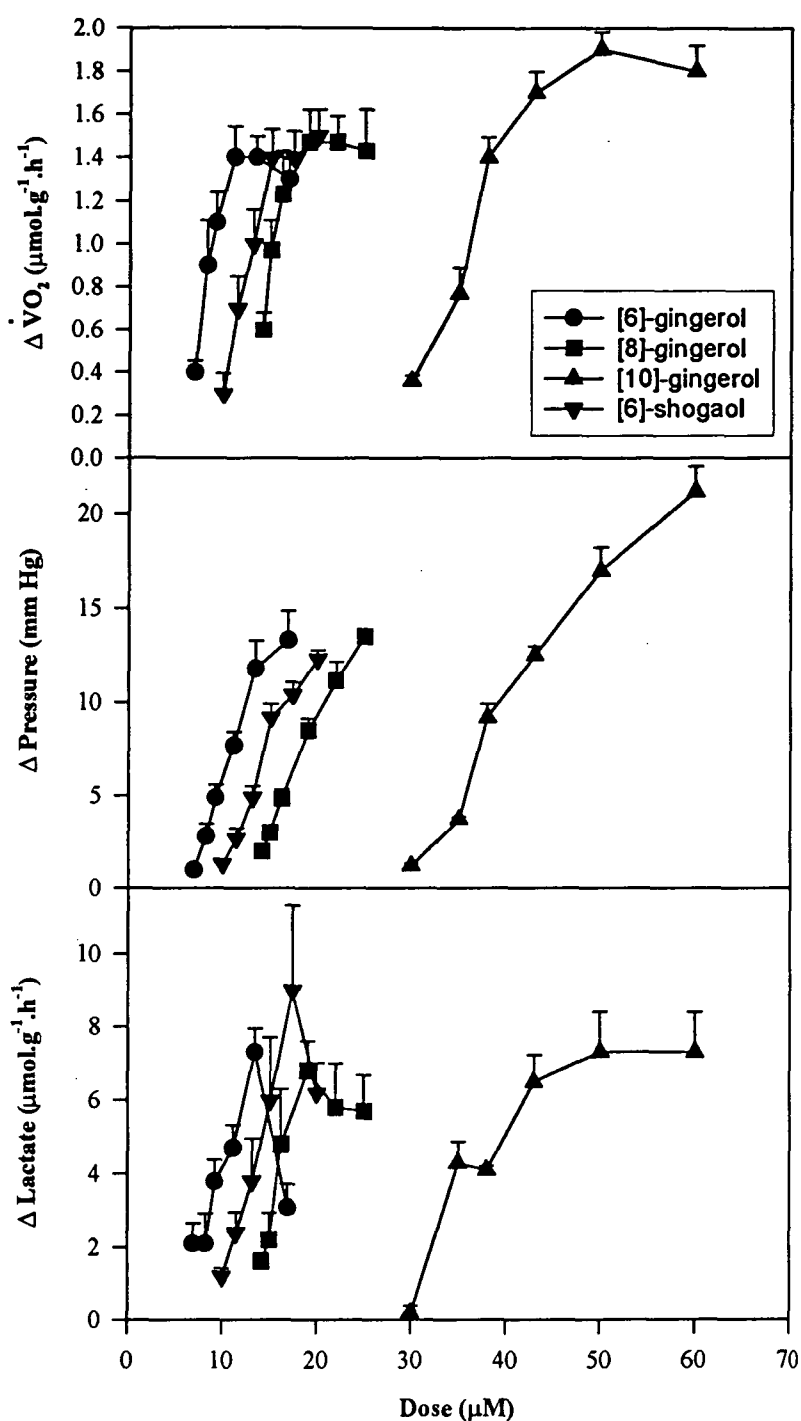
Fig. 2a.5. Dose response curves for changes in $\dot{V}O_2$, perfusion pressure, and lactate efflux in response to [6]-gingerol ($n = 7$), [6]-shogaol ($n = 5$), [8]-gingerol ($n = 3$) and [10]-gingerol ($n = 3$). Basal $\dot{V}O_2$ was 6.6 ± 0.2 $\mu mol \cdot g^{-1} \cdot h^{-1}$ ($n = 22$) and basal perfusion pressure was 26.0 ± 0.6 mm Hg ($n = 22$). Basal lactate efflux rate was 5.4 ± 0.3 $\mu mol \cdot g^{-1} \cdot h^{-1}$ ($n = 19$). Each value is the mean $\pm$ SE. Where error bars are not visible they are within the symbol.

Increasing the doses of all the ginger principles beyond the levels shown in Fig. 2a.5 led to a progressive inhibition of steady-state oxygen consumption (Fig. 2a.6). In the extreme case (Fig. 2a.6, 45 µM [6]-gingerol), oxygen consumption was inhibited to sub-basal levels following initial transient stimulation. Perfusion pressure continued to increase towards a plateau. Removal of ginger principles during high dose treatment resulted in large dose-dependent transient increases in oxygen consumption (Fig. 2a.6), whilst perfusion pressure (Fig. 2a.6) and lactate production (data not shown) levels returned to basal.
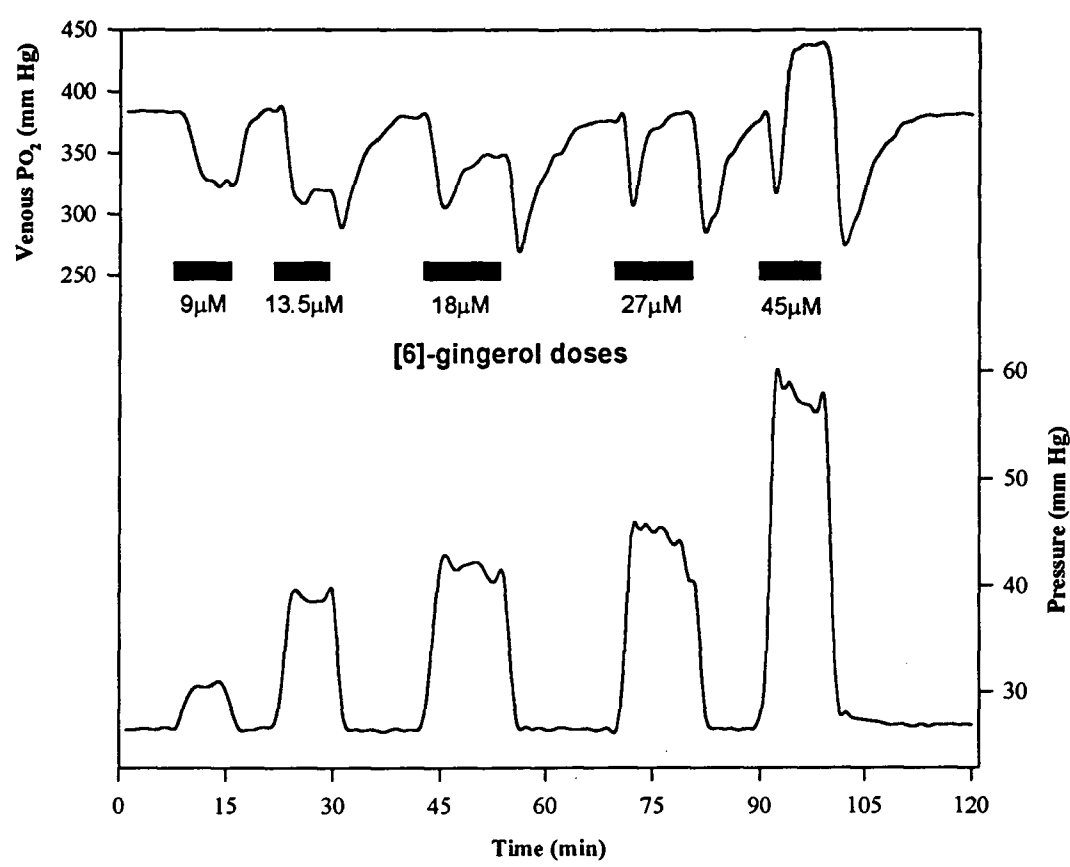
Fig. 2a.6. Typical dose response tracing of changes in venous PO₂ and perfusion pressure in perfused rat hindlimb preparations subjected to increasing high concentrations of [6]-gingerol (9-45 µM).

A half-maximal dose of [6]-shogaol (13.2 µM) was chosen to investigate the effects of specific nitrovasodilation, α–adrenergic blockade, and β–adrenergic blockade on the stimulated hindlimb (Fig. 2a.7). Neither 5 µM prazosin nor 5 µM

propranolol (supramaximal effective concentrations as determined by previous perfusion experiments, data not shown) inhibited the [6]-shogaol-induced response. The slight potentiation induced by each antagonist were not statistically significant. 5 µM GTN (maximal effective concentration) significantly (P < 0.05) inhibited the increases in oxygen uptake (56%) and perfusion pressure (72%) induced by [6]-shogaol (Fig. 2a.7). Typical oxygen and pressure traces shown in Fig. 2a.8 illustrate the effect of direct addition of 5 µM GTN to a half-maximal dose of [6]-shogaol.
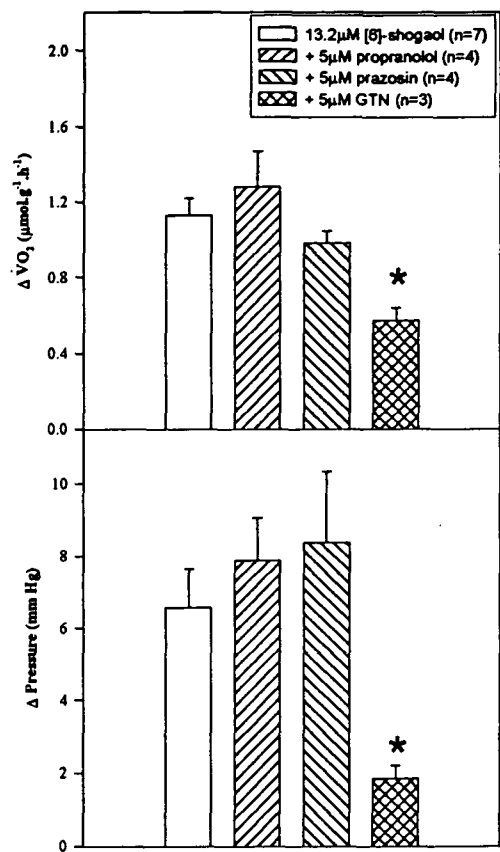
Fig. 2a.7. Effects of propranolol (PROP, 5 µM), prazosin (PRAZ, 5 µM) and glyceryl trinitrate (GTN, 5 µM) on steady state (5 min) changes in $\dot{V}O_2$ and perfusion pressure in perfused rat hindlimb preparations stimulated with 13.2 µM [6]-shogaol. Results are shown as the mean ± SE percentages of the responses relative to those using [6]-shogaol alone. Statistically significant (P < 0.05) effects are indicated (*). None of the antagonists alone had any effect on basal (unstimulated) hindlimb preparations.
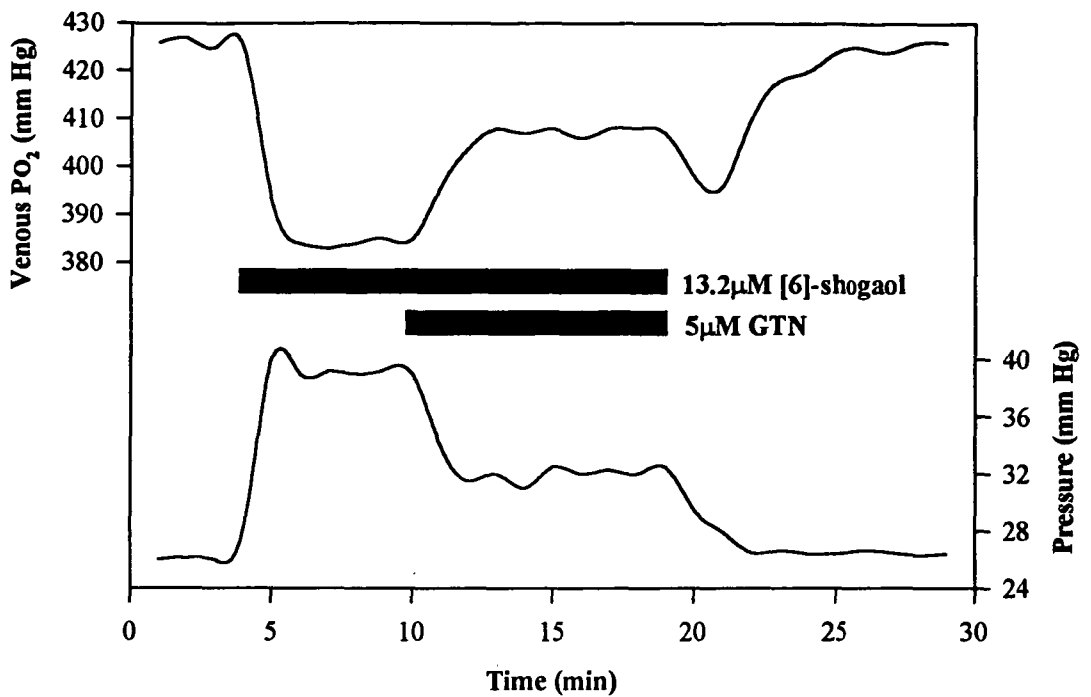
Fig. 2a.8. Typical tracing of the effect of glyceryl trinitrate (GTN, 5 μM) on changes in venous PO₂ and perfusion pressure in perfused rat hindlimb preparations stimulated with 13.2 μM [6]-shogaol.

Since the gingerols, shogaols, and capsaicinoids all bear the 4-methoxy, 3-hydroxy phenyl moiety, experiments were conducted to evaluate the thermogenic potential of other compounds bearing this vanillyl group. The structures selected (Fig. 2a.9) were vanillin, curcumin (from turmeric) and eugenol (cloves). Infusion of each over the range 0-10 mg·ml⁻¹ had no effect on perfusion pressure nor V̇O₂.

VANILLIN

EUGENOL

CURCUMIN
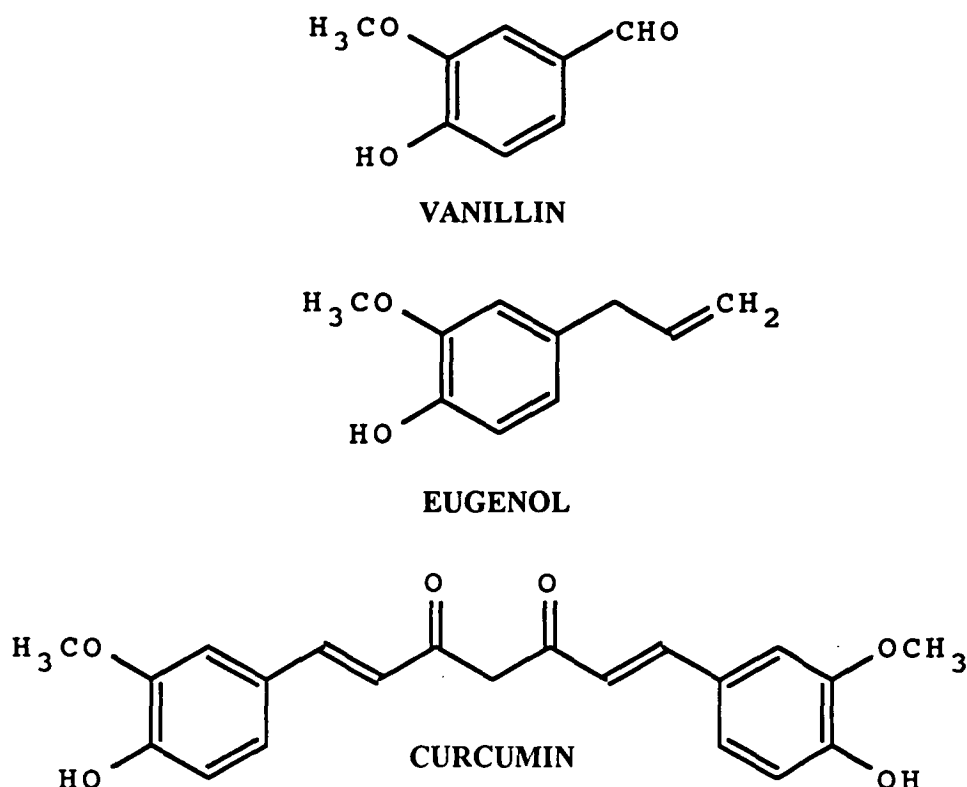
Fig. 2a.9. Structures of natural vanilloid molecules found to be inactive in the hindlimb perfusion model.

2a.4 Discussion

In general, perfused hindlimb tissue has proved to be responsive to thermogenic agents and hormones (Colquhoun *et al.*, 1988, 1990; Ye *et al.*, 1990a, 1990b; Janský and Hart, 1963; Mejsnar and Janský, 1971; Ruderman *et al.*, 1971; Grubb and Folk, 1976; Richter *et al.*, 1982a; Côté *et al.*, 1985). For the perfused rat hindlimb, the use of a non-erythrocyte perfusate at 25°C (Colquhoun *et al.*, 1988; Côté *et al.*, 1985) is comparable to constant flow perfusion with erythrocyte-containing medium at 37°C (Mejsnar and Janský, 1971; Grubb and Folk, 1976; Richter *et al.*, 1982a) for assessing noradrenaline-induced oxygen uptake; the technique has allowed the identification of vasopressin and angiotensin (Colquhoun *et al.*, 1988) and also the capsaicinoids (Cameron-Smith *et al.*, 1990) as potential thermogenic substances.

In the present study, the gingerols and shogaols were found to stimulate the hindlimb in a manner similar to that reported for the capsaicinoids (Cameron-Smith *et al.*, 1990). The observed responses were not related to the order of dose infusion (data not shown). Although the ginger principles do not possess an acylamide linkage, they bear two major structural similarities to the capsaicinoids, a 4-hydroxy, 3-methoxy phenyl (vanillyl) `head' and a carbonyl-containing alkyl `tail'. The failure of eugenol and vanillin, structures possessing only the vanillyl moiety (Fig. 2a.1), to induce a response in the perfused hindlimb suggests that both features are necessary for thermogenic activity. Curcumin, containing two vanillyl groups with a bridging, rather than a tailing, alkyl section was also inactive. Szolcsányi and Janscó-Gabor (1975) investigated the effect of altering the aromatic ring substituents, as well as the length and nature of the alkyl chain, on relative pungency of a range of vanillyl-derived compounds. The aromatic substituents - particularly the hydroxyl group - were of critical importance, whilst the chain length affected pungency in a more subtle manner. The overall trend was that relative pungency increased with chain length to a maximum at around 8-10 carbon atoms. Subsequent increases in chain length led to progressive pungency decreases. In the present study, the latter trend was expressed by the gingerol homologues, [6]-gingerol (10 carbon chain) having the highest molar potency. Maximal oxygen uptake, however, increased with alkyl chain length (Fig. 2a.5). [4]-gingerol (8 carbon chain) may have still greater molar potency, but its effect is of little consequence to the overall activity of ginger due to its trace presence (Harvey, 1981).

Although the reported ratios of the principles in ginger are somewhat variable, the [6]-homologue is consistently found to be the major compound. Connell and Sutherland (1969) found the [6]-, [8]-, [10]-gingerol ratio to be 53:17:30 respectively, whilst Chen *et al.* (1986) reported the same ratio to be 119:17:24. The [6]-gingerol content in ginger varies as a function of growth time, location, and storage period - a typical value being 1.5% of dry weight (Baranowski, 1986).

Dehydration of the alkyl chain (conversion of gingerol to shogaol) resulted in a small decrease in molar potency whilst maximal oxygen uptake was not significantly affected. Relatively few studies have directly compared the magnitude of the physiological effects induced by gingerols with those induced by shogaols. Suekawa

et al. (1984) found [6]-gingerol to have a lower LD$_{50}$ in rats, yet [6]-shogaol was reported to be more active on both the CNS and the digestive system.

It has previously been demonstrated that noradrenaline-induced oxygen uptake and perfusion pressure increases in the rat hindlimb are blocked both by phentolamine and high dose propranolol (Grubb and Folk, 1977). In the present study the effects of [6]-shogaol were not significantly altered by either of these antagonists (Fig. 2a.7), suggesting that the ginger principles were not acting directly via adrenergic receptors, nor by secondary catecholamine release. This latter phenomenon has been reported to be activated *in vivo* by a number of pungent principles including capsaicin (Watanabe *et al.*, 1987) and, to a lesser extent, zingerone (Kawada *et al.*, 1988).

In the present study, GTN (a specific nitrovasodilator) was used to inhibit both the oxygen and pressure responses to [6]-shogaol. This implies that the mechanism of action is closely related to the vascular system. Szallasi and Blumberg (1990a, 1990b) have reported the existence of a specific "vanilloid" receptor following studies using cultured nerve cells and radiolabelled ligands. The possibility of the presence of such a receptor on vascular smooth muscle cannot be overlooked since work from this laboratory has provided strong evidence of a link between the vascular system and hindlimb thermogenesis. Increases in perfusion pressure and oxygen uptake induced by vasopressin, angiotensin II and noradrenaline were inhibited by sodium nitroprusside (Colquhoun *et al.*, 1988), another specific nitrovasodilator. In addition, oxygen consumption by electrically stimulated skeletal muscle was found to be additive to that associated with vasoconstriction (Colquhoun *et al.*, 1990), whilst variable flow experiments showed that all flow-induced increases in oxygen uptake were enhanced by noradrenaline infusion but blocked by sodium nitroprusside (Ye *et al.*, 1990b).

Previous studies from this laboratory have reported that the vasoconstrictors norephedrine (Hettiarachchi *et al.*, 1991), vasopressin, angiotensin II and methoxamine (Hettiarachchi *et al.*, 1992) increase lactate release from perfused hindlimb preparations in association with increases in oxygen consumption and perfusion pressure. The present study has found that ginger principles induce similar dose-related lactate release concomitant with vasoconstriction and oxygen uptake. If such lactate production occurs *in vivo*, it could be part of a significant long-loop

thermogenic mechanism due to the high energy phosphates required for the resynthesis of lactate back to glucose in the liver (Cori cycle). In the case of [6]-gingerol, for example, the mean increase in lactate release associated with the mean maximal $\dot{V}O_2$ is 4.7 $\mu mol \cdot g^{-1} \cdot h^{-1}$. This rate of production would require an increase of 2.4 $\mu mol \cdot g^{-1} \cdot h^{-1}$ in oxygen consumption for full conversion to glucose in the liver. Experiments using both [6]-gingerol and [6]-shogaol found that there was no increase in lactate release associated with the large transient increases in $\dot{V}O_2$ following removal of high doses of ginger principles (data not shown). This indicated that these transient periods of greater $\dot{V}O_2$ were not associated with the reperfusion of hypoxic tissue.

Studies from this laboratory have found that serotonin (an endogenous vasoconstrictor) inhibited perfused hindlimb $\dot{V}O_2$ in a dose-dependent manner, but stimulated isolated mesenteric artery $\dot{V}O_2$ (Dora *et al.*, 1991). It was proposed that serotonin-induced vascular shunting was masking vascular thermogenesis. In the present study high doses of ginger principles, after initial stimulation, led to sub-basal $\dot{V}O_2$ (Fig. 2a.6), regardless of the order of dose infusion (data not shown). This phenomenon may also be due to vascular shunting. However, unlike serotonin the removal of high doses of ginger principles is followed by pronounced (but temporary) periods of low venous PO_2 (i.e. increased $\dot{V}O_2$) not associated with vasoconstriction (Fig. 2a.6).

Chudapongse and Janthasoot (1976) studied the effects of the analogous principle capsaicin on the energy-linked functions of isolated rat liver mitochondria. At lower doses with glutamate as substrate, capsaicin inhibited oxidative phosphorylation. At higher doses with succinate as substrate, capsaicin uncoupled mitochondrial respiration. High doses of capsaicinoids and ginger principles may be inducing similar effects within the hindlimb. Experiments performed in this laboratory have shown that hindlimb oxygen consumption induced by the known metabolic uncoupler sodium azide was inhibited by serotonin (Dora *et al.*, 1992b). High doses of ginger principles may have caused perfusate to be shunted away from uncoupled tissue associated with microvasculature, thereby masking net oxygen uptake by the hindlimb as a whole. Removal of the shunting might allow the oxygen-depleted

perfusate to depart the microvascular beds, resulting in the observed apparent increase in $\dot{V}O_2$. Testing these hypotheses will require further experimentation.

Henry and Piggott (1987) have examined the effect on human subjects of consuming a ginger sauce (containing unspecified amounts of ginger principles) with a meal. They concluded that metabolic rate was not significantly enhanced relative to subjects who consumed a control meal. Although little is known about the passage of ginger principles across the gut wall, the gingerol analogues capsaicin and dihydrocapsaicin are rapidly absorbed from the rat stomach and small intestine - both *in vivo* and *in vitro* (Kawada *et al.*, 1984). Results of pharmacokinetic studies (Ding *et al.*, 1991) have reported the half life of [6]-gingerol in rat plasma to be relatively short (7.2 minutes). The hindlimb perfusion results of this study found that the final concentration range of [6]-gingerol required for thermogenic responses was relatively narrow (7-15 μM). Concentrations below this range had no effect, whilst concentrations above this range gave either a reduced effect, or a negative thermogenic effect (Fig. 2a.6). In comparison, the concentration range for noradrenaline to increase oxygen consumption in this system is approximately 1-100 nM (Colquhoun *et al.*, 1988). Thus the ginger dose used by Henry and Piggott (1987) may have resulted in final *in vivo* concentrations outside any thermogenic range.

If ginger principles are subsequently found to have whole body thermogenic effects, the mechanism of action will be a moot point. The pressor response to [6]-shogaol in the whole rat body has been related to both central and autonomic (sympathetic) nervous system activity (Suekawa *et al.*, 1984, 1986a, 1986b). Similarly, postprandial thermogenesis in dogs has been decisively linked to autonomic activation due to oropharangeal sensory inputs (Diamond *et al.*, 1985; Diamond and LeBlanc, 1987). In the rat, similar sensory input increased brown adipose tissue thermogenesis except when sympathetic innervation was deactivated (Saito *et al.*, 1989). Thus any thermogenic effect of ginger principles *in vivo* may be attributable to central or autonomic nervous system activity, or a combination of both.

The present study, however, has shown that the perfused hindlimb thermogenic responses were intimately involved with the vasculature, and were not significantly altered by $\alpha-$ and β-adrenergic receptor blockade. These findings

therefore warrant future investigations of the actions of ginger and its principles in alternative vascular beds and ultimately *in vivo* with rats and humans in order to assess the potential of ginger as a dietary anti-obesity agent.

Chapter 2b

Resiniferatoxin and piperine: further direct-acting natural vanilloids.

2b.1 Introduction

Resiniferatoxin (RTX) and piperine are further members of the vanilloid family of naturally-occurring capsaicin-like compounds. Of these molecules, resiniferatoxin and capsaicin have in particular attracted escalating interest in recent years (Bevan and Szolcsányi, 1990; Szallasi and Blumberg, 1990b; Dray, 1992; Craft and Porreca, 1992; Maggi, 1992). Recent work with vanilloid molecules has increasingly focused on the neuropharmacological aspects of their activity (Bevan and Szolcsányi, 1990; Szallasi and Blumberg, 1990b; Dray, 1992).

RTX (Fig. 2b.1) is a diterpene present in the latex of some members of the genus *Euphorbia* (*E. resinifera*, *E. poissonii* and *E. unispina*; Hergenhahn *et al.*, 1975; Schmidt and Evans, 1976). The compound was first isolated after plant extracts were found to have unusually high activity in a mouse ear irritant assay (Hecker *et al.*, 1966). RTX has structural similarities to the phorbol esters, a group of compounds which act chiefly via their ability to stimulate protein kinase C (Castagna *et al.*, 1982). However, the mechanism of RTX-induced irritation has subsequently been shown to differ from that of the phorbol esters, such as phorbol 12-myristate 13-acetate (PMA; Evans and Taylor, 1983). Structurally, RTX is distinguished by the presence of a 4-hydroxy 3-methoxy phenyl acetate moiety in the 20 position. This homo vanillyl group has been shown to be essential for the potent, yet transient (Hergenhahn *et al.*, 1975), irritant activity exhibited by RTX (Adolf *et al.*, 1982; Schmidt and Evans, 1979). A number of studies have reported that RTX acts as an ultra potent capsaicin analogue (reviewed by Bevan and Szolcsányi, 1990; Szallasi and Blumberg, 1990b), its potency generally ranging from 10 to 10,000 times that of capsaicin for responses such as induced hypothermia, neurogenic inflammation, and stimulation (followed by desensitisation) of specific sub populations of sensory neurons.

Piperine (Fig. 2b.1) is best known as the pungent principle of black pepper (*Piper nigrum*). Both piperine and RTX have been found to stimulate some capsaicin-sensitive afferent neurons (Miyauchi *et al.*, 1988, 1989; Szolcsányi, 1983). In a relatively wide spectrum of pharmacological activity, other noteworthy actions of piperine include the stimulation of serotonin synthesis in the rat brain (Liu *et al.*, 1984), inhibition of smooth muscle nerve stimulation (Cole, 1985), anticonvulsant activity (Pei and Tas, 1974), and modulation of glucuronidation activity (Singh *et al.*, 1986).

CAPSAICIN

PIPERINE

RESINIFERATOXIN
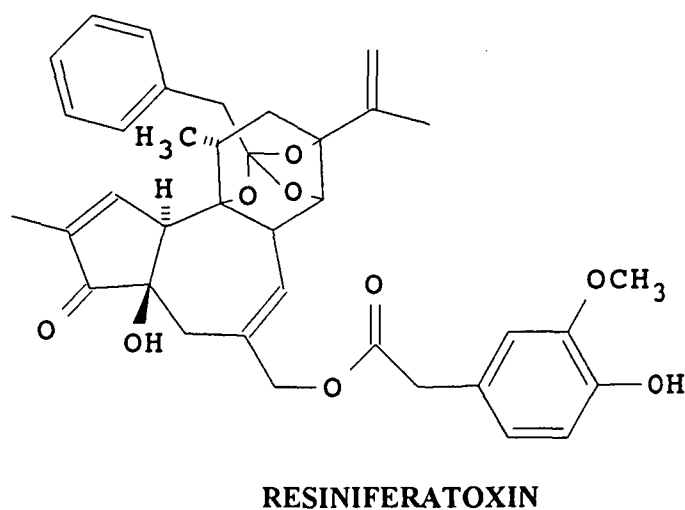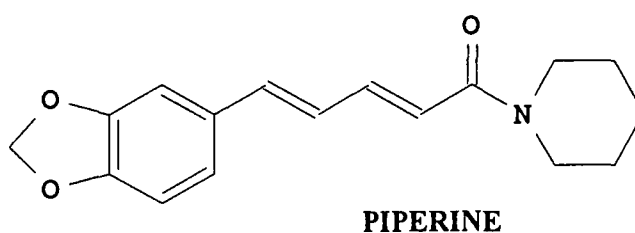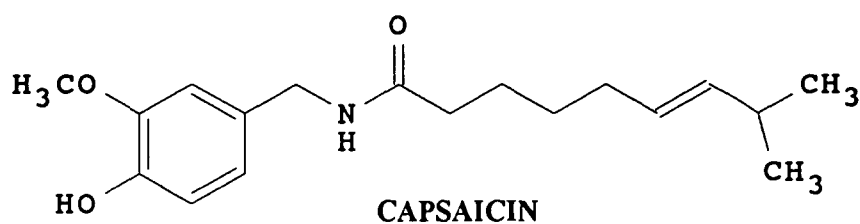
Fig. 2b.1. Structures of capsaicin and the capsaicin-like (vanilloid) agents discussed in the text.

Previous studies using neural tissue (reviewed by Szallasi and Blumberg, 1990b) have reported cross-tolerance of RTX and capsaicin, as well as piperine and capsaicin (Patacchini *et al.*, 1990). Such findings are consistent with a common mechanism of action. In addition, receptor-binding experiments using [^{3}H]-RTX have provided direct evidence of specific binding by sensory ganglion membranes not associated with protein kinase C (Szallasi and Blumberg, 1990a). Furthermore, alternative vanilloid compounds have been shown to inhibit the binding of [^{3}H]-RTX (reviewed by Szallasi and Blumberg, 1990b). The putative vanilloid receptor on specific subsets of afferent neurons is thought to be a ligand-gated non-specific cation channel (Marsh *et al.*, 1987).

In Chapter 2a it was demonstrated that vanilloid principles in ginger, the gingerol and shogaol homologues, were responsible for the direct-acting thermogenic activity of ginger extracts in the perfused rat hindlimb. The effects were similar to those mediated by capsaicin and dihydrocapsaicin (Cameron-Smith *et al.*, 1990) but of less magnitude than those mediated by infusion of catecholamines, angiotensin (I-III), or vasopressin in the same system (Colquhoun *et al.*, 1988). Prazosin and propranolol infusion showed that the responses to the vanilloid principles were not due to stimulation of adrenoreceptors, However, the responses were significantly impeded by nitrovasodilators. These findings highlighted the possibility of direct stimulus of vascular smooth muscle via vanilloid receptors on the smooth muscle itself, or alternatively via the release of non-adrenergic vasoactive agents by autonomic neurons embedded in the vessel walls.

In the present chapter, the investigation of thermogenesis mediated by vanilloid agents is extended by examining the effects of both RTX and piperine in the perfused rat hindlimb model.

2b.2 Methods

The methods used were essentially those described in Chapter 2a. Variations are listed below.

2b.2.1 Materials

Piperine was purchased from Sigma (St. Louis, Missouri, USA), whilst RTX was a generous gift from Dr D.J. de Vries, Australian Institute of Marine Science, Townsville, Australia.

2b.2.2 Agent infusion

Agents were infused using glass syringes with teflon tubing in a Sage Instruments syringe pump (model 355, USA). Ethanolic piperine solutions were infused at 5 $\mu l \cdot min^{-1}$. RTX, in 20% ethanol solutions, was infused at rates between 10 $\mu l \cdot min^{-1}$ and 40 $\mu l \cdot min^{-1}$. Stock ethanolic GTN solutions were diluted with saline and infused at 5 $\mu l \cdot min^{-1}$. Vehicle infusions had no effect on basal conditions.

2b.2.3 Statistics

The data are expressed as means ± standard errors. Regression curves were fitted using the Sigma-Plot program (Jandel Scientific, USA). Significance of differences ($P \leq 0.05$) was assessed using Student's unpaired two-sided t-test. In general, a minimum of five animals were used to determine a single data point.

2b.3 Results

The isolated perfused rat hindlimb was initially allowed to reach steady state perfusion pressure and venous PO_2. The mean arterial and venous PO_2 values were 666 ± 11 mm Hg ($n = 24$) and 375 ± 19 mm Hg ($n = 24$) respectively. The mean basal $\dot{V}O_2$ was therefore 7.1 ± 0.4 $\mu mol \cdot g^{-1} \cdot h^{-1}$ ($n = 24$). The mean basal perfusion pressure was 23.6 ± 0.9 mm Hg ($n = 24$). These values are consistent with those reported in Chapter 2a and those observed during other studies performed in this

laboratory (Cameron-Smith *et al.*, 1990; Colquhoun *et al.*, 1988, 1990; Ye *et al.*, 1990a, 1990b).

The perfusion pressure and $\dot{V}O_2$ responses were rapid for both piperine (Fig. 2b.4, $\dot{V}O_2$-stimulatory dose) and RTX (data not shown) with steady state conditions being attained within 5 minutes of commencing infusion of each agent. Similarly, basal conditions were re-established rapidly, within 10 minutes of agent removal. The traces for both $\dot{V}O_2$ and perfusion pressure were similar to those of the ginger vanilloids featured in Chapter 2a for both $\dot{V}O_2$-stimulatory doses (Chapter 2a, Fig. 2a.4) and $\dot{V}O_2$-inhibitory doses (Chapter 2a, Fig. 2a.6) As previously noted for other vanilloid molecules (Chapter 2a and Cameron-Smith *et al.*, 1990), blockade of α_1 and β_1/β_2 adrenoreceptors using 5 μM prazosin and 5 μM propranolol respectively had no inhibitory effects on the increased $\dot{V}O_2$ caused by either RTX or piperine (data not shown).

The effects of RTX and piperine on steady state $\dot{V}O_2$ and perfusion pressure as a function of concentration are shown in Fig. 2b.2. Concentration-response curves for capsaicin (Cameron-Smith *et al.*, 1990) and [6]-gingerol (Chapter 2a) are also included. RTX was clearly the most potent, and piperine the least potent agent used in this study. The molar potency of RTX was approximately 500-fold that of capsaicin, which in turn was 150-fold more potent than piperine (Fig. 2b.2).

The effects of glyceryl trinitrate (GTN) on stimulation of $\dot{V}O_2$ and perfusion pressure at steady state are shown using time course plots (all $n = 3$) in Fig. 2b.3. Values immediately prior to GTN infusion were significantly different ($P < 0.05$) from those at the end of the GTN infusion period in which $\dot{V}O_2$-stimulatory concentrations of either RTX or piperine were used. Higher concentrations of GTN resulted in no further inhibitory effects (data not shown).

The effects of a series of prolonged infusions with $\dot{V}O_2$-stimulatory concentrations of piperine are shown in Fig. 2b.4. There were no apparent changes in magnitude of the stimulated $\dot{V}O_2$ nor the increase in perfusion pressure throughout the series of infusions.
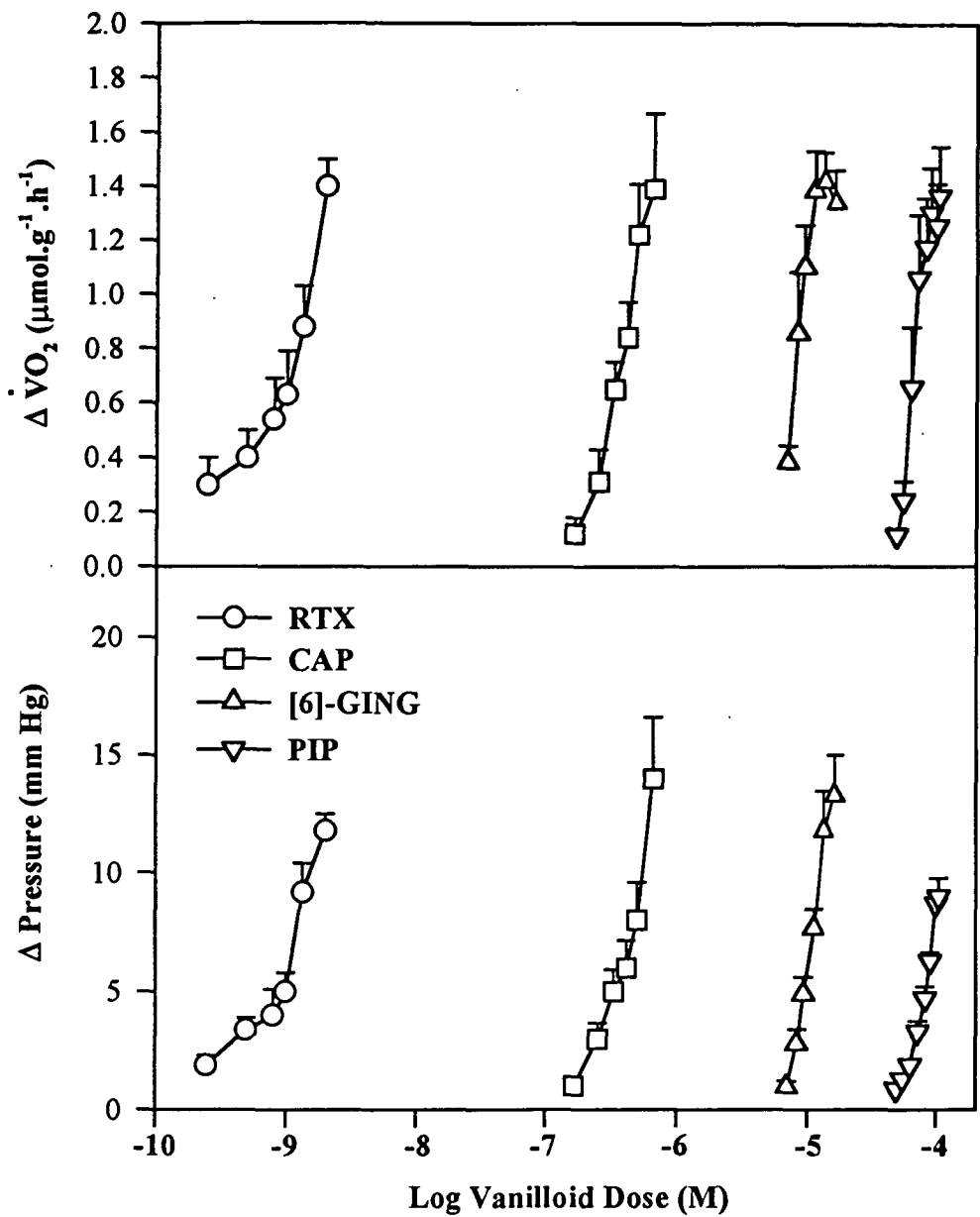
Fig. 2b.2. Dose-response curves for changes in oxygen uptake ($\Delta \dot{V}O_2$) and perfusion pressure in response to RTX (n = 8), capsaicin (CAP, n = 5-13, data taken from Cameron-Smith et $al.$, 1990), [6]-gingerol ([6]-GING, n = 7, data taken from Chapter 2a), and piperine (PIP, n = 8). The basal $\dot{V}O_2$ was 7.1 ± 0.4 $\mu mol \cdot g^{-1} \cdot h^{-1}$ (n = 24) and the basal perfusion pressure was 23.6 ± 0.9 mm Hg (n = 24). Each value represents the mean ± SE Where error bars are not visible they are within the symbol.
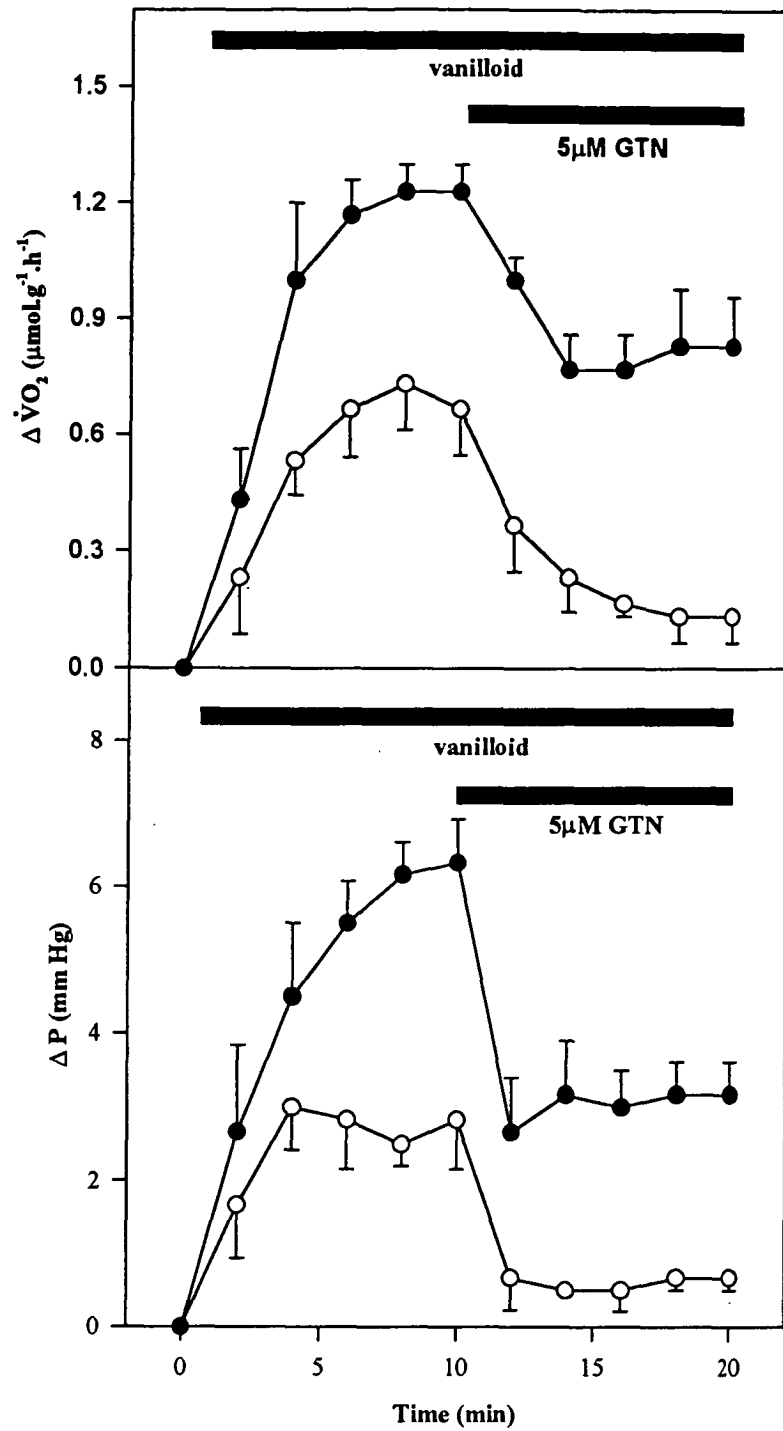
Fig. 2b.3. Time course plots showing the effect of 5 μM GTN on changes in oxygen uptake ($\Delta\dot{V}O_2$) and perfusion pressure (ΔP) induced by $\dot{V}O_2$-stimulatory concentrations of RTX (●, 1 nM, $n = 3$) and piperine (○, 75 μM, $n = 3$).
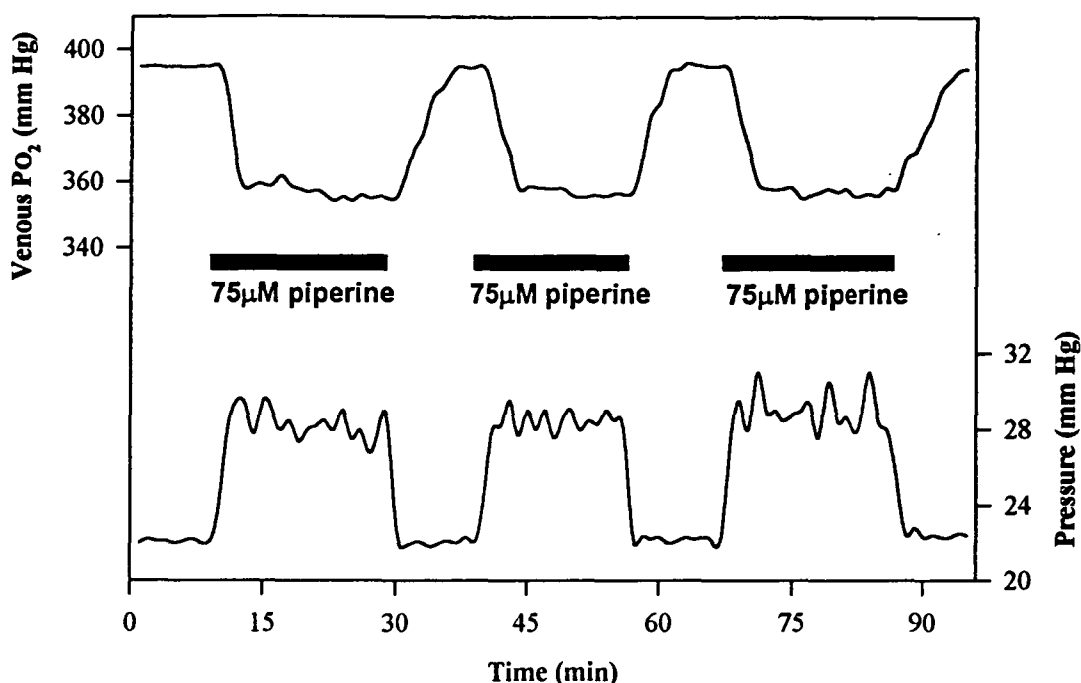
Fig. 2b.4. Time course tracings showing the effects of a series of prolonged infusions of piperine (75 μM) on venous PO₂ and perfusion pressure.

2b.4 Discussion

Both RTX and piperine stimulated the hindlimb to consume oxygen and the vascular bed to constrict in a manner similar to that of other active vanilloid compounds in this system (Chapter 2a, this study; Cameron-Smith *et al.*, 1990). However, the molar potencies of RTX and piperine were vastly different. RTX, containing a classical vanillyl (3-methoxy 4-hydroxy phenyl) group (Fig. 2b.1), was around 500-fold more potent than capsaicin, a result consistent with other studies which report RTX to be an ultra potent capsaicin analogue (reviewed by Szallasi and Blumberg, 1990b). Piperine, on the other hand, contains a vanillyl-like moiety in which the substituent groups form a secondary ring, giving a benzodioxolane fused ring system (Fig. 2b.1). The lower potency of piperine (approximately 150-fold less potent than capsaicin) may reflect the altered structure of this group.

High concentrations of both agents resulted in a steady-state inhibition of $\dot{V}O_2$ associated with increased perfusion pressure (type B effects). Such effects have previously been noted for the endogenous vasoconstrictors serotonin (Dora *et al.*,

1991) and high concentrations of noradrenaline (Dora *et al.*, 1992a). It has been proposed that type B effects for these agents are the result of a redistribution of vascular flow by functional flow shunts in the microvasculature (Dora *et al.*, 1991, 1992a). The vanilloid-induced effects may be due to the operation of a similar mechanism.

Concurrent infusion of GTN significantly blocked the $\dot{V}O_2$ and pressure increases induced by low doses of both RTX and piperine, indicating that the $\dot{V}O_2$ is somehow related to the vasoconstriction. This result is consistent with previous work in this laboratory which has repeatedly demonstrated such an association (Colquhoun *et al.*, 1988, 1990; Ye *et al.*, 1990a, 1990b).

RTX and capsaicin are known to stimulate a specific group of primary afferent neurons to release the vasodilatory neuropeptides substance P and calcitonin gene-related peptide (CGRP) from their peripheral endings (Szolcsányi *et al.*, 1990). However, in the perfused rat hindlimb active vanilloid compounds cause net vasoconstriction. Other studies have found capsaicin to have either contractile or relaxing endothelium-independent effects on vascular smooth muscle (Saito *et al.*, 1988; Duckles, 1986). The likely explanation is that constriction and relaxation are both components of acute *in vitro* capsaicin treatment (Duckles, 1986) Chronic pre treatment with capsaicin *in vivo* (thereby ablating capsaicin-sensitive sensory neurons) has resulted in isolated guinea pig vessels constricting rather than dilating when subsequently challenged with capsaicin *in vitro* (Duckles, 1986). This suggested that contraction was the result of a direct action on vascular smooth muscle, whilst relaxation was due to the release of neuropeptides (Saito *et al.*, 1988). The stimulatory responses induced by RTX and piperine in the present study are not subject to changes in magnitude following prolonged stimulation nor by repeated stimulation (Fig. 2b.4). Such changes might be expected if afferent nerve fibres in the vessel walls were being subject to progressive depletion of vasodilatory neuropeptides.

Although the participation of receptors has yet to be unequivocally established, the relative potencies of the vanilloid compounds in the perfused rat hindlimb are consistent with those reported in neuropharmacological studies (reviewed by Szallasi and Blumberg, 1990b) where direct evidence of a vanilloid

receptor has been obtained. Furthermore, the rapid kinetics of the responses (Fig. 2b.2) is consistent with a receptor-mediated mechanism in the hindlimb vasculature. Previous work using the perfused rat hindlimb model has shown that noradrenaline-induced increases in oxygen uptake and perfusion pressure were blocked by adrenoreceptor antagonists (Grubb and Folk, 1977). The use of specific α_1 (prazosin) and β_1/β_2-(propranolol) adrenoreceptor antagonists did not result in any diminution of the responses to RTX and piperine (data not shown), the ginger-derived vanilloids (Chapter 2a), nor the capsaicinoids (Cameron-Smith *et al.*, 1990). These findings suggest that vanilloids do not act directly on adrenoreceptors, nor via the secondary release of catecholamines.

Despite the thermogenic activity of capsaicin-like compounds *in vitro*, many such agents are reported to cause hypothermia *in vivo* in a variety of species (reviewed by Szolcsányi, 1982). The hypothermic response is thought to be the due to stimulation of the warm-sensors of the preoptic/anterior hypothalamic area, thus impairing body temperature regulation (reviewed by Szolcsányi, 1982). However, desensitisation of these warm-sensors is readily achieved; a single subcutaneous dose of capsaicin (50-75 $mg \cdot kg^{-1}$) in the rat results in the absence of capsaicin-induced hypothermia for several months. Furthermore, such desensitisation results in pyrexia at room temperature and long periods of hyperthermia at high ambient temperatures. In addition, desensitised rats showed a pyrogen-induced increase in core temperature associated with increased $\dot{V}O_2$ and vasoconstriction. Attempts to synthesise analogues with antinociceptive but not hypothermic properties have met with some success (Hayes *et al.*, 1984), suggesting that hypothermia need not necessarily be a feature of all active capsaicin analogs.

Studies in the human in the absence of any prior desensitisation procedures have found that meals containing capsaicin caused elevated body temperature during the first sleep cycle (Edwards *et al.*, 1992) and an increase in metabolic rate relative to a non-spicy control meal (Henry and Emery, 1986).

The notion of a vanilloid-sensitive thermogenic mechanism in the rat hindlimb raises the intriguing possibility of developing capsaicin analogs without hypothermic actions as anti-obesity agents *in vivo*. Such selectivity may be possible given that the

afore-mentioned hypothermic effects are both readily desensitised and separable from other actions by means of structural manipulations.

Chapter 3

Evidence for Vanilloid Receptor Subtypes (VN₁/VN₂) in Perfused Rat Hindlimb

3.1 Introduction

Most research interest into capsaicin and other vanilloids has centered on their well known actions on unmyelinated sensory nerves in the periphery as well as in the spinal cord and the brain. Recently, the role of vanilloid or capsaicin-like molecules in the depolarisation of these nerves and the mediation of pain and other effects has been extensively reviewed (Wood, 1993). The ability of capsaicin to desensitise a subpopulation of primary sensory neurones has led to its therapeutic use for the treatment of neuropathic pain (reviewed by Carter, 1991). Moreover, there is currently a great deal of interest in the potential of vanilloids as non-narcotic, non-steroid antiinflammatory and analgesic agents (reviewed by Szolcsányi, 1991; Maggi, 1992; Szallasi and Blumberg, 1993).

Capsaicin-sensitive primary afferent neurones release a number of neuropeptides when stimulated by capsaicin or other active vanilloids. These include substance P, neurokinin A, calcitonin gene-related peptide, galanin, dynorphin, cholecystokinin, vasoactive intestinal peptide and somatostatin (Holzer, 1991). These peptides all appear to play a role in the communication of primary sensory neurones with other neuronal and non-neuronal cells.

The sensitivity of the capsaicin-sensitive neurones to vanilloids is most likely due to the presence of a cation channel which, when stimulated by capsaicin, allows the influx of calcium and sodium ions and the efflux of potassium ions (Bevan and Szolcsányi, 1990). Capsaicin and other vanilloids are relatively lipophilic molecules and it is suggested that capsaicin may have a binding site on the surface of the cation channel proteins within the lipid bilayer (James *et al.*, 1993). The binding of capsaicin then results in the opening of the channel and the initiation of an impulse, the release of neuropeptides and ultimately the acute, painful, burning sensation associated with capsaicin (Buck and Burks, 1986).

The acute actions of capsaicin are not restricted to neurones; there are a number of reports of capsaicin influencing non-neuronal systems. These effects of capsaicin and functional analogues include inhibition of cardiac muscle excitability, inhibition of visceral smooth muscle activity, and contraction of vascular smooth muscle (Holzer, 1991).

In contrast to the findings of Kawada *et al.* (1988) and Watanabe *et al.* (1991) in whole rats, neither α- nor β-adrenergic antagonists significantly altered the vanilloid-induced effects in the perfused rat hindlimb (Chapters 2a and 2b; Cameron-Smith *et al.*, 1990). However, the vanilloid actions were largely - but not completely - blocked by the vascular smooth muscle relaxants nitroprusside and glyceryl trinitrate (GTN). Thus it appears that the vanilloids may act via a non-adrenergic mechanism, perhaps similar to their actions on the cation channel in sensory nerves. Such an action might be directly on smooth muscle or alternatively by the release of tachykinins from perivascular nerves which then cause vasoconstriction and produce either a stimulation of oxygen consumption at lower doses, or an inhibition of $\dot{V}O_2$ at higher doses.

The bidirectional effect on $\dot{V}O_2$ found with each vanilloid (Chapters 2a and 2b; Cameron-Smith *et al.*, 1990) suggests that two different vanilloid receptors might be present on or near vascular smooth muscle, or that the same receptor could be located on different responsive cell types having different post-receptor events. This chapter presents further functional and metabolic evidence showing two distinctly different sets of actions of capsaicin in the perfused rat hindlimb.

3.2 Methods

Methods are largely described in Chapter 2a. Variations are outlined below.

3.2.1 Materials

Capsaicin, ethylene-glycol-bis(β-aminoethyl-ether) N,N-tetraacetic acid (EGTA), xylazine, polyoxyethylene sorbitan monooleate (Tween 80) were supplied by Sigma (USA); sodium azide was from Merck (Germany); potassium cyanide from B.D.H. Laboratory Chemicals Division (England); paracetamol drops from Mead Johnson (Australia) and ketamine from Aldrich Chemical Co. (USA).

3.2.2 Rat hindlimb perfusion

During hypoxic perfusions, 95% N_2-5% CO_2 replaced the 95% O_2-5% CO_2 mixture after initial steady state conditions were obtained using 95% O_2-5% CO_2. Calcium-free ("zero calcium") perfusions were performed by omitting calcium from the perfusate and adding 0.1 mM Na_2EGTA.

When required, oxygen and perfusion pressure data were calculated from both peak and steady state values on the chart recorder.

The infusion of the various agents into the rat commenced only after the hindlimb had reached steady state $\dot{V}O_2$ and pressure values (approximately 30 min). Agents infused during the perfusion were freshly prepared prior to use. Due to the lipophilic nature of vanilloids and their apparent affinity for silastic tubing, capsaicin was dissolved in 50% ethanol and infused using a syringe pump (Model 355, Sage Instruments, Orion Research Inc., USA) driving a 1.0 ml glass syringe equipped with teflon tubing. All other agents were dissolved in isotonic saline and infused using a LKB 2132 Microperpex peristaltic pump (Bromma, Sweden) at rates between 5 and 40 ml·min⁻¹. Controls were conducted using vehicle alone.

3.2.3 Statistical analysis

The statistical significance of differences between groups of data was assessed by Student's unpaired t test. Significant differences were recognised at $P \leq 0.05$ and $P \leq 0.01$. All values given are the mean ± SE and are generally determined from a minimum of five animals.

3.3 Results

After perfusions had reached steady state, the mean arterial PO_2 was 672.5 ± 8.0 mm Hg ($n = 31$) and the unstimulated mean venous PO_2 was 372.7 ± 7.9 mm Hg ($n = 31$) with a basal $\dot{V}O_2$ of 7.0 ± 0.2 μmol·g⁻¹·h⁻¹ ($n = 31$) and a mean perfusion pressure of 24.5 ± 0.6 mm Hg ($n = 31$). Infusion of capsaicin over its effective range yielded similar data to that of Cameron-Smith *et al.* (1990). At the lower end of the dose range (0.125 μM), capsaicin showed a monophasic stimulation of oxygen consumption (Fig. 3.1A.) and the expected vasoconstriction-induced rise in perfusion

pressure (Fig. 3.1B). Infusion of higher doses of capsaicin (> 0.5 μM) led to further vasoconstriction (Fig. 3.1B). However, the effects on $\dot{V}O_2$ became triphasic with an initial stimulation followed by a steady state inhibition and a third phase of transient stimulation of $\dot{V}O_2$ upon cessation of the infusion of capsacin (Fig. 3.2A) as previously observed by Cameron-Smith *et al.* (1990). Steady state values for the high dose inhibition are shown in Fig. 3.1A. Other vanilloids show similar triphasic effects at high dosage (Chapters 2a and 2b).
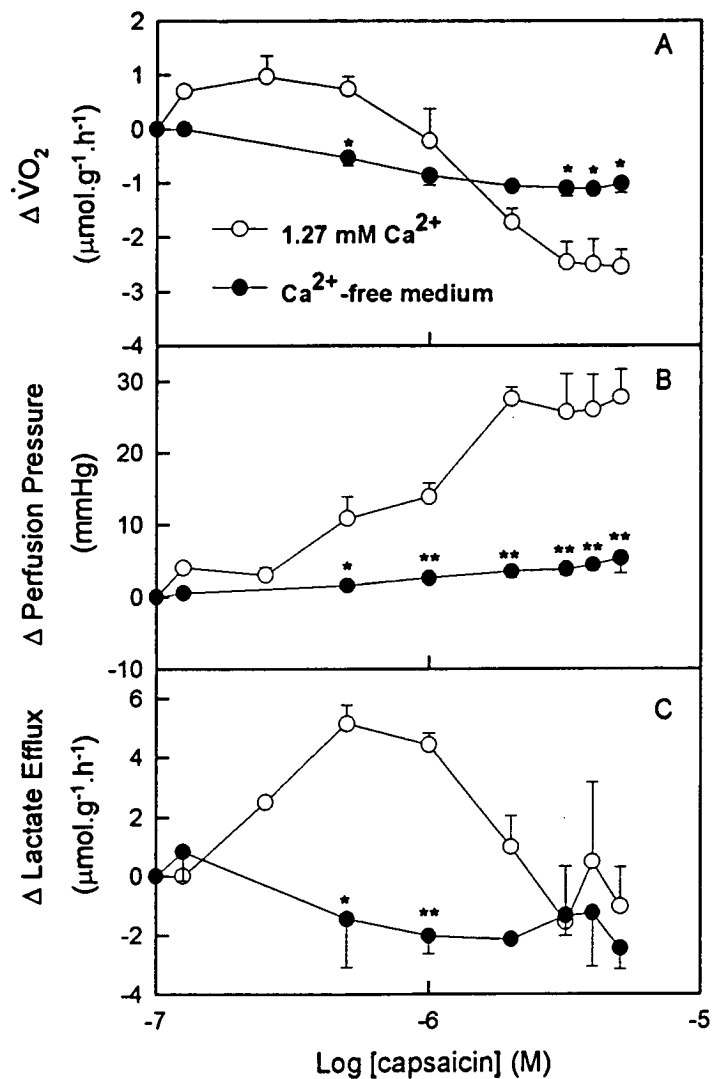
Fig. 3.1. Dose response curve for changes in steady state $\dot{V}O_2$ (panel A), plateau perfusion pressure (panel B) and lactate efflux (panel C) in response to capsaicin in perfused rat hindlimbs perfused with medium containing 1.27 mM calcium or with medium containing 0.1 mM EGTA and no added calcium (Ca^{2+}-free medium). Points are the mean ± SE of 3-5 observations. Where error bars are not visible they are within the symbol. *P < 0.05 **P < 0.01
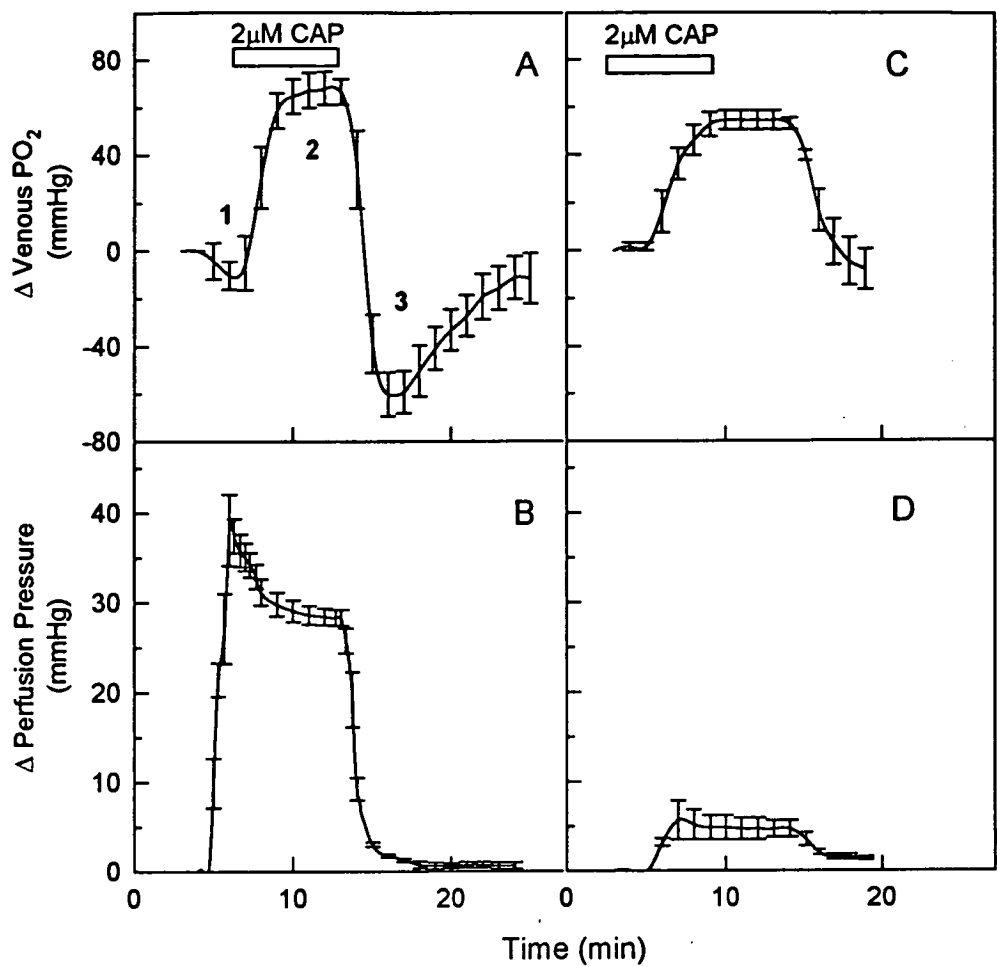
Fig. 3.2. Time courses of the oxygen and pressure responses exhibited by 2 μM capsaicin in the hindlimb perfused with: medium containing 1.27 mM calcium (n = 5) (A,B) or medium containing 0.1 mM EGTA and no added calcium (n = 3) (C,D). Values are the mean ± SE. Resting values of venous PO_2 were (A) 395.9 ± 24.7 mm Hg and (C) 394.4 ± 21.9 mm Hg. Basal perfusion pressures were (B) 21.6 ± 0.9 mm Hg and (D) 23.7 ± 0.9 mm Hg.

Infusion of large concentrations (2 μM) of capsaicin increased the perfusion pressure (Fig. 3.2B) by a maximum of 39.1 ± 2.4 mm Hg (173.4 ± 12.3% above basal, n = 5) followed by a steady change of 29.4 ± 1.1 mm Hg (130.1 ± 6.2% above basal, n = 5). In association with the rise in perfusion pressure, 2 μM capsaicin exhibited a triphasic oxygen consumption response. Initially $\dot{V}O_2$ increased (PvO_2 decreased) transiently above basal oxygen consumption during Phase 1, and was then

inhibited to below basal (PvO$_2$ increased) by 1.9 ± 0.5 μmol·g^{-1}·h^{-1} ($27.3 \pm 5.3\%$, $n =$ 5) during steady state (Phase 2). High dose capsaicin-induced effects approached steady state $\dot{V}$O$_2$ inhibition within 5 min of infusion and remained constant provided capsaicin was not withdrawn. The removal of capsaicin resulted in a period of increased oxygen uptake (Phase 3). The $\dot{V}$O$_2$ transiently increased above basal by 1.5 ± 0.1 μmol·g^{-1}·h^{-1} ($22.2 \pm 2.3\%$, $n = 5$), while perfusion pressure rapidly fell to resting values. The steady state dose response curves for $\dot{V}$O$_2$, perfusion pressure and lactate efflux in response to capsaicin are shown in Fig. 3.1. In general, the pattern of lactate efflux (Fig. 3.1C) followed the oxygen consumption; efflux was increased during stimulatory phases and inhibited in Phase 2 in which steady state $\dot{V}$O$_2$ was inhibited.

The infusion of capsaicin in the presence of an effectively zero external concentration of Ca^{2+} (0.1 mM EGTA) led to marked diminution of the perfusion pressure (Figs. 3.1B and 3.2D) and changes in the $\dot{V}$O$_2$ (Figs. 3.1A and 3.2A, C) and lactate efflux responses (Fig. 3.1C). Vasoconstriction was less at all effective concentrations of capsaicin, and the steady state $\dot{V}$O$_2$ (normally either Phase 1 for low dose capsaicin, or Phase 2 for higher inhibitory doses in the presence of Ca^{2+} ions, Fig. 3.2A) was either zero at low doses, or inhibited (PvO$_2$ increased) at higher doses (see Fig. 3.1), However the inhibition was less in magnitude than the inhibition of $\dot{V}$O$_2$ observed in the presence of Ca^{2+} ions. Zero Ca^{2+} reduced the steady-state efflux of lactate during infusion of 2 μM capsaicin (Fig. 3.1C) followed by a transient increase after capsaicin removal (data not shown).

The effects of hypoxia, cyanide, and azide on resting hindlimb $\dot{V}$O$_2$ and perfusion pressure are summarised in Fig. 3.3 and Table 3.1. Changes in $\dot{V}$O$_2$ were calculated from the basal values of those perfusions before additions. No significant effect on basal perfusion pressure was observed for any of these treatments. Infusion of 1 μM potassium cyanide inhibited $\dot{V}$O$_2$ by 4.6 ± 0.4 μmol·g^{-1}·h^{-1} ($77.9 \pm 5.2\%$, $n = 5$) below the basal value. Infusion of sodium azide increased $\dot{V}$O$_2$ by 4.4 ± 0.1 μmol·g^{-1}·h^{-1} ($73.2 \pm 7.1\%$, $n = 5$) above the $\dot{V}$O$_2$ basal value. Gassing the perfusion medium with 95% N$_2$-5% CO$_2$ decreased the arterial partial pressure of oxygen (PO$_2$) from 652 mm Hg to 18.5 ± 2.6 mm Hg. Representative traces for the action of high dose capsaicin in the rat hindlimb when mitochondrial respiration was impaired are shown in Fig. 3.3.

When steady state inhibition of oxygen consumption was reached with 1 mM potassium cyanide, shown in Fig. 3.3C as an increase in venous PO_2, 2 μM capsaicin was infused. This resulted in a transient and rapid increase in perfusion pressure of approx. 19.2 ± 1.1 mm Hg before falling by approx. 14 mm Hg to be maintained at 4.8 ± 0.3 mm Hg above the basal value (see Table 3.1). In addition, there was a small increase in oxygen consumption of 0.3 ± 0.1 $μmol·g^{-1}·h^{-1}$ (n = 5) that was not sustained but rapidly returned to the level existing prior to the infusion of capsaicin. Upon removal of capsaicin, the perfusion pressure returned to the basal value.

At the peak stimulation of $\dot{V}O_2$ (deepest trough in venous PO_2) achieved by 1 mM sodium azide (Fig. 3.3E), subsequent infusion of 2 μM capsaicin similarly resulted in a rapid and transient increase in perfusion pressure of 24.9 ± 1.9 mm Hg. As with the cyanide experiments, the pressure was not sustained and fell by approx. 12 mm Hg to a steady state of 12.5 ± 1.5 mm Hg above basal (n = 5). Capsaicin clearly induced a biphasic inhibition of azide-stimulated oxygen consumption. In the first rapid phase, $\dot{V}O_2$ was inhibited by 0.9 ± 0.2 $μmol·g^{-1}·h^{-1}$ before the second phase of inhibition of $\dot{V}O_2$ which was decreased by 1.0 ± 0.4 $μmol·g^{-1}·h^{-1}$. Removal of capsaicin resulted in the pressure returning to the basal value and the $\dot{V}O_2$ returning to the azide-alone stimulated value observed prior to capsaicin infusion.

Under hypoxic conditions achieved by gassing with 95% N_2-5% CO_2, 2 μM capsaicin induced a maximal perfusion pressure increase of 19.2 ± 3.2 mm Hg above basal, as indicated in Table 3.1 and Fig. 3.3H. The pressure fell back to be maintained for the remainder of the infusion period at a value of 40.9 ± 2.7 mm Hg or 15 ± 2.7 mm Hg above basal. Once the capsaicin was removed, pressure again returned to resting values. In all three methods of disturbing mitochondrial action in the perfused hindlimb, the infusion of a low dose of capsaicin (0.25 μM) had no discernible effect on perfusion pressure nor oxygen uptake (data not shown).

Table 3.1. Effects of 2 μM capsaicin on oxygen uptake and perfusion pressure during cyanide, azide and hypoxia.

Treatment	phase	Arterial PO$_2$ (mm Hg)	Venous PO$_2$ (mm Hg)	$\dot{V}O_2$ (μmol·g^{-1}·h^{-1})	Perfusion pressure (mm Hg)	(n)
Vehicle only	control	662.1±10.9	359.8±15.6	7.2±0.4	23.1±1.2	(8)
+2μM Capsaicin	phase 1			7.8±0.5	58.6±2.1	(8)
	phase 2			5.3±0.4	50.8±1.7	(8)
	phase 3			8.6±0.5	24.6±1.8	(8)
	control	710.5±17.6	414.4±16.6	5.9±0.3	26.9±0.6	(5)
Cyanide (1mM)	alone	670.7±4.7	613.3±12.2	1.3±0.3	25.3±0.8	(5)
+2μM Capsaicin	phase 1			1.6±0.3	48.5±2.5	(5)
	phase 2			1.0±0.3	34.1±2.9	(5)
	control	681.3±14.1	396.3±16.4	6.3±0.6	24.9±1.8	(5)
Azide (1mM)	alone	680.7±14.5	239.3±13.9	10.1±0.5	26.1±0.5	(5)
+2μM Capsaicin	phase 1			9.1±0.6	54.0±1.9	(5)
	phase 2			9.1±0.8	42.1±3.9	(5)
	phase 3			9.9±0.5	35.8±4.3	(5)
	control	666.6±26.1	359.2±12.5	6.8±0.8	25.5±0.8	(5)
N$_2$:CO$_2$ (95%:5%)alone		18.5±2.6	5.8±1.5	0.3±0.1	25.9±1.1	(5)
+2μM Capsaicin	phase 1			0.3±0.1	45.1±3.5	(5)
	phase 2			0.3±0.1	40.9±2.7	(5)

Values are means ± SE; n = number of hindlimb perfusions. The effects of cyanide, azide and hypoxia on basal venous and arterial oxygen tension, $\dot{V}O_2$, and perfusion pressure are shown. A gas mixture of 95% N$_2$-5% CO$_2$ was used to induce hypoxia. Control values are basal levels before treatment with cyanide, azide or nitrogen. Phase 1 is the initial stimulatory phase of oxygen uptake associated with peak pressure development. Phase 2 is steady state. Phase 3 is following the removal of capsaicin. (See also Fig. 3.3.)
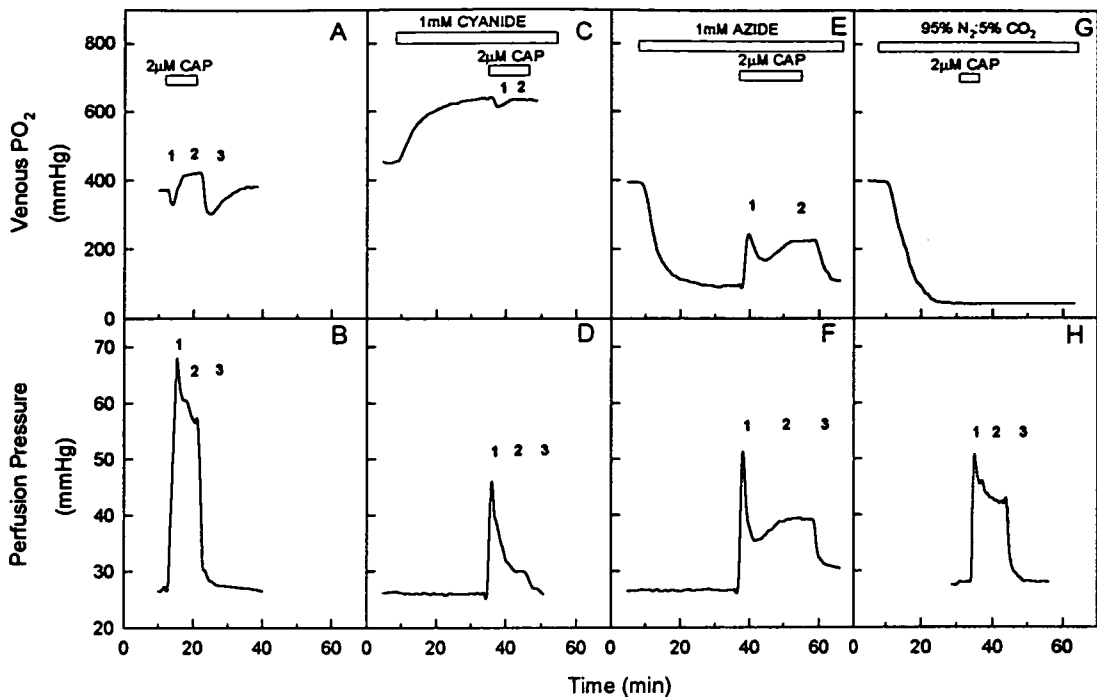
Fig. 3.3. Representative tracings of changes in venous PO₂ and perfusion pressure in response to infusion of 2 μM capsaicin (CAP) in the presence of potassium cyanide (1 mM), sodium azide (1 mM) or hypoxia. Perfusion medium, initially equilibrated against 95% O₂-5% CO₂, was either maintained (A-F) or switched to one equilibrated against 95% N₂-5% CO₂ (G,H) as shown. Capsaicin was infused for varying times until apparent steady state conditions of oxygen consumption were obtained. Phases of oxygen consumption and pressure change are labelled as follows: initial response, phase 1; steady-state response, phase 2; recovery phase, phase 3. Mean values from all experiments are given in Table 3.1.

3.4 Discussion

3.4.1 Hindlimb vanilloid receptor heterogeneity

The findings reported in this chapter have extended the findings of previous work with vanilloids in the perfused rat hindlimb model (Chapters 2a and 2b; Cameron-Smith *et al.*, 1990;) by examining the responses to both high and low dose vanilloid stimulation under conditions of metabolic challenge. These included low

external calcium concentration, hypoxia, and disruption to mitochondrial function using both cyanide and azide. The main finding to emerge from the data outlined in the present chapter is that the stimulation of $\dot{V}O_2$ at low concentrations of capsaicin and the inhibition of $\dot{V}O_2$ at high concentrations of capsaicin appear to result from activation of two different mechanisms. Both responses are independent of secondary release of catecholamines (Chapters 2a and 2b; Cameron-Smith *et al.*, 1990). It is conceivable that these different actions of capsaicin are activated through two receptor types (presumptive VN_1 and VN_2). The key reasons for the proposed classification into VN_1 and VN_2 receptors are summarised in Table 3.2 and discussed in the remainder of this section.

Table 3.2. Proposed classification criteria for VN_1 and VN_2 vanilloid receptors in perfused muscle.

Receptor type	VN_1	VN_2
Oxygen consumption	increased	decreased
Vasoconstrictor	strong	moderate
Affinity for vanilloid	high	low
Dependent on external Ca^{2+}	yes	no[1]
Dependent on O_2	yes	no
Lactate production (steady state)	increased	decreased[2]

[1] Independent of $[Ca^{2+}]$ but may require some Ca^{2+} for full agonist effect as inhibition of $\dot{V}O_2$ is less than in the presence of Ca^{2+}.
[2] After removal of capsaicin there is a "wash-out" peak of lactate.

Both mechanisms of vanilloid action are vasoconstrictive and appear to be additive because the perfusion pressure continues to rise with increasing capsaicin concentrations despite $\dot{V}O_2$ becoming inhibitory (Fig. 3.1). Similar patterns are seen with other active vanilloids such as gingerols and shogaols (Chapter 2a) and piperine and resiniferatoxin (Chapter 2b). The two oxygen consumption responses of

stimulation and inhibition occur at low and high doses respectively, suggesting differing affinities of the two presumptive receptors for capsaicin. Thus the receptors stimulated by lower concentrations of capsaicin (high affinity) and which stimulate $\dot{V}O_2$ are nominated as VN_1 and the receptors stimulated by higher concentrations of capsaicin (low affinity) and which inhibit $\dot{V}O_2$ are nominated as VN_2. The generation of bell-shaped response curves or related curves by the overlapping actions of both stimulatory and inhibitory receptors has been reviewed recently by Rovati and Nicosia (1994) and previously by Szabadi (1977). Such ideas are consistent with the premise of two vanilloid receptors acting in concert in the hindlimb to firstly stimulate, and then inhibit oxygen consumption with increasing dose of vanilloid.

The absence of external calcium inhibited the observed maximal capsaicin-induced vasoconstriction and shifted the dose curve markedly to the right (Figs. 3.1 and 3.2). Remarkably, the absence of external Ca^{2+} ions led only to an inhibition (Phase 2) of both $\dot{V}O_2$ and lactate efflux in response to capsaicin. Neither Phase 1 nor Phase 3 stimulation of $\dot{V}O_2$ were observed. However, inhibition of $\dot{V}O_2$ at high doses of capsaicin was less pronounced than observed with equivalent doses of capsaicin in the hindlimb perfused with buffer containing 1.27 mM Ca^{2+}. Thus, this vasoconstriction effect was substantially independent of Ca^{2+} but required external calcium for full agonist effect.

The proposed VN_1 site appears to be calcium dependent and to stimulate increases in $\dot{V}O_2$ and lactate production, whilst the proposed VN_2 receptor inhibits $\dot{V}O_2$ and lactate efflux and is largely independent of the need for external calcium ions. The simultaneous increase in lactate and $\dot{V}O_2$ without hypoxia has been seen previously in the perfused rat hindlimb in response to a number of different vasoconstrictors (Hettiarachchi *et al.*, 1992)

Hypoxia (N_2 gas), cyanide (cytochrome oxidase inhibitor), and azide each block some (but not all) of the perfusion pressure. Azide at 1 mM acts as though it is an uncoupler of mitochondria in the perfused hindlimb (Dora *et al.*, 1992a). These data, taken together, suggest that the inhibitory receptor or site for $\dot{V}O_2$ (VN_2) is not functionally dependent on O_2, even in the presence of O_2.

3.4.2 Other reports of vanilloid receptor/mechanism heterogeneity

Radioligand binding experiments with [^{3}H]RTX have not only permitted the biochemical characterisation of the vanilloid receptor (Szallasi and Blumberg, 1990a, 1990b, 1993), but have demonstrated species receptor heterogeneity, as well as possible intraspecies receptor subtypes (Szallasi, 1994). More recently, parallel assays for cooperative binding and functional potency (Acs *et al.*, 1995b) have yielded data which can be interpreted in terms of vanilloid receptor heterogeneity (see section 3.4.3). Lou *et al.* (1992) have described two mechanisms of action of capsaicin in the perfused lung which depend on the concentration of capsaicin present. Low dose effects (10^{-8} M capsaicin) were blocked by tetrodotoxin (TTX) whereas high dose effects (10^{-6} M capsaicin) were not. These authors modified an earlier suggestion that there were two mechanisms of action of capsaicin in which low concentrations of capsaicin stimulated the influx of limited amounts of Na^+ or Ca^{2+} ions which then triggered voltage sensitive Na^+ channels to conduct depolarisation to other varicosities or collaterals (Maggi *et al.*, 1989) whereas high dose capsaicin stimulated sufficient influx of ions to cause depolarisation without the need for Na^+-induced depolarisation. Recently, using patch-clamp methods, rat trigeminal cells have been shown to exhibit two different capsaicin-induced currents - one being fast and the other, slow (Liu and Simon, 1994).

Implicit in these observations is the premise that there may be two receptor types, one of which has a higher affinity for capsaicin and the other with lower affinity for capsaicin. Alternatively, there may be only one receptor type which is coupled to two post receptor mechanisms. However, differing affinities of a single receptor type requires that the receptors be situated in differing microenvironments. Such conditions result in alternative receptor protein conformations, giving affinity differentiation.

3.4.3 Cooperative binding of vanilloid ligands

The vanilloid receptor associated with neural tissue is reported to contain a non-selective, ligand-gated cation channel (reviewed by Bevan and Szolcsányi, 1990; James *et al.*, 1993). All such ion channels possess a multisubunit structure. The radiation inactivation size of the neural vanilloid receptor (Szallasi and Blumberg, 1991) is consistent with an oligomeric structure, a prerequisite for positive or negative

cooperative binding behaviour. Indeed neural preparations have been found to bind vanilloid ligands in a cooperative fashion (Szallasi *et al.*, 1993b; Acs *et al.*, 1995a), although the cooperative binding appears to be ligand-induced as opposed to being an inherent property of vanilloid receptors (Szallasi *et al.*, 1996).

Cooperative vanilloid binding can be demonstrated by monitoring [^{3}H]RTX binding in the presence of vanilloid ligands (e.g. RTX, capsaicin, and capsazepine; Szallasi *et al.*, 1993b; Acs *et al.*, 1995a; Szallasi *et al.*, 1996). Positive binding cooperativity is characterised by an initial enhancement of [^{3}H]RTX binding, followed by inhibition with increased ligand concentrations. The resultant binding curves bear a remarkable resemblance to vanilloid $\dot{V}O_2$ dose-response curves outlined in this and previous chapters, raising the possibility of such cooperative binding behaviour, and not receptor heterogeneity, being implicated in the biphasic $\dot{V}O_2$ dose-response curves. However, experiments in this laboratory with PPAHV (phorbol 12-phenylacetate 13-acetate 20-homovanillate), a vanilloid ligand demonstrated to abolish positive binding cooperativity in neural preparations (Szallasi *et al.*, 1996), give qualitatively similar biphasic $\dot{V}O_2$ dose-response curves to those noted for other vanilloid agents (unpublished observations by C.D. Griffiths). It is postulated that PPAHV acts to block the conformational changes leading to positive cooperativity (Szallasi *et al.*, 1996). Thus if the putative peripheral vanilloid receptors of the rat hindlimb are assumed to be related to those found in the CNS, cooperative binding effects seem an unlikely explanation for the biphasic nature of the vanilloid $\dot{V}O_2$ dose-response curves. Indeed, other workers have found that an absence of binding cooperativity, in addition to lower binding affinities, associated with peripheral vanilloid receptors distinguishes these binding sites from central vanilloid receptors in the rat (reviewed by Szallasi, 1994).

Acs *et al.* (1995b) have directly compared vanilloid binding affinities with functional potencies (in Ca^{2+} uptake assays) in neural tissue. The stimulation of Ca^{2+} uptake was reported to be non-cooperative, whilst binding behaviour was cooperative. The favoured explanation for the differing kinetics was that the two assays were detecting responses mediated by two distinct receptor classes, with differing structure-activity profiles, on primary sensory neurons (Acs *et al.*, 1995b; Szallasi *et al.*, 1996). Such vanilloid receptor heterogeneity has been previously

proposed to account for the diverse relative potencies of vanilloids (Szallasi and Blumberg, 1990b; Szallasi, 1994).

3.4.4 Parallel subtype/mechanism heterogeneity in other receptor systems

The use of alterations in external Ca^{2+} ions to discriminate between α-adrenergic receptor subtypes has been used by Minneman (1988) and by Han et al. (1987, 1990). They have suggested that the α_{1a} subtype is coupled to external Ca^{2+} ions and that the α_{1b} subtype is coupled to internal Ca^{2+} stores. Similarly, studies in this laboratory have shown that the presence or absence of external Ca^{2+} can distinguish between two presumptive α_1-adrenoceptor subtypes in the control of oxygen uptake by noradrenaline in the perfused rat hindlimb (Dora et al., 1992a).

On the other hand, Ruffolo and coworkers (1991) have argued strongly that the same α-adrenoceptor may be present, but coupled to different effector mechanisms. Were a similar arrangement to underlie the present study, with one post receptor mechanism stimulating $\dot{V}O_2$ and another inhibiting $\dot{V}O_2$, it is hard to reconcile how such receptors could exist simultaneously on the same cell. It would thus seem more appropriate to postulate the presence of the same receptor on two different smooth muscle cell types. As both sites are associated with vasoconstriction, the receptors may thus be on different calibre arteries or arterioles. Supporting this idea is the general correlation between artery size and dependency on external calcium ions for contraction, with smaller vessels showing the greatest dependency (Tayo and Bevan, 1987). This is also true in the rat hindlimb (Sutter et al., 1977).

The notion of presumptively different VN_1 and VN_2 recognition sites being distributed on vessels of different calibre, is consistent with reports by others of different anatomical distribution of $5HT_1$ and $5HT_2$ receptors (Blackshear et al., 1985; Lamping et al., 1989) and of α_1 and α_2 (Ruffolo et al., 1991; Dora et al., 1992b) adrenoceptors on the arterial vasculature.

3.4.5 Implications of potential vanilloid receptor subtype selectivity

The presence of an inhibitory receptor for the vanilloids may explain why the consumption of such spice principles has not uniformly shown a thermogenic or weight-loss effect, despite the peripheral thermogenic activity demonstrated in this

and previous chapters. The data suggest that it might be possible to synthesise agents that selectively stimulate VN_1 or inhibit VN_2 putative subtypes which would thus have important thermogenic or weight loss potential, particularly if central neurological effects can be avoided. The results of a structure-activity study conducted using a range of vanilloid agents modelled and synthesised at this University are presented in Chapter 4.

Chapter 4

Structural Requirements of Synthetic Vanilloid Agents for Hindlimb Thermogenesis

4.1 Introduction

Vanilloids possess a wide spectrum of pharmacological activity, particularly with respect to both central and peripheral neural tissue (reviewed by Holzer, 1991; Buck and Burks, 1986). As a result of their ability to desensitise a subset of small diameter unmyelinated sensory neurones, vanilloids have been identified as a new group of non-narcotic, non-steroidal analgesic and antiinflammatory agents (reviewed by Szolcsanyi, 1991; Maggi, 1992; Szallasi and Blumberg, 1993). This potential therapeutic application has provided the major impetus for investigations of vanilloid receptor(s) and structure-activity relationships of vanilloid agents.

The results outlined in Chapters 2a and 2b identify naturally-occurring vanilloids as agents capable of stimulating thermogenic responses under controlled conditions *in vitro*. Evidence presented in Chapter 3 supported the postulate that specific receptor subtypes may be mediating the observed responses. Present vanilloid structures possess an inherent lack of pharmacological selectivity. If, for example, the positive and negative thermogenic actions reported in Chapters 2a, 2b and 3 are to be separated from each other and from the CNS-mediated effects on thermoregulatory control (e.g. hypothermia) resulting from *in vivo* vanilloid administration (reviewed by Buck and Burks, 1986), a detailed knowledge of the range of vanilloid receptor subtypes and the structure-activity relationships of vanilloid ligands is required.

The earliest vanilloid structure-activity studies linked pungency and structure (Jones and Pyman, 1925). However the first proposal of a capsaicin receptor followed the evaluation of a series of capsaicin analogues using eye-wipe assays (Szolcsányi and Jansco-Gabor, 1975). More recently, resiniferatoxin (Szallasi and Blumberg, 1990a) and the competitive antagonist capsazepine (Bevan *et al.*, 1992) were introduced as tools to characterise the neural vanilloid receptor. Studies performed using [^{3}H]RTX have not only succeeded in demonstrating the existence of vanilloid

receptors, believed to be ligand-gated cation channels (James *et al.*, 1993), but have implied that the receptor system is heterogeneous, with receptor types, subtypes and marked species-related differences (reviewed by Szallasi, 1994).

Recent vanilloid structure-activity investigations have centred on refining the pharmacophore for anti-nociceptive activity (Janusz *et al.*, 1993; Walpole *et al.*, 1993a-c). The general approach has been to consider vanilloid molecules in terms of three regions (Fig. 4.1), each of which plays an important part in determining the structure-activity profile of this group of agents. Using this regional classification, systematic structural changes have been confined to a single region of a given synthetic molecule, the structure of the remaining two regions being conserved.

A Region   B Region   C Region
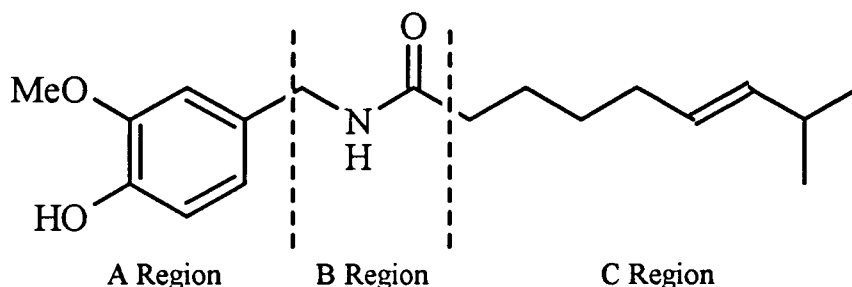
Fig. 4.1. Structure of capsaicin showing the three regions generally considered when examining structure-activity relationships of vanilloid molecules.

Chapter 3 presented evidence for the presence of two vanilloid receptor subtypes in the perfused rat hindlimb preparation. It has already been noted that natural vanilloid molecules differ in terms of maximal $\dot{V}O_2$ responses (e.g. [6]-gingerol and [10]-gingerol, Chapter 2a). Such differences may be due to receptor subtype selectivity. Given that evidence exists for vanilloid receptor heterogeneity (Chapter 3; Szallasi, 1994; Acs *et al.*, 1995b), an improved understanding of receptor-ligand interactions may in future enable pharmacological selectivity for a number of vanilloid responses. The existing literature contains some evidence to support such selectivity. Attempts to separate antinociceptive and hypothermic properties by Hayes *et al.* (1984) were partially successful using a range of synthetic capsaicin analogues.

Structure-activity studies by Brand *et al.* (1987) found that certain long chain C-regions reduced or abolished activity in pungency assays, whilst antinociceptive and 'desensitising' properties were retained.

The broad objective of the studies reported in this chapter was to conduct a systematic structure-activity investigation of synthetic vanilloid molecules in order to determine the structural features and configurations required for thermogenic activity, as measured by increased oxygen consumption, in perfused rat hindlimb preparations. Comparison of such a structure-activity profile with those developed in alternative bioassays may allow the identification of structural features unique to *in vitro* thermogenic activity, thereby enhancing the possibility of developing agents as selective anti-obesity drugs.

4.2 Methods

4.2.1 Materials

Synthetic vanilloid analogues were synthesised in the Department of Chemistry, University of Tasmania. The synthetic methods used are outlined by Mackey (1992), Reardon (1994), and Clippingdale (1995) [Honours theses, University of Tasmania]. HPLC purification of synthetic compounds, when required, was performed in the author's laboratory using the preparative HPLC apparatus and methods given in Chapter 2a. Compound purity was assessed by GC-MS, and compounds were characterised by ^{1}H nuclear magnetic resonance (NMR) spectroscopy, ^{13}C NMR, GC-MS, and high resolution direct-insertion mass spectroscopy. Spectroscopic analyses were performed by the Central Science Laboratory and the Department of Chemistry, University of Tasmania.

Ethanol and dimethyl sulphoxide used as solvents for synthetic vanilloid infusions and subcutaneous injections respectively were both AR grade (Ajax, Australia). Other materials used for perfusion experiments are outlined in Chapter 2a.

4.2.2 Perfusion experiments

The protocols for the perfusion experiments were the same as those given in Chapter 2a.

4.2.3 Infusion of synthetic vanilloid compounds

Synthetic vanilloids were infused as concentrated ethanol solutions. Infusions of vehicle alone up to a final concentration of 0.25% ethanol had no effect on basal perfusion conditions. In the rare instances that the ethanol final concentration did exceed 0.25% during the infusion of synthetic agents (due to low agent solubility), comparable vehicle alone infusions were performed to confirm the vehicle plus vanilloid result. Synthesised compounds were considered inactive if concentrations of 300 μM did not alter basal perfusion pressure or oxygen consumption. The isolated perfused hindlimb has been well established as a reliable preparation in this laboratory. As a result, compounds found to be inactive were generally not re-tested in a further perfusion experiment, but were assessed using a number of concentrations (generally 5-8 concentrations up to 300 μM) in a single perfused preparation. Capsaicin (0.5 μM) was used to confirm the vanilloid-responsiveness of a hindlimb preparation following a series of negative results. Active compounds, on the other hand, were assessed in 2-5 hindlimb preparations. The construction of concentration-response curves allowed the determination of half-maximal concentrations and maximal $\dot{V}O_2$ increases (given in the Figures).

4.2.4 Rat whole body calorimetric measurement

Whole body calorimetry was performed using the apparatus and methods similar to those described by Ye *et al.* (1995). Male hooded Wistar rats (approximately 250 g) were anaesthetised with Saffan (9 mg·ml^{-1} alphaxalone, 3 mg·ml^{-1} alphadolone acetate, Pitman-Moore, Australia; 830 μl·kg^{-1} *i.v.* bolus, followed by continuous *i.v.* administration of 83 μl·kg^{-1} via the tail vein). Vanilloid analogues were injected subcutaneously as 100% dimethyl sulphoxide solutions. Whole body heat production was calculated from measured $\dot{V}O_2$ according to the method of Weir (1949).

4.3 Results and Discussion

4.3.1 Phenyldecane derivatives

A series of phenyldecane derivatives (Fig. 4.2) with 3,4- A-region substitution and carbonyl or hydroxyl chain substitution were assessed for hindlimb thermogenic activity.

2A (- , oedema) R = OH
2B (-) R = OCH$_3$

2C (-)

2D (160, 14.6%)
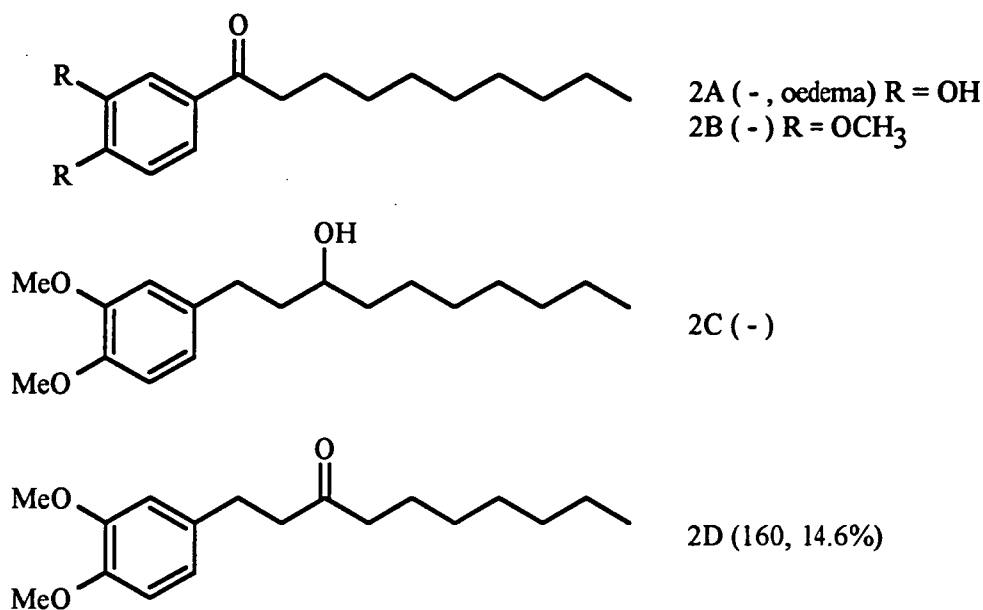
Fig. 4.2. Phenyldecane derivatives tested for thermogenic activity. Numbers in brackets represent the concentration required for a half-maximal $\dot{V}O_2$ response (µM), followed by the maximal $\dot{V}O_2$ response above basal ($\Delta \dot{V}O_{2\,max}$, %). Lack of activity (no change in $\dot{V}O_2$ or perfusion pressure at concentrations ≤ 300 µM) is indicated by (-). Where 'oedema' is indicated, compound activity could not be fully assessed due to the induction of tissue oedema (i.e. swelling due to an abnormal accumulation of fluid in extracellular spaces).

The activity of 2D, although lower than that of the natural vanilloids (Chapters 2a, 2b and 3), demonstrates that the vanillyl A-region of the natural vanilloids is not an absolute prerequisite for activity. The lack of activity of 2C suggests that the carbonyl substitution in the chain cannot simply be replaced by another oxygen-containing moiety. Comparison of 2B and 2D reveals that positioning the carbonyl group adjacent to the aromatic ring abolishes activity.

4.3.2 Ester B-regions

A series of compounds with ester B-regions replacing the amide linkage of the capsaicinoids were examined (Fig. 4.3). A similar ester linkage is a feature of RTX (Chapter 2b, Fig. 2b.1), the most potent of the natural vanilloids in this (Chapter 2b) and the majority of other vanilloid bioassays (Bevan and Szolcsányi, 1990; Szallasi and Blumberg, 1990b).

3A (50, 15.3%) R = n-octyl

3B (65, 16.5%) R = H₂C

3C (-) R = H₂C

3D (-)

3E (-)

3F (oedema)
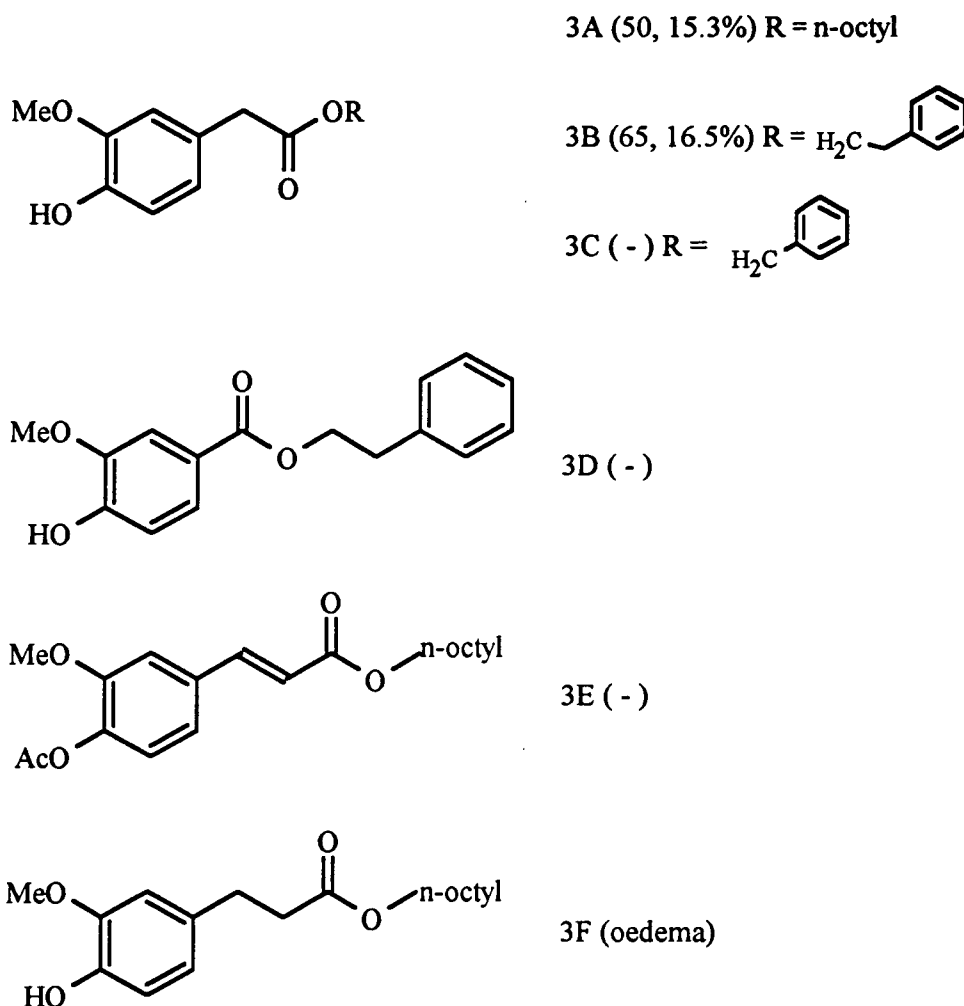
Fig. 4.3. Ester-linked vanilloids tested for thermogenic activity. Figures in brackets refer to the same parameters given in Fig. 4.2.

Structures 3A and 3B have A and B-regions identical to those of RTX (Chapter 2b, Fig. 2b.1), but are around 50 000 times less potent. Clearly the C-region is an important determinant of overall thermogenic activity. The n-octyl C-region

(3A) conferred a higher potency than either phenylethyl (3B) or phenylmethyl (3C, inactive) moieties. If the phenyl ring of the C-region is considered to contribute 3 carbon atoms to the overall length, the relative lengths of the C-region in 3A, 3B and 3C are 8, 5, 4 carbon atoms respectively. Using a similar approach with RTX (Chapter 2b, Fig. 2b.1), the length of the C-region is 7 carbon atoms. Taken together, these conclusions are consistent with those of Walpole *et al.* (1993c) who found that the optimal chain length in neural tissue, regardless of B-region structure, was 8-10 carbon atoms.

Placement of the carbonyl group adjacent to the A-region ring (3D) abolishes activity, as noted for 2A and 2B. It appears likely that the inactivity of 3E is due to the 4-acetoxy substituent in the A-region rather than the unsaturated A-region/B-region bridge, since similar unsaturation in the 'reverse amide' structures given in Fig. 4.4 has not removed activity.

4.3.3 'Reverse amides' with an unsaturated A-region/B-region bridge

The series of structures shown in Fig. 4.4 are referred to as 'reverse amides' (Walpole *et al.*, 1993b) due to the reversed positioning of the nitrogen atom and the carbonyl group relative to the A-region when compared to the capsaicin structure (Fig. 4.1).
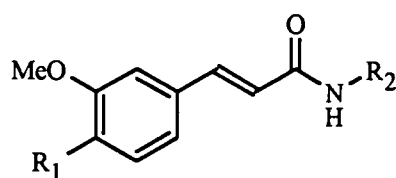
4A (150, 18.1%) R1 = OH, R2 = n-octyl

4B (280, 16.8%) R1 = OH, R2 = H_2C

4C (350, 16.8%) R1 = OH, R2 = H_2C

4D (-) R1 = AcO, R2 = H_2C

4E (-) R1 = AcO, R2 = n-octyl

Fig. 4.4. Reverse amides with an unsaturated A-region/B-region bridge. Figures within the brackets refer to the same parameters as given for Fig. 4.2.

Structures 4A-C have the same series of C-regions as 3A-C (Fig. 4.3). The same order of potency is observed for each set of structures, whilst the differences in $\Delta\dot{V}O_2$ max are negligible. The unsaturated bridge configuration appears to markedly reduce potency, but does not entirely abolish activity. Walpole *et al.* (1993b) reported a similar phenomenon in neural tissue structure-activity studies, and suggested that sp^2 hybridisation of the carbon adjacent to the aromatic ring always resulted in little or no activity. As noted with 3E (Fig. 4.3), 4-acetoxy substitution of the A-region abolishes activity (4D and 4E).

4.3.4 Reverse amides

A further series of reverse amides with variations to A-region substituents, the A-B-region bridge, and the C-region is presented in Fig. 4.5.
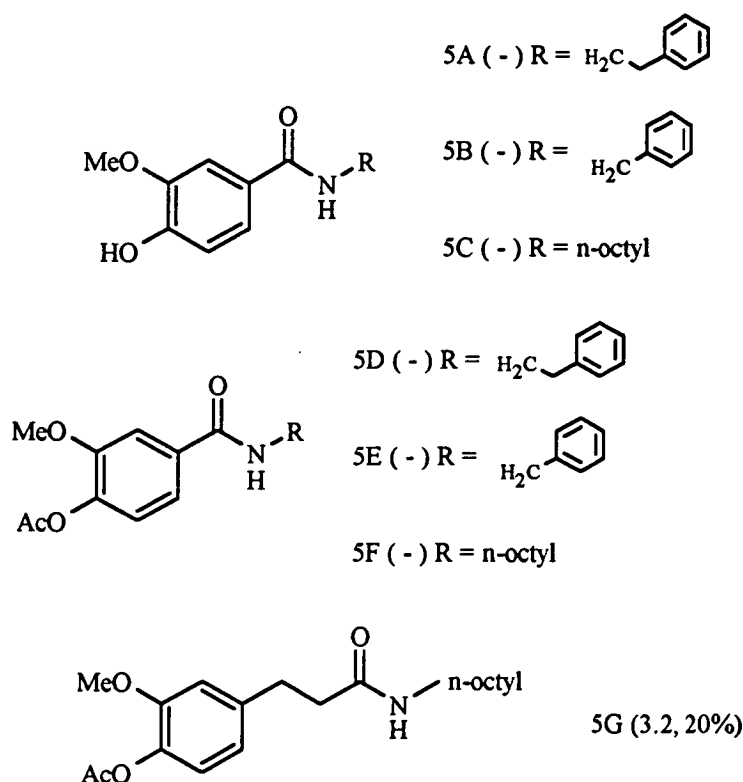
Fig. 4.5. Structure variations of reverse amide vanilloids. Figures within the brackets refer to the same parameters as given for Fig. 4.2.

Positioning the carbonyl group on the carbon adjacent to the A-region ring removes activity (5A-C), as noted for 2A and 2B (Fig. 4.2) as well as 3D (Fig. 4.3). This observation is again consistent with the notion that any marked activity requires the carbon adjacent to the A-region ring to be sp^3 hybridised. Compound 5G not only fulfills this criterion, but is in fact substantially more potent with a higher $\dot{V}O_{2\,max}$ than the synthetic agents thus far considered. This activity is despite the presence of 4-acetoxy substitution of the A-region, which eliminated activity in compounds 3E (Fig. 4.3) and 4D and 4E (Fig. 4.4). However, it is possible that esterase enzyme activity promotes rapid hydrolysis of the acetoxy moiety within the hindlimb preparation, giving a 3-methoxy-4-hydroxy configuration, despite the single pass (non-recirculating) perfusion protocol. Acetylcholine has been shown to be relatively ineffective vasodilator in perfused hindlimb preparations for similar reasons (data not shown).

4.3.5 Dimethoxyphenyl reverse amides

Conserving the A and B-region structure in a dimethoxyphenyl reverse amide configuration (Fig. 4.6) enabled a series of C-region structures to be assessed.
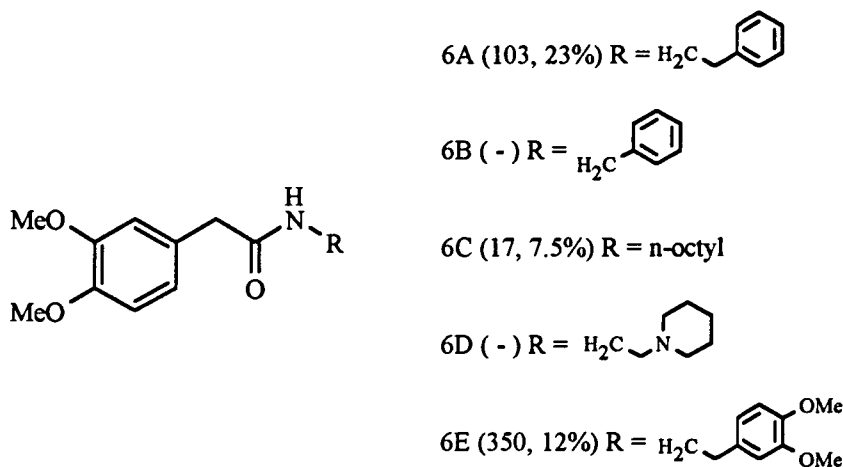
Fig. 4.6. Dimethoxyphenyl reverse amides with a number of C-region structural variations. Figures within the brackets refer to the same parameters as given for Fig. 4.2.

The potency of the compounds with n-octyl, phenylethyl, and phenylmethyl C-regions (6C, 6A and 6B respectively) followed the same order as the other synthetic agents with the corresponding series of C-regions (3A-C, Fig. 4.3; 4A-C Fig. 4.4). However, efficacy in terms of $\Delta \dot{V}O_{2 \, max}$ appeared to be more variable. Compounds 3A, 3B and 4A-C all had $\Delta \dot{V}O_{2 \, max}$ values in the range 15-18%, whereas the values for 6A and 6C were 23 ± 3% at 125 μM (n = 3) and 7.5% at 60 μM respectively. One possible explanation for such variation in $\Delta \dot{V}O_{2 \, max}$ is differing selectivity for the putative VN_1 and VN_2 receptors discussed in Chapter 3.

The remaining C-region variations examined showed little (6E) or no activity (6D). The poor responses may be related to the presence of electronegative heteroatoms in a C-region requiring a highly lipophilic nature for optimal activity at the recognition site.

4.3.6 Dimethoxyphenyl amides

In order to compare amides with reverse amides, a series of compounds with the same B-region as capsaicin (Fig. 4.1) were assessed for hindlimb thermogenic activity (Fig. 4.7).
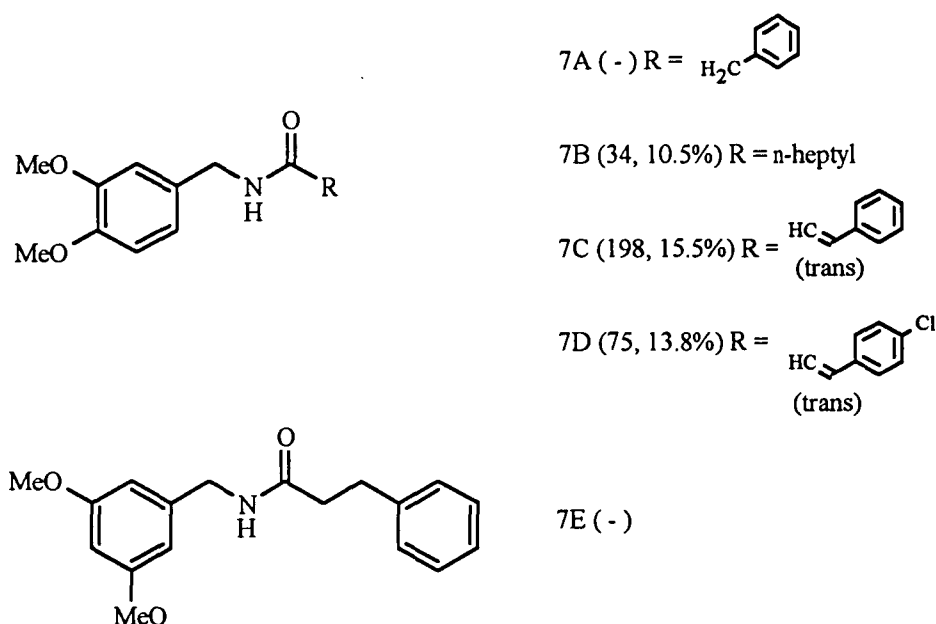
Fig. 4.7. Dimethoxyphenyl amides tested for hindlimb thermogenic activity. Compounds 7C and 7D have *trans* stereochemistry. Figures within the brackets refer to the same parameters as given for Fig. 4.2.

The amide 7B (n-heptyl side chain) and the analogous reverse amide 6C (Fig. 4.6, n-octyl side chain) were approximately equipotent, a result matching findings with amide and reverse amide analogues in neural tissue (Walpole *et al.*, 1993b). The phenylmethyl C-region of 7A again resulted in the abolition of activity, as was observed for 3C, 4D, 5B, 5E and 6B. The 4-Cl phenyl substitution of 7D increased potency by nearly 3-fold over 7C. Similar substitution by Walpole *et al.* (1993c) increased the EC_{50} for calcium influx into neural tissue around 9-fold. Replacing the 3,4-substitution pattern of the A-region with 3,5-substitution abolished activity (7E).

4.3.7 Reverse amides with a vanillyl A-region

A number of C-region variations were examined in structures with a vanillyl reverse amide A and B-region configuration (Fig. 4.8).

8A (10, 23.0%) R = H_2C

8B (3.3, 9.8%) R = H_2C

8C (0.8, 31.1%) R = n-octyl

8D (-) R = H_2C N

8E (3.2, 35.5%)

KR-25003
(Park et al., 1995)
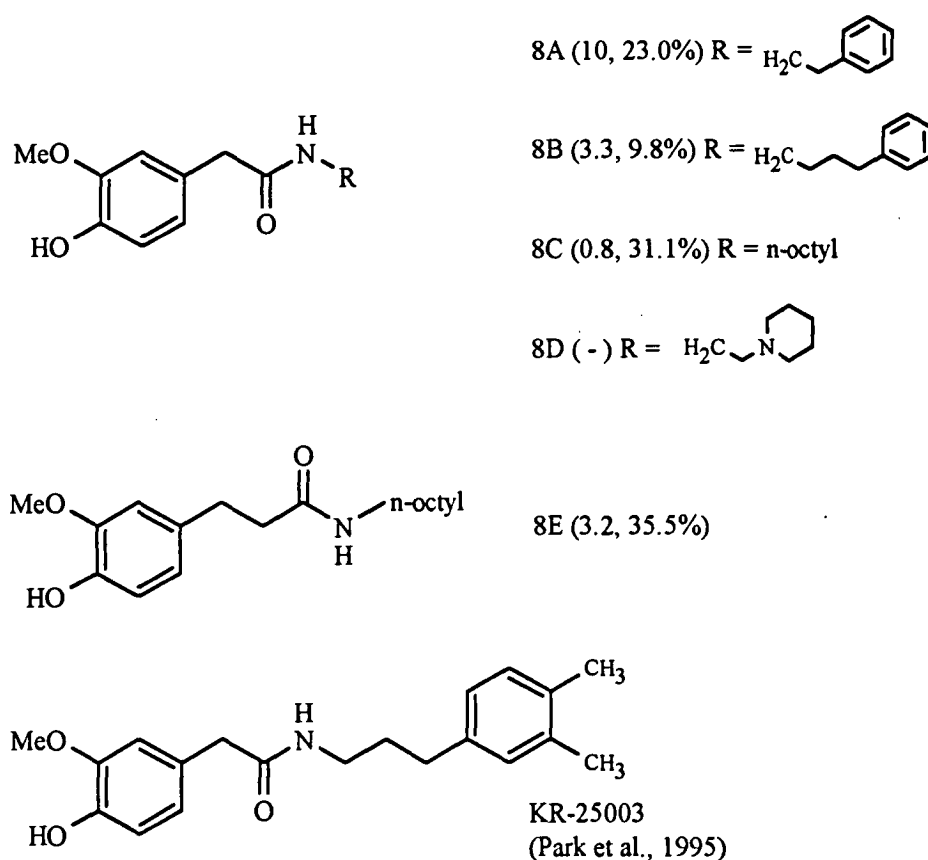
Fig. 4.8. Reverse amide vanillyl compounds assessed for hindlimb thermogenic activity. Figures within the brackets refer to the same parameters as given for Fig. 4.2. The structure of a recently-described potent analgesic agent, KR-25003 (Park *et al.*, 1995) is also shown.

8A is around 10-fold more potent with a comparable $\Delta \dot{V}O_{2max}$ (23 ± 2% at 18 μM, $n = 5$) than the dimethoxy analogue 6A. Similarly, 8C has around 20-fold higher potency than the dimethoxy analogue 6C. Interestingly, 8C (31.1%) has a 4-fold higher $\Delta \dot{V}O_{2\,max}$ than 6C (7.5%). This value is also markedly higher (around 50%) than the $\Delta \dot{V}O_{2\,max}$ for the structurally analogous capsacin and dihydrocapsaicin molecules (23% and 20% respectively, Cameron-Smith *et al.*, 1990). Similarly, the increased C-region length of 8B increases potency but reduces efficacy relative to 8A. The greatest $\Delta \dot{V}O_{2max}$ was achieved by 8E (35.5%), the increased A-region/B-region bridge length giving lower potency but greater efficacy than 8C. Walpole *et al.* (1993b) report that the optimal length of the bridge between the A-region and the dipolar part of the B-region is a single carbon atom in neural tissue. The present results suggest a similar trend if potency is used as the activity index.

It is noteworthy that structures 8A-E (Fig. 4.8) are structurally similar to KR-25003 (Fig. 4.8, Park *et al.*, 1995), a potent analgesic agent. KR-25003 (*N*-[3-(3,4-dimethylphenyl)propyl](4-hydroxy-3-methoxyphenyl)acetamide) has similar A- and B-regions to structures 8A-E, but is distinguished by a 3,4-dimethylphenyl C-region. Park *et al.* (1995) suggest that the overall conformation of KR-25003 is somewhat different to other vanilloids in the crystalline state.

4.3.8 A-region halogen substitution

Halogen groups were substituted in the 3- and/or 5- positions of the A-region ring whilst conserving the 4-hydroxyl substitution (Fig. 4.9).

9A (63, 24.7%) X = Cl, R = H_2C—

9B (56, 20.8%) X = Br, R = H_2C—

9C (250, 29.3%) X = Cl, R = n-octyl

9D (118, 12.5%)
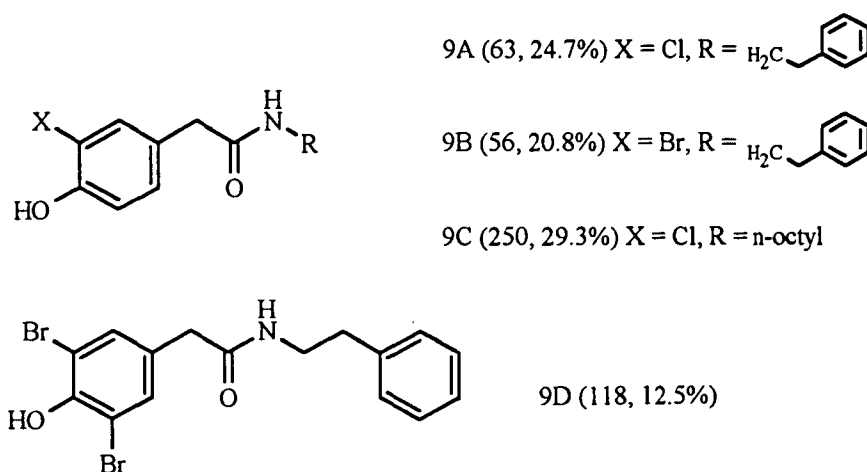
Fig. 4.9. Halogen substitution of the A-region. Figures within the brackets refer to the same parameters as given for Fig. 4.2.

Replacement of the 3-methoxy substituent with either a chloride or bromide substituent reduces the potency around 6-fold in the case of 9A and 9B relative to the 3-methoxy analogue 8A. Efficacy in terms of $\Delta \dot{V}O_{2\,max}$ is not altered. Interestingly, replacing the 3-methoxy substituent with chlorine in reverse amides with n-octyl C-regions (9C and 8C) results in a 300-fold potency reduction without a change in $\Delta \dot{V}O_{2\,max}$. In light of the relatively minor potency differences between 9A and 8A, this result is difficult to explain. Adding a further halogen substituent in the 5- position of the A-region (9D) halved both the potency and the efficacy relative to 9A.

4.3.9 Reverse amides with single-substituent A-regions

Reverse amides with substitution only in the 4- position of the A-region were generally active with relatively low $\Delta \dot{V}O_{2\,max}$ efficacy (Fig. 4.10).

10A (107, 10.6%) R1 = HO, R2 = H₂C

10B (21, 11.9%) R1 = HO, R2 = n-octyl

10C (83, 17.4%) R1 = AcO, R2 = H₂C

10D (27, 14.2%) R1 = AcO, R2 = n-octyl

10E (-) R1 = MeO, R2 = H₂C
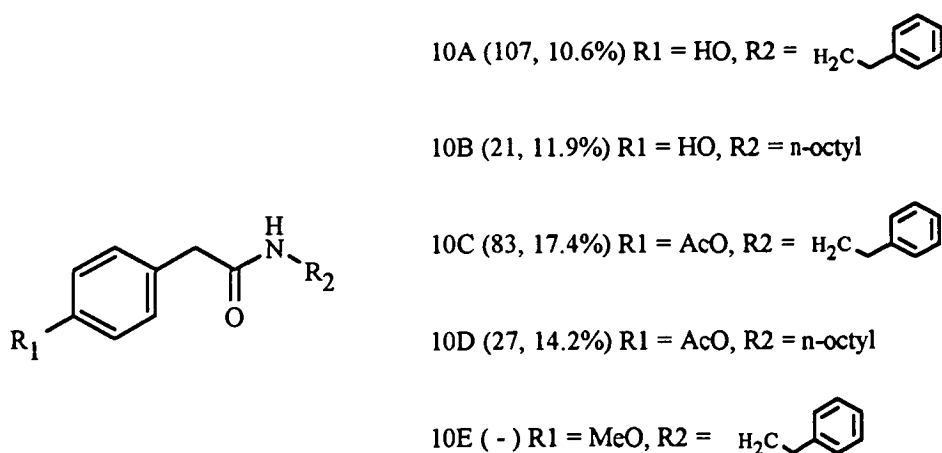
Fig. 4.10. Single 4- substituent reverse amides. Figures within the brackets refer to the same parameters as given for Fig. 4.2.

Structures with both hydroxyl (10A, 10B) and acetoxy 4- substituents (10C, 10D) were active. However it is conceivable that esterase enzyme activity in the hindlimb preparation results in hydrolysis of acetoxy groups to hydroxyl groups. Such activity renders acetylcholine virtually inactive in the hindlimb preparation (data not shown). As noted for previous analogues (e.g. 4A and 4B), an n-octyl C-region

confers greater potency than a phenylethyl C-region (10A and 10B; 10C and 10D). 4-Methoxy substitution abolished activity.

4.3.10 Pharmacophore for thermogenesis

A molecular pharmacophore represents a set of common features which characterise active molecules. It is derived from the common geometry's of a number of active compounds and therefore, in theory, acts as a template for activity. Pharmacophore determination is generally achieved by performing conformer searches on known active agents. Specialised computer software compares common geometric elements of these structures to allow calculation of the three-dimensional template. The pharmacophore concept can improve the efficiency of drug development programs, since improving structural features to comply with a known pharmacophore reduces both the time and expense associated with synthesis and bioassay procedures.

In the present study, the program PHARM, developed at this University (Martin, R.J., 1993; Marks, C.J., 1995, Honours theses, University of Tasmania) was used to compare the 16 most active synthetic conformers from the structures presented in Figs. 4.2 - 4.10 and calculate a pharmacophore for oxygen consumption in the perfused rat hindlimb model (Fig. 4.11).

Previous work by Klopman and Li (1995) has resulted in the development of a partial pharmacophore for receptor binding in neural tissue, based on the structure-activity data of Walpole and colleagues (1993a-c). However this study was unable to report the distance and angle of the C-region relative to the remaining regions due to difficulties associated with the recognition of this region by the software.

The synthesis of a number of analogues with aromatic groups (recognised as anchor groups by PHARM) in the C-region enabled PHARM to calculate a pharmacophore encompassing the geometry of the entire molecule in the present study. The calculated angle between of the A- and C-region planes is 57°-87°, confirming that the C-region is bent back over the A-region in active vanilloid structures. Consequently, the distance between the A- and C-region aromatic ring centres (in structures with aromatic C-regions) was calculated to be only 4.7 ± 0.2Å. This conformation is clearly illustrated by the 3-D global minimised structure of

compound 9C shown in Fig. 4.12. The work of Klopman and Li (1995) calculated distances of 7.78Å and 8.71Å from the B-region (thiourea sulfur atom) to the A-region oxygen atoms. The pharmacophore of the present work places the single oxygen atom on the aromatic ring only 7.1 ± 0.1Å from the B-region, indicating a greater angle between the A- and B-region planes. Whilst this result supports the previous findings in neural tissue which suggest a requirement for non-coplanarity of the regions (Klopman and Li, 1995; Walpole and Wrigglesworth, 1993), the finding nevertheless provides further indications that alternative pharmacophores may exist for different vanilloid recognition sites.
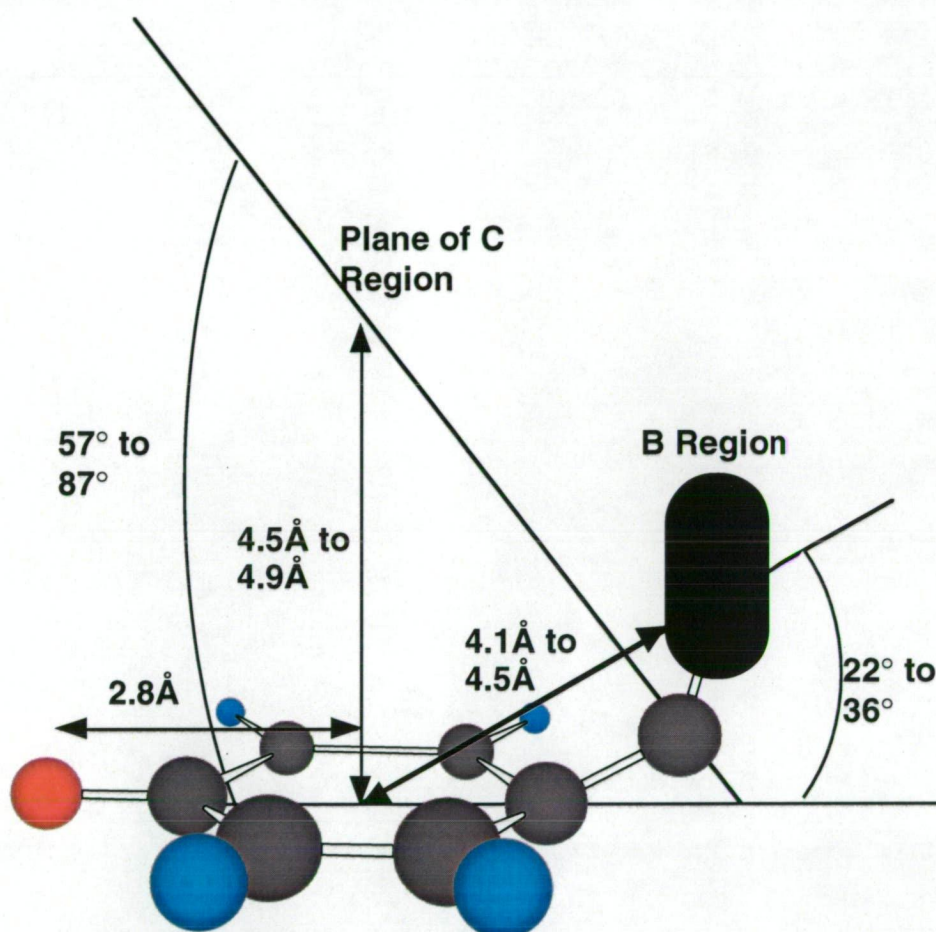
Fig. 4.11. Pharmacophore for oxygen consumption. Redrawn from Eldershaw *et al.*, (1995) and Clippingdale (1995, Honours thesis, University of Tasmania). Colour identification: red, oxygen; grey, carbon; blue, hydrogen. Distance from A- to C-region is from aromatic ring centres. Full details of the calculation are reported in Clippingdale (1995, *op. cit.*). Note that the pharmacophore angle of the A- and C-region planes only applies to structures with aromatic ring-containing C-regions.
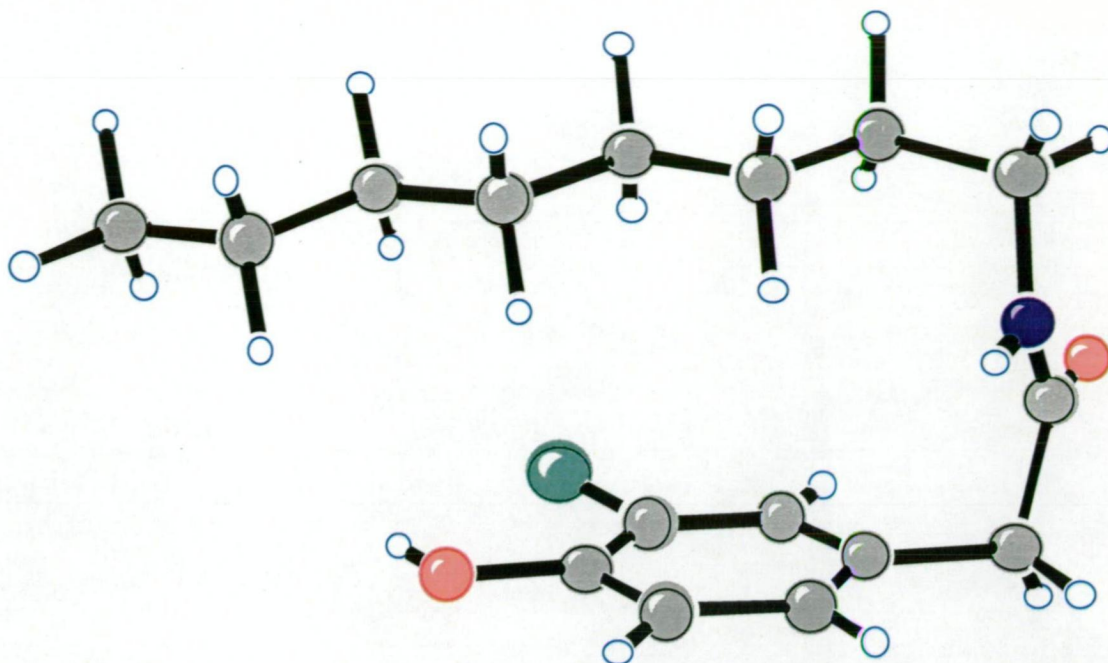
Fig. 4.12. Global minimum conformer of compound 9C (steric energy = 29.5134 kJ·mol^{-1}) redrawn from Clippingdale (*op. cit.*). Conformational analysis was performed using PCMODEL 5.0, PKM, PKIN (all Serena software, Bloomington, USA, 1994), and MM3 (N.L. Allinger, University of Georgia, 1992). Full details are reported in Clippingdale (1995, *op. cit.*). Note that the angle of the A- and C-region planes is reduced due to the absence of the aromatic C-region.

4.3.11 Dose-response curves of synthetic analogues in perfused hindlimb preparations

At the appropriate concentrations, all active synthetic analogues were capable of inducing the triphasic VO_2 response (data not shown) noted for the natural vanilloid agents (e.g. capsaicin, Chapter 3, Fig. 3.2) in association with increased perfusion pressure. No active analogue was able to demonstrate exclusive selectivity for stimulatory or inhibitory VO_2 responses, although the marked variation of the ΔVO_{2max} values could be interpreted as preliminary evidence for differential receptor selectivity. The VO_2 and perfusion pressure dose-response curves (Fig. 4.13) were therefore both qualitatively and quantitatively similar to those for the natural vanilloids in the perfused rat hindlimb model (Chapters 2a, 2b, and 3). The VO_2 dose-response curves were typically biphasic, possessed narrow concentration ranges, and typically gave ΔVO_{2max} values of between 1.0 and 1.5 μmol·g^{-1}·h^{-1} (Fig. 4.13).
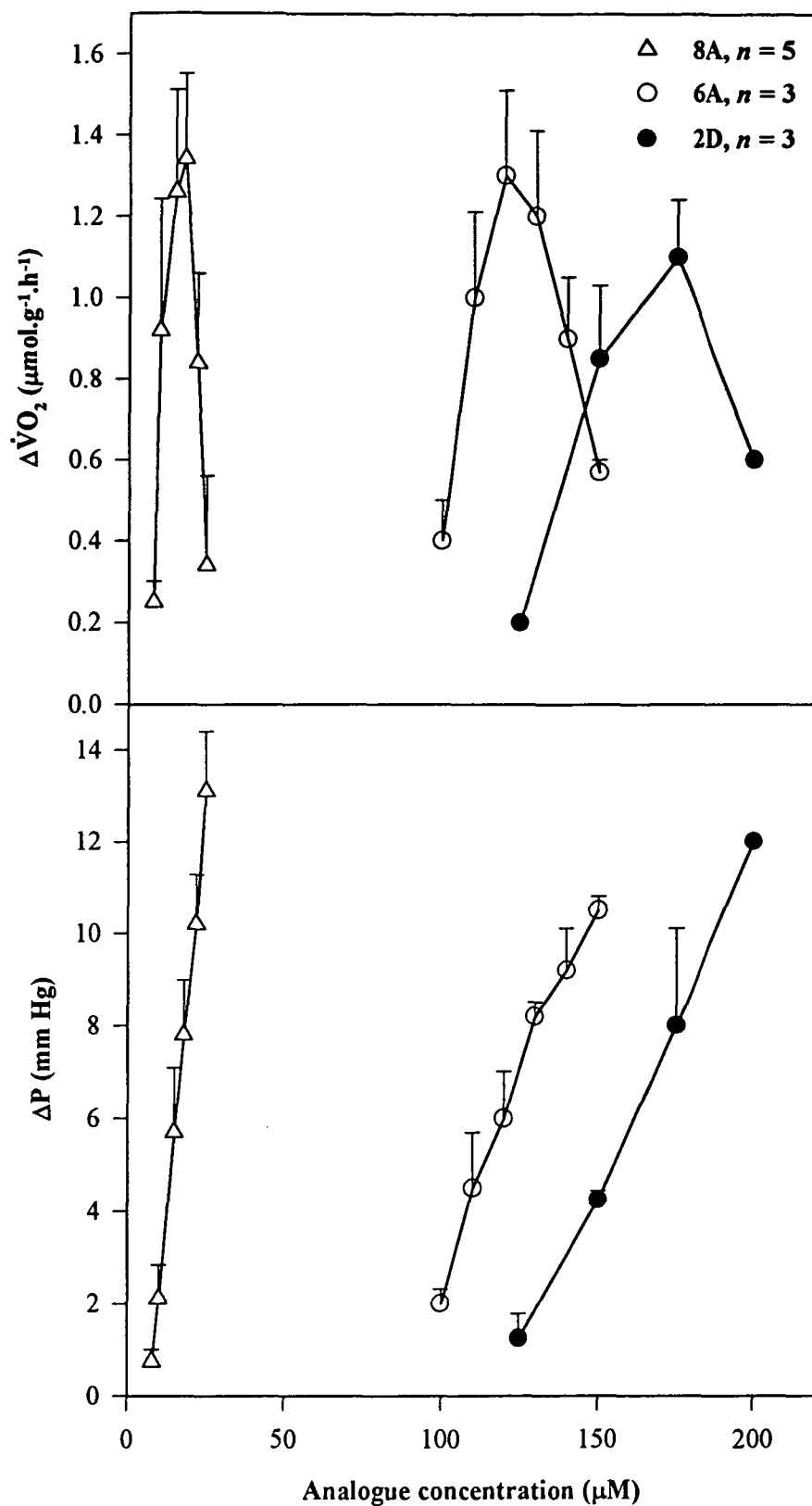
Fig. 4.13. Change in $\dot{V}O_2$ and perfusion pressure (ΔP) dose-response curves for a selection of synthetic vanilloid analogues (8A, 6A and 2D). Data points are means ± SE.

4.3.12 Preliminary in vivo *thermogenic testing*

Following encouraging results in the perfused rat hindlimb preparation in terms of $\Delta\dot{V}O_{2\ max}$, compound 6A (Fig. 4.6) was selected for preliminary *in vivo* thermogenic testing. Using anaesthetised rats, positive thermogenic effects were observed following subcutaneous injection of 6A (Fig. 4.14).
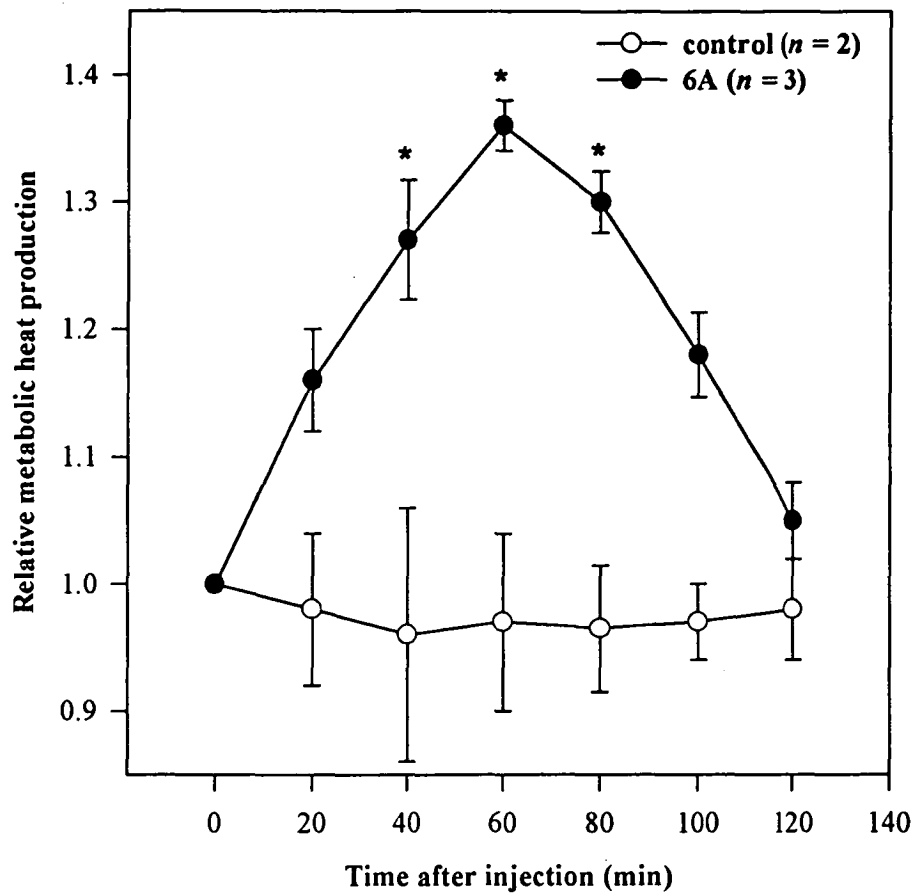
Fig. 4.14. Effect of subcutaneous injection of analogue 6A (116 mg·kg^{-1}) on metabolic heat production (relative to the pre-injection period) in 250 g anaesthetised hooded Wistar rats. Each data point represents the mean ± SE for a 20 minute period. The control injection was 100% dimethyl sulphoxide (0.5 ml). *Significantly greater than the 40 min control data point (P < 0.05, Student's unpaired two-tailed *t*-test). Experiments were performed in conjunction with Dr S.J. Edwards in this laboratory.

Although analogue 6A gave a significant thermogenic response in the anaesthetised whole rat (Fig. 4.14), similar experiments in conscious whole rats (performed in conjunction with Dr S.J. Edwards in this laboratory) failed to show any increase in metabolic rate. The effects shown in Fig. 4.14 may therefore be artefacts occurring as a result of a reduction in the degree of anaesthesia. However, previous experiments in this laboratory (data not shown) using capsaicin and extracts of ginger did not result in similar *in vivo* thermogenic responses using anaesthetised rats. A comprehensive *in vivo* thermogenic testing program is required to fully assess the potential of vanilloid analogues as lead thermogenic agents.

4.3.13 Relationship between thermogenic and analgesic structural requirements

The studies of Walpole *et al.* (1993a-c), Chen *et al.* (1992) and Park *et al.* (1991) combine to build a comprehensive structure-activity picture for the analgesic actions of vanilloid analogues. However, the present study is the first to examine the structural requirements for thermogenic activity in muscle.

Walpole and Wrigglesworth (1993) have noted that there is good correlation between the $^{45}Ca^{2+}$ influx assay used with cultured dorsal root ganglion neurones to indicate potential analgesic activity, and the ability to induce contraction of guinea-pig ileum. The parallel structural requirements for activity dictated by the present data suggests that the same correlation may exist between these two indices and the vasoconstrictor actions in perfused rat hindlimb. As noted in this and previous chapters, vanilloid-induced $\dot{V}O_2$ is associated with increased perfusion pressure, and the blockade of vasoconstriction with nitrovasodilators (Chapters 2a and 2b) simultaneously reverses $\dot{V}O_2$ increases.

On the evidence presented in this chapter, it must be concluded that there are no major differences in the pharmacophores for analgesic activity and hindlimb thermogenesis. Given that the increases in thermogenesis may be mediated by a putative VN_1 receptor (Chapter 3), it must be assumed that this is the same or similar recognition site as that which appears on sensory neurones. Alternatively, the thermogenic effects may be the direct result of stimulating neurones associated with the hindlimb vascular bed, possibly via the release of neurokinins. However, it must be taken into account that despite the evidence presented for a Ca^{2+}-independent VN_2

receptor (Chapter 3), the synthetic analogues failed to effectively separate the actions attributed to these putative subtypes. It remains possible, therefore, that different vanilloid recognition sites do exist on various tissues and that separation of the various pharmacological actions of these agents may be achievable given further refinements of the current structure-activity picture.

Chapter 5

Vascular Control in Comparative Perfusion Models.
A. Perfused Chicken (*Gallus domesticus*) Muscle Thermogenesis.

5.1 Introduction

When exposed to the cold, endotherms increase their facultative heat production (see Appendix 1) by either shivering thermogenesis, nonshivering thermogenesis, or a combination of both. Whilst shivering is a function of skeletal muscle, the site(s) and mechanism(s) of NST are less certain. In many eutherian mammals, a large proportion of the NST is thought to occur in brown adipose tissue (BAT) but increasingly, skeletal muscle is seen as an important NST effector tissue (Chapter 1). Studies in this laboratory have led to the proposal that skeletal muscle NST is regulated by its own microcirculation, and that the vascular smooth muscle itself may even significantly contribute to the observed thermogenesis (reviewed in Chapter 1 and by Clark *et al.,* 1995). The vanilloid group of molecules have been identified as a new group of vasoconstrictors capable of controlling skeletal muscle thermogenesis, as measured by the oxygen uptake of perfused rat hindlimb preparations (Chapters 2a, 2b, 3 and 4). Whilst this vascular control phenomenon has been demonstrated to exist in representatives of both eutherian mammals (rat, Chapter 1) and metatherian mammals (i.e. marsupials, Tasmanian bettong, Ye *et al.,* 1995), there have been no attempts to identify similar vascular control mechanisms in the skeletal muscle of birds, the remaining major endothermic taxon.

Birds are a large Class of effective endothermic animals in which BAT is most likely absent. Indeed, although cold-acclimated birds have a multi-locular fat tissue, this tissue does not have the numbers of mitochondria, the Krebs cycle enzymes, the cytochromes (Barré *et al.,* 1987a), the uncoupling protein (Saarela *et al.,* 1991), nor the sympathetic innervation of true BAT (Saarela *et al.,* 1989) to provide the observed NST. Due to the absence of BAT, the capacity for true NST in birds remains in dispute (Marsh, 1993). Nevertheless, a growing body of evidence supports the existence of avian NST (Duchamp *et al.,* 1993a). The ability of birds to raise their

metabolic rate in a facultative manner without shivering in the face of a rapid or acute fall in temperature has been established in long-term cold-acclimated or -acclimatized birds such as chickens (El-Halawani *et al.*, 1970), muscovy ducklings (Barré *et al.*, 1986) and king penguin chicks (Duchamp *et al.*, 1989). In the absence of BAT, skeletal muscle has emerged as a major potential site of NST in view of catecholamine-induced stimulation of muscle $\dot{V}O_2$ *in vitro* (Hissa *et al.*, 1975b) and more recent measurements of muscle blood flow and arteriovenous differences in $\dot{V}O_2$ *in vivo* (Duchamp and Barré, 1993). However, the signal molecules, receptors, and the exothermic reactions of avian NST remain contentious or unknown.

In eutherian mammals, the stimulus for NST in many species appears to be the activation of the sympathetic nervous system (SNS) and its stimulation of BAT, which can be mimicked by exogenous noradrenaline (NOR; Girardier and Stock, 1983). By contrast, exogenous catecholamines usually induce little if any thermogenesis in birds. For example, Hissa (1988) concluded that exogenous NOR led to either no change or a fall in core body temperature, a fall in oxygen consumption, and a decrease in shivering in cold-exposed birds. Nevertheless, there are some reports of *in vivo* calorigenic effects of catecholamines in birds (Freeman, 1966; Hissa *et al.*, 1975a; Barré and Rouanet, 1983). These effects are small (10-40% above basal), and are usually observed only at or above thermoneutral ambient temperatures, although significant effects in king penguin chicks have been noted in the cold (Barré and Rouanet, 1983). Below thermoneutrality, catecholamine-induced inhibition of shivering (Hissa, 1988 and references therein) may be masking underlying (if any) calorigenic effects. However, if catecholamines are detrimental in the cold, it is puzzling to observe increased plasma catecholamine levels in cold-acclimated chickens (Lin and Sturkie, 1968), and increased sympathetic nervous system activity and catecholamine turnover in a number of cold-exposed birds (El-Halawani *et al.*, 1970; Saarela and Hissa, 1977; Koban and Feist, 1982). Furthermore, the NOR-induced calorigenesis is more pronounced in cold- than in warm-acclimated pigeons above thermoneutrality (Hissa *et al.*, 1975a), although this effect is not invariably found (reviewed by Chaffee and Roberts, 1971). Taken together, these apparently conflicting data may be related to species differences, animal ages, ambient temperatures, catecholamine doses, method of injection, or to the combined effects of

several hormonal actions *in vivo*. There could presumably also be major interactions between the vascular and metabolic actions of catecholamines, but neither this aspect, nor the thermogenic site has been directly addressed in birds.

The potential of glucagon as a calorigenic agent in birds has been disputed in the literature. Workers such as Hohtola *et al.* (1977, pigeon) and Palokangas *et al.* (1973, chicken) found no evidence of any *in vivo* thermogenic responses. However, others have identified glucagon as a potential thermogenic enhancer (Barré and Rouanet, 1983; Keller, 1980; Krimphove and Opitz, 1975). Barré *et al.* (1987b) have shown that chronic injection of twice daily glucagon into ducklings induces physiological changes similar to those of cold-acclimation, and that the injection of glucagon into cold-acclimated ducklings stimulates increased oxygen consumption (the latter also reported by Duchamp *et al.*, 1993b). A marked increase in oxygen consumption in response to exogenous glucagon was also observed *in vivo* in young chickens (Barré, 1983; Keller, 1980), as well as in three-month-old Japanese quail (Krimphove and Opitz, 1975). Skeletal muscle may be a site of such glucagon-induced calorigenesis *in vivo* (Duchamp *et al.*, 1993b) although it is not known whether the observed actions of glucagon are direct or indirect. There are apparently no reports of glucagon-induced calorigenesis in adult birds.

In view of the controversy surrounding the existence of NST in birds, and the fact that resting perfused skeletal muscle preparations from both eutherians (Chapter 1) and marsupials (Ye *et al.*, 1995) can show significant potential for thermogenesis, the primary aims of the present chapter were firstly to examine whether perfused resting chicken muscle would respond to putative thermogenic hormones such as NOR and glucagon, or other known vasoactive agents (e.g. vanilloids), and secondly to examine the possibility of a link between thermogenic and vascular effects in a comparative perfusion model.

5.2 Methods

5.2.1 Animals

Birds were cared for under the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (1990) and the experimental protocols were approved by the University of Tasmania Ethics Committee (Animals).

Chickens (male and female) of local Hyline and Leghorn strains were obtained from a commercial hatchery and kept for 2-14 days in a retaining pen at 21 ± 1 °C with *ad libitum* access to commercial pellets and water. At experiment, body masses were in the range of 400-800 g. The supply of birds was variable and limited over the period of the study, hence the strain, sex and age of chickens used was not necessarily uniform for any series of experiments. Although the hatchery employed mechanisms to control temperature, seasonal influences could not be discounted as birds were hatched at various times throughout the year.

5.2.2 Materials

Bovine serum albumin fraction V was from Boehringer (Australia); noradrenaline bitartrate, adrenaline bitartrate, arginine vasopressin, human angiotensin II, prazosin hydrochloride, *d,l*-propranolol hydrochloride, capsaicin, oleic acid, serotonin creatinine sulfate, *d*-tubocurarine chloride and Evans blue were from Sigma (USA); porcine glucagon hydrochloride (with lactose) and porcine insulin (Actrapid MC, 100 units·ml^{-1}) were obtained from Novo-Nordisc (Denmark); heparin sodium was from David Bull Laboratories (Australia); and sodium nitroprusside was from Merck (Germany). All other chemicals and solvents were AR grade from Ajax (Australia). Drug solutions for infusion were prepared daily using 0.1% ascorbic acid in isotonic saline. Capsaicin solutions were prepared from a stock solution of 5 mg·ml^{-1} in ethanol using 0.1% ascorbic acid in isotonic saline.

5.2.3 Surgical procedures

After a number of preliminary experiments with differing anaesthetic agents, birds were satisfactorily anaesthetised with 60 mg·kg^{-1} of intraperitoneal sodium pentobarbitone. During induction of anaesthesia, particular care was taken to cover the cage and keep the birds in a warm and quiet environment.

Once anaesthetized, the leg was plucked free of feathers, the major skin vessels of the lower limb were ligated, and the popliteal fossa incised to expose the popliteal artery and vein. The popliteal nerve was divided and the hamstring muscles were ligated and resected proximal to the fossa to give good access for cannulation of the popliteal artery and vein with teflon cannulae (Terumo 20G, 1.25") which were

bent to approximately 60° for better access to the vessels in the fossa. Heparin (2000 $IU \cdot kg^{-1}$) was administered intravenously into the brachial vein prior to cannulation. After cannulation, tight ligatures were placed around the ankle and the lower thigh just above the cannulation site in order to prevent flow to other tissues. Evans blue dye (1%) was infused to confirm that perfusate flow was confined to the lower limb in both hormone-stimulated and non-stimulated preparations. During perfusion, the animal was laid on its back and the limb supported partially aloft. Exposed muscle was covered in plastic cling wrap. Where nerve stimulation was performed, the distal end of the divided popliteal nerve was positioned in a suction electrode. This involved drawing the nerve through an annulus formed in one platinum electrode into plastic tubing containing the other platinum electrode. Negative pressure supplied by a syringe held the nerve in the tubing. The stimulation was a continuous train of 5 ms pulses at 5 V and 1 Hz. After commencement of perfusion, the other leg was ligated similarly and then excised and the muscles removed and weighed to allow calculation of required perfusate flow to the perfused limb. This method of estimating perfused muscle mass was validated by infusing 1% Evans blue dye followed by removxal and weighing of stained muscle to determine the mass of muscle perfused, generally in the range 14-17 g.

5.2.4 Perfusion protocols

The perfusion system was similar to that described previously for the rat (Chapter 2a). The non-recirculating constant flow system was maintained at 25°C for all experiments. The perfusate was the same composition as outlined in Chapter 2a. Agent infusions were made continuously at ≤ 1% of the total flow directly into a combined mixing chamber/bubble trap. In each series of experiments, infusion of vehicle alone had no effect on oxygen uptake nor perfusion pressure.

Perfusions were conducted at flow rates of 0.27 $ml \cdot min^{-1} \cdot g^{-1}$ or 0.33 $ml \cdot min^{-1} \cdot g^{-1}$. The responses to NOR alone were determined at both flow rates. The experiments with 5-HT were conducted at 0.27 $ml \cdot min^{-1} \cdot g^{-1}$, whilst all other experiments were conducted at the higher flow rate. The data for perfusion pressure and $\dot{V}O_2$ represent the mean ± SE of steady-state values, generally attained within five minutes of agent infusion. $\dot{V}O_2$ calculations were performed as previously described

for the rat (Chapter 2a). Perfused muscle mass was estimated by the method outlined in section 5.2.3.

Experiments to determine NOR dose-response curves in the presence or absence of glucagon were performed as paired comparisons in the same preparation at 0.33 ml·min^{-1}·g^{-1}. The order of treatment was reversed in each experiment. When glucagon was present, it was infused prior to the NOR treatment. Washout periods of 25-30 minutes were included in experiments where the NOR + glucagon treatment preceded the NOR alone treatment. These experiments were conducted around 12 months after the previous set of experiments to determine responses to NOR at 0.33 ml·min^{-1}·g^{-1}. The birds were supplied from a different hatchery which employed upgraded temperature control mechanisms.

5.2.5 Metabolite assays

Muscle metabolite concentrations were determined in samples which were freeze-clamped with liquid nitrogen-cooled aluminium tongs, powdered under liquid nitrogen, and stored at -85°C prior to rapid perchloric acid extraction and neutralization with K_2CO_3. Precipitates were removed by centrifugation at 4°C prior to analysis by HPLC. Creatine compounds (Cr and CrP) and adenine nucleotides (ATP, ADP, AMP) were measured simultaneously by using a modified reverse-phase isocratic HPLC method of Sellevold et al. (1986). In brief, the aqueous mobile phase contained KH_2PO_4 (215 mM), tetrabutylammonium phosphate (2.3 mM) and CH_3CN (3.5% v/v) and the pH adjusted to 6.25. A Waters Radial-Pak C18 radial compression column was used with a flow rate of 2 ml·min^{-1}. Creatine compounds were detected by absorbance at 214 nm, adenine moieties by absorbance at 254 nm. The specific detection limits of CrP and ATP were 1.5 μM and 2.0 μM respectively (Sellevold et al., 1986). Similar detection limits applied to the other phosphagen compounds.

The HPLC method of Sellevold et al. (1986) gave distinct peak separation, allowing specific identification of single pure peaks. The total run time was less than 10 minutes. Repeated trials using muscle extracts gave identical peak heights (data not shown). Similar reproducibility and stability were reported by Sellevold et al. (1986) for heart extracts.

Wet weight/dry weight ratios were determined using separate powdered muscle samples dried at 80°C to a constant weight.

5.2.6 Statistical analysis

Statistical significance was determined using Student's unpaired t-test or repeated measures analysis of variance (ANOVA). Significant differences were recognised for $P \leq 0.05$. In general, 3-7 animals were used to determine individual data points.

5.3 Results

5.3.1 Perfusion validation

Perfusions at both flow rates (0.27 ml·min^{-1}·g^{-1} and 0.33 ml·min^{-1}·g^{-1}) were stable with respect to resting oxygen consumption (6.3 ± 0.3 µmol·g^{-1}·h^{-1}, n =17 and 7.4 ± 0.3 µmol·g^{-1}·h^{-1}, n = 31 respectively) and perfusion pressure (44.0 ± 3.6 mm Hg, n = 17 and 44.8 ± 2.2 mm Hg, n = 42 respectively) for periods up to 3 hours or greater after reaching an initial steady state. Muscle phosphagens were determined on freeze-clamped samples taken at the end of the perfusions or immediately after anaesthesia without perfusion ("*in vivo*" samples). Muscle energy charge of the adenylate system (Atkinson, 1968), defined as {[ATP] + 0.5 [ADP]} / {[ATP] + [ADP] + [AMP]}, remained at the *in vivo* value regardless of the perfusion flow rates, duration or presence of hormonal stimulation (Fig. 5.1A). The CrP:Cr ratio was significantly reduced after both 60 minute and 180 minute perfusion periods under basal conditions at 0.27 ml·min^{-1}·g^{-1} (Fig. 5.1B). However, preparations perfused for 180 minutes and stimulated with vasoconstrictors did not show a reduction in CrP:Cr ratio (Fig 5.1B). Increasing the flow by 20% to 0.33 ml·min^{-1}·g^{-1} was sufficient to maintain CrP:Cr ratio after 180 minutes under basal conditions (Fig. 5.1B).

Muscle wet weight/dry weight ratios increased slightly from *in vivo* values (4.32 ± 0.06, n = 8) over 3 hours without stimulation to 5.55 ± 0.15 (n = 3) at 0.27 ml·min^{-1}·g^{-1} and 4.77 ± 0.09 (n = 4) at 0.33 ml·min^{-1}·g^{-1}, indicating some oedema formation although this was not visible to the naked eye. Basal $\dot{V}O_2$ and perfusion pressure were not altered over the 3 hour perfusion period.
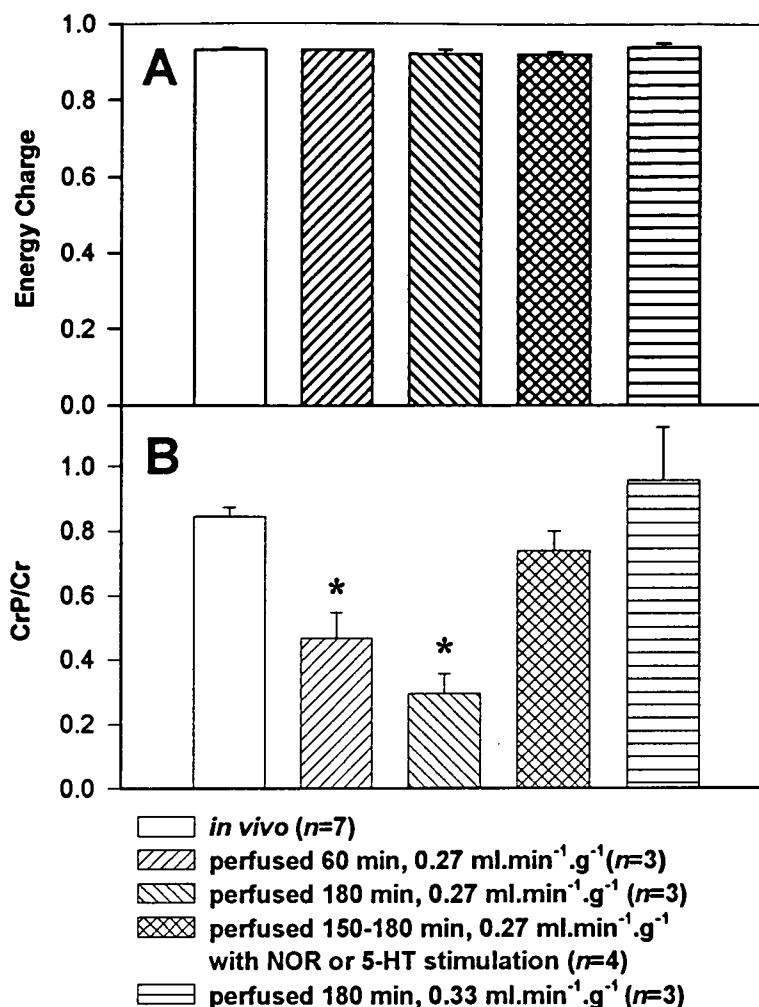
Fig. 5.1. Energy charge (A) and creatine phosphate:creatine ratio (B) of freeze clamped *in vivo* and perfused muscle samples. Data are the mean ± SE. * P < 0.0005.

5.3.2 Agonist infusions

Infusion of NOR gave similar effects at both 0.27 ml·min^{-1}·g^{-1} (Fig. 5.2, high dose data not shown) and 0.33 ml·min^{-1}·g^{-1} (Fig. 5.3). The agent induced a rapid onset of sustained vasoconstriction accompanied by a sustained increase in oxygen consumption at lower doses (1-333 nM) as shown by a fall in the monitored venous PO_2 (Fig. 5.2). Both effects were rapidly reversed on cessation of NOR infusion. The changes were significant at low concentrations of NOR (10 nM, Fig. 5.3). Basal values of $\dot{V}O_2$ and perfusion pressure were unaffected by prolonged periods of NOR stimulation (Fig. 5.2). The maximal NOR-stimulated increase in $\dot{V}O_2$ at 0.33 ml·min

1·g⁻¹ was 2.6 ± 0.3 µmol·g⁻¹·h⁻¹ (35%, $n = 5$, Fig. 5.3). At higher doses (>100 nM) of infused NOR, there was a steady fall in the dose-dependent steady state value for stimulated oxygen consumption until a true inhibition of oxygen consumption relative to control was observed (Fig. 5.3). This was despite a dose-dependent monotonically increasing rise in perfusion pressure. The effects of infused ADR were similar to those of NOR, although the inhibitory VO_2 phase commenced at a lower agonist concentration (Fig. 5.3A), and the changes in perfusion pressure were generally larger (Fig. 5.3B). The changes were also significant at low ADR concentrations (10 nM). 5-HT infusion led to less marked effects on oxygen consumption (Fig. 5.3A). Rises in perfusion pressure in response to infusion of 5-HT were approximately half of those associated with NOR at any given agonist concentration (Fig. 5.3B).
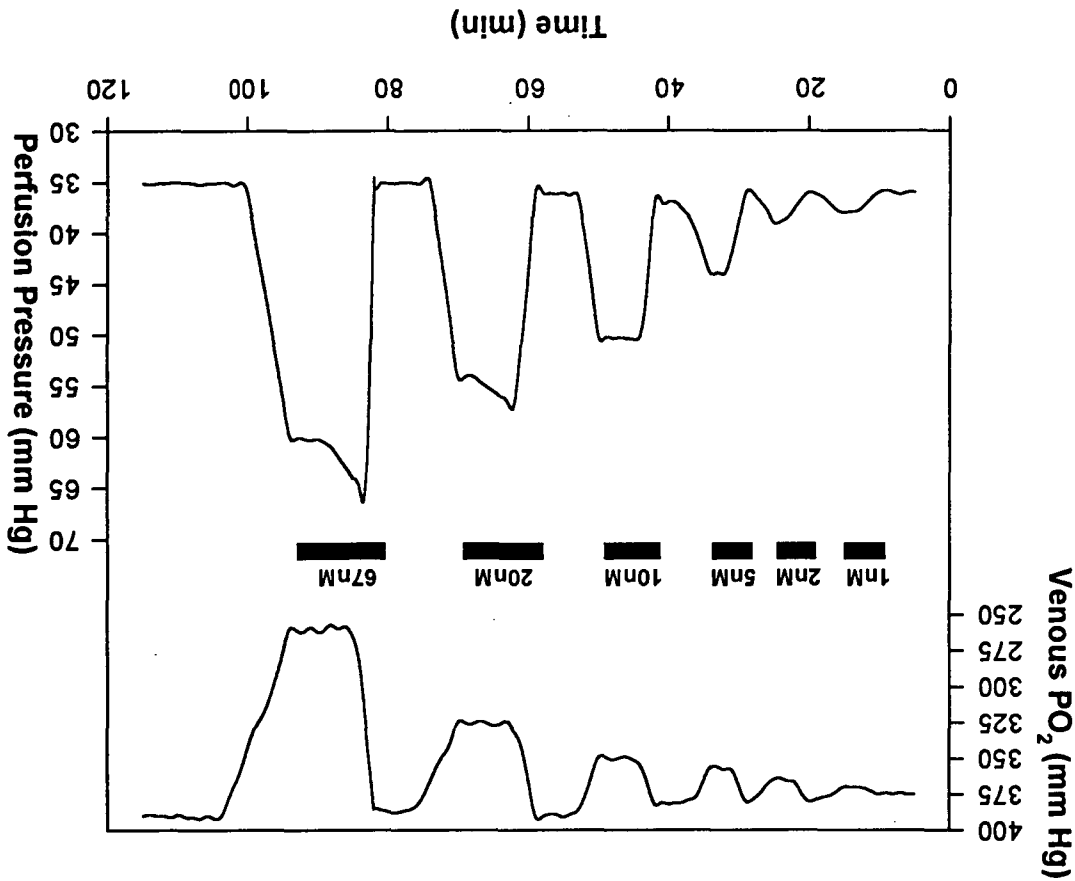
Fig. 5.2. Typical tracings of venous PO_2 (upper) and perfusion pressure (lower) for a perfusion preparation at 0.27 ml·min⁻¹·g⁻¹ stimulated with a series of NOR doses.
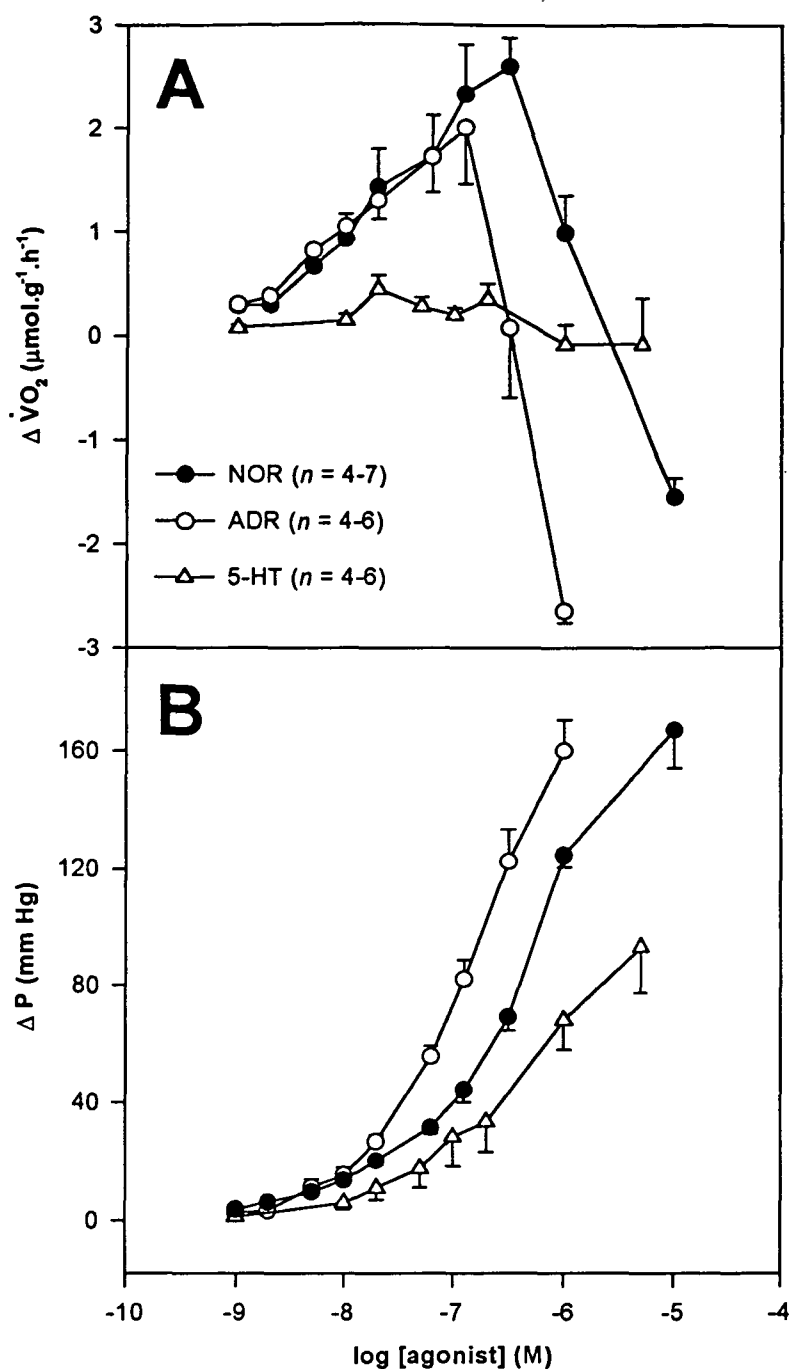
Fig. 5.3. Concentration-response curves for (A) change in $\dot{V}O_2$ and (B) change in perfusion pressure in response to infusion of NOR, ADR and 5-HT. The flow rate was 0.33 ml·min^{-1}·g^{-1} for the NOR and ADR experiments, and 0.27 ml·min^{-1}·g^{-1} for the 5-HT experiments. All points are the mean ± SE. Perfusion pressure and $\dot{V}O_2$ increases were significant (P < 0.05) at 10 nM concentrations of both NOR and ADR (Student's unpaired t-test). Repeated measures analysis of variance (ANOVA) showed that for all values up to 1 μM (all n = 6) there was a significant effect of 5-HT on $\dot{V}O_2$ (P ≤ 0.05).

5.3.3 Agonist blockade

The effects of adrenergic antagonists and nitrovasodilation on responses to low-dose NOR (50 nM) are shown in Fig. 5.4. Propranolol (10 μM) had no significant effect, but prazosin (10 μM) and nitroprusside (0.5 mM) significantly blocked the induced changes in $\dot{V}O_2$ and perfusion pressure. Nitroprusside blocked both parameters to below commencing steady-state values, indicating the presence of basal vascular tone with associated $\dot{V}O_2$.
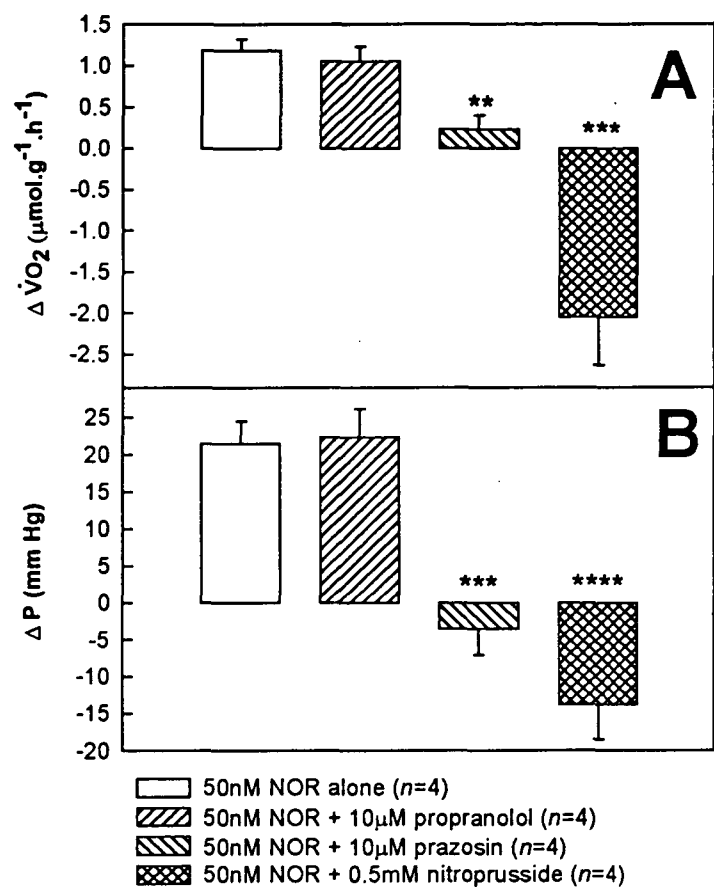
Fig. 5.4. Effects of adrenergic antagonists and nitrovasodilatation on (A) $\dot{V}O_2$ and (B) perfusion pressure induced by 50 nM ($\dot{V}O_2$-stimulatory) NOR in preparations at 0.33 ml·min^{-1}·g^{-1}. Data are the mean ± SE. Significantly different to 50 nM NOR alone, **P < 0.01; *** P < 0.005; **** P < 0.001.

5.3.4 Glucagon infusions

Infusion of glucagon alone (100 nM - 1 μM) had no significant effect on basal $\dot{V}O_2$ nor perfusion pressure (Fig. 5.5). However when glucagon was infused following establishment of NOR-induced $\dot{V}O_2$ inhibition (2 μM NOR), there was a

dose-dependent fall in perfusion pressure and, at 1 µM glucagon, a reversal (to varying extents) of the inhibited $\dot{V}O_2$ induced by the infusion of high dose NOR alone (Fig. 5.5). When glucagon (1 µM) was infused continuously prior to and during the full concentration range of NOR (3 nM - 10 µM), the observed effects were more consistent. Increases in $\dot{V}O_2$ were significantly greater than those mediated by NOR alone, and the $\dot{V}O_2$-stimulatory concentration range was increased (Fig. 5.6A). The changes in perfusion pressure were significantly lower in the NOR + glucagon experiments at the corresponding NOR concentrations (Fig. 5.6B).
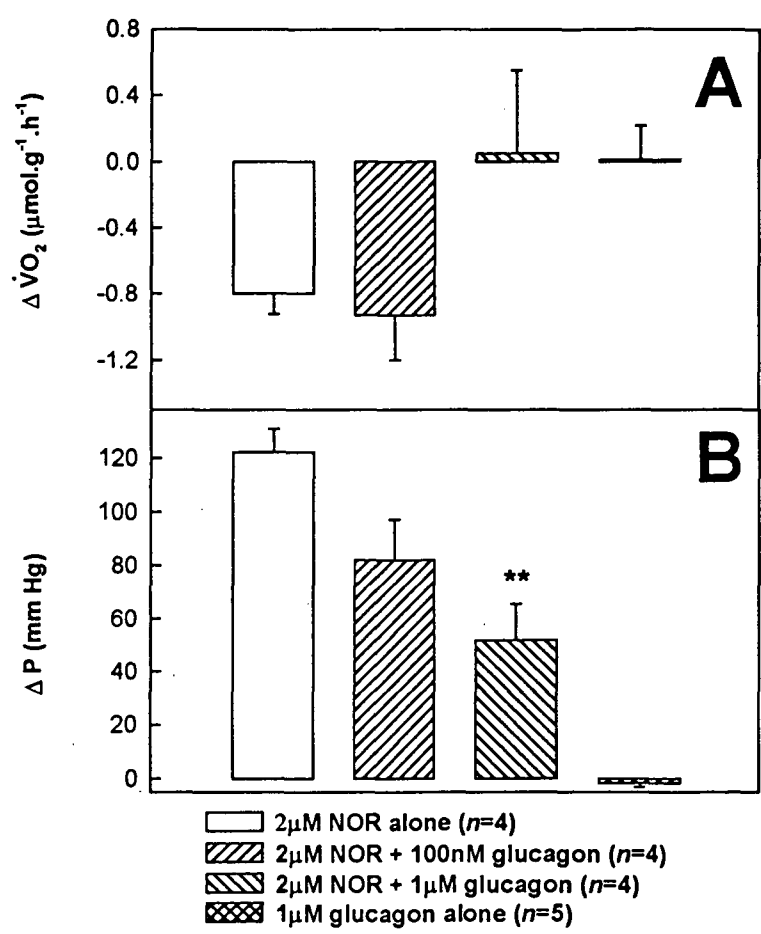
Fig. 5.5. Effects of glucagon on (A) change in $\dot{V}O_2$ and (B) change in perfusion pressure in basal preparations and NOR (2 µM, $\dot{V}O_2$ inhibitory)-treated preparations. When co-infused with NOR, glucagon was infused after steady-state responses to NOR had been attained. Data are the mean ± SE. ** Significantly different to 2 µM NOR alone, P < 0.01.
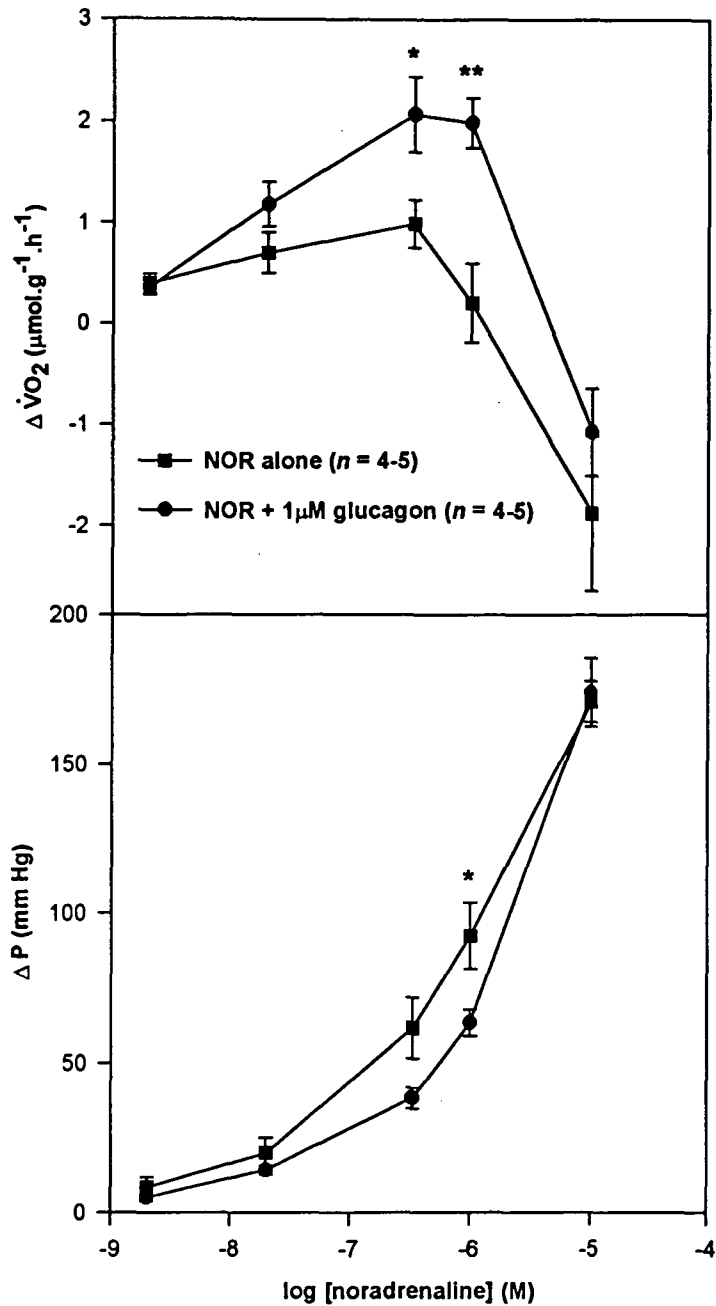
Fig. 5.6. Concentration-response curves for (A) change in $\dot{V}O_2$ and (B) change in perfusion pressure in response to NOR alone, and NOR in the presence of 1 μ M glucagon. NOR responses both with and without glucagon were determined in each perfusion preparation. The order of treatment was alternated between experiments. Glucagon infusion, when present, was continuous and commenced prior to NOR infusions. Points are means ± SE. Significantly different to NOR alone, * P < 0.05; ** P < 0.01. Experiments were conducted around 12 months after those used for Fig. 5.3, using birds from an upgraded hatchery with improved temperature control.

5.3.5 Other stimuli

The qualitative effects of a range of other agents and stimuli on the perfused chicken muscle preparation are shown in Table 5.1. Of these, only motor nerve stimulation produced a response, giving marked vasodilatory effects in addition to large increases in $\dot{V}O_2$ associated with muscle contraction.

Table 5.1. Effects of other potentially active agents and stimuli.

Agent	Vasoconstriction	Dilatation (NOR preconstricted*)	$\dot{V}O_2$ effects	Remarks
Angiotensin II (0.01-5 nM)	0	0	0	co-infused with 333 nM NOR
Vasopressin (0.3-5 nM)	0	0	0	co-infused with 333 nM NOR
Capsaicin (2.5-5 µM)	0	not tested	0	-
Insulin (15-100 nM)	0	0	0	tested with high (0.5-1.2 µM) and low (5-50 nM) dose NOR
Popliteal Nerve Stimulation (1Hz, 5V, width 5ms)	0	+++	+++	contracting skeletal muscle
Tubocurarine (1µM)	0	0	0	neuromuscular blockade does not change 5-HT-induced $\dot{V}O_2$
Oleic acid (1µM-1.3 mM)	0	not tested	0	no effects alone or in combination with glucagon (100 nM-1 µM)

* preconstriction with $\dot{V}O_2$ -stimulatory doses of NOR

5.4 Discussion

The present studies examining the constant flow perfused chicken popliteal muscle bed extend the previous findings using the perfused hindlimb muscle beds of two other endothermic taxonomic groups: eutherians (rats, Chapter 1) and marsupials (Tasmanian bettong, *Bettongia gaimardi;* Ye *et al.*, 1995). These perfused preparations have been shown to be positively thermogenic in response to NOR and a

number of other vasoconstricting agents in this laboratory. The present study represents the first report of both vasoconstrictor-induced thermogenesis and dose-dependent dual effects of catecholamines in avian skeletal muscle.

The decision to perfuse at 25°C was taken in order to be consistent with previous work and to eliminate the need for erythrocytes in the perfusate. This avoids potential problems associated with high flow rates such as the need - for economic reasons - to recirculate perfusate which results in the accumulation of lactate and other metabolites. The lower flow rate of 0.27 $ml\cdot min^{-1}\cdot g^{-1}$ wet weight of muscle perfused in the popliteal bed was chosen in view of microsphere studies of blood flow to the chicken pectoral muscle of 0.15 $ml\cdot min^{-1}\cdot g^{-1}$ (Wolfensen *et al.*, 1978) and to duckling limb muscles of 0.3 to 0.36 $ml\cdot min^{-1}\cdot g^{-1}$ (Duchamp and Barré, 1993). However, the decline in CrP:Cr ratio at this flow rate, despite the maintenance of energy charge (Fig. 5.1A) prompted an increase of 20% to 0.33 $ml\cdot min^{-1}\cdot g^{-1}$ in all subsequent experiments. Studies in this laboratory using the rat have shown the CrP:Cr ratio to be an effective early index of muscle hypoxia (Ye *et al.*, 1996). At the higher flow rate, the ratio remained at the *in vivo* value for at least three hours. The experiments using NOR were repeated and a similar response pattern observed. The fact that the increases in $\dot{V}O_2$ were still observed in a preparation with a well maintained CrP:Cr ratio strongly discounts the possibility that the $\dot{V}O_2$ increases are due to vasoconstrictors artefactually redirecting flow to zones of regional skeletal muscle hypoxia in the perfused vascular bed.

The oxygen consumption of resting perfused chicken muscle at 25°C (6.3 ± 0.3 and 7.4 ± 0.3 $\mu mol\cdot g^{-1}\cdot h^{-1}$ at 0.27 and 0.33 $ml\cdot min^{-1}\cdot g^{-1}$ respectively) is similar to that of the rat (6.4 ± 0.2 $\mu mol\cdot g^{-1}\cdot h^{-1}$ at 0.27 $ml\cdot min^{-1}\cdot g^{-1}$, Colquhoun *et al.*, 1988), but greater than that of the bettong (4.18 ± 0.35 $\mu mol\cdot g^{-1}\cdot h^{-1}$ at 0.28 $ml\cdot min^{-1}\cdot g^{-1}$, Ye *et al.*, 1995). The maximal NOR-induced increase in $\dot{V}O_2$ in the chicken hindlimb (35% to 9.9 $\mu mol\cdot g^{-1}\cdot h^{-1}$ at 0.33 $ml\cdot min^{-1}\cdot g^{-1}$ in the absence of glucagon) occurred in a perfused muscle bed with surgically divided somatic motor and sensory nerve connections (via the popliteal nerve). These nerve connections therefore cannot be responsible. Nor is it likely that the observed responses are related to sympathetic outflow as the popliteal artery and hence the sympathetic fibres in its wall is crushed by two ligatures in the cannulation procedure. Such damage to sympathetic nerve

fibres effectively halts nerve transmission (Cowen *et al.*, 1982). Furthermore, experiments performed with tubocurarine (1 μM, Table 5.1) demonstrated that 5-HT-induced $\dot{V}O_2$ and perfusion pressure increases were unaffected by neuromuscular junction blockade. These findings eliminate the possibility of any form of shivering being implicated in the observed thermogenesis.

Nitroprusside (0.5 mM, Fig. 5.4) blocked the NOR-induced stimulatory effects, thereby demonstrating a relationship between $\dot{V}O_2$ and vasoconstriction similar to that observed in the rat (Chapters 2a, 2b, and Colquhoun *et al.*, 1988). However, an effect of nitroprusside to lower both $\dot{V}O_2$ and perfusion pressure to below commencing steady-state values is generally not observed in the rat, indicating the presence of higher basal tone with associated $\dot{V}O_2$ in perfused chicken muscle. Prazosin (10 μM, Fig. 5.4) inhibited the NOR stimulation to near basal levels, indicating that this stimulation was largely due to α_1-adrenergic receptors. However, the failure of prazosin to reduce the $\dot{V}O_2$ and pressure to the sub-basal levels seen with nitroprusside suggested that the basal tone is not mediated by release of endogenous catecholamines acting on α_1-adrenergic receptors. Since 10 μM propranolol did not significantly alter the observed responses (Fig. 5.4), the role of β-adrenergic receptors in the NOR-induced effects appears to be minimal.

5-HT infusion resulted in vasoconstriction associated with small $\dot{V}O_2$ increases at the lower end of the concentration-response curve (Fig. 5.3). The $\dot{V}O_2$ response is therefore different to that observed in perfused rat hindlimb and bettong hindlimb where 5-HT inhibited $\dot{V}O_2$ at all effective doses. This behaviour has previously been suggested to be due to the operation of functional flow shunts in the microvasculature, effectively short-circuiting the respiring tissue (discussed in Chapter 1). This reasoning can be extended to suggest that the inhibitory effects of high catecholamine doses in perfused chicken muscle (Fig. 5.3) may also be due to flow redistribution caused by vascular shunting as proposed in the rat (Chapter 1) and in the bettong (Ye *et al.*, 1995). However, the biphasic $\dot{V}O_2$ responses to 5-HT suggest that the control of these flow patterns may be quite different to that of the mammalian models.

Angiotensin II (alone and with NOR), vasopressin and capsaicin, all potent vasoconstrictors in perfused rat muscle (reviewed by Clark *et al.*, 1995), were inactive

in the present study (Table 5.1). Previous studies using birds have suggested that a variety of responses to capsaicin are absent or substantially reduced relative to the rat (Pierau *et al.*, 1987), presumably due to the absence of vanilloid receptors. This conclusion is supported by other studies in which no specific [³H]resiniferatoxin binding to chicken dorsal root ganglia was reported (Szallasi and Blumberg, 1990b). However, given that angiotensin II is reported to have vasodepressor effects in chickens *in vivo* and induce relaxation of chicken aortic rings (Yamaguchi and Nishimura, 1988) and that chickens possess the vasopressin-like hormones arginine vasotocin and arginine vasopressin (Choy and Watkins, 1986), the inactivity of these hormones in either pre-constricted or unstimulated preparations was somewhat surprising. In intact pigeons, intravenous administration of vasotocin and angiotensin II have been reported to respectively reduce and enhance oxygen consumption (Hassinen *et al.*, 1994). However, these effects occurred in parallel with changes in shivering intensity.

The effect of glucagon on perfused muscle oxygen consumption may be related to its vasodilatory action. Fig. 5.5 shows that 1 µM glucagon alone has little effect, regardless of the presence of a suitable substrate (oleic acid, Table 5.1). However both 100 nM and 1 µM glucagon exhibit a marked vasodilatory action in the presence of NOR (Figs. 5.5 and 5.6). Furthermore, when infused against a concentration of NOR that induces a type B ($\dot{V}O_2$-inhibitory) effect, glucagon not only reduces the perfusion pressure, but increases the oxygen consumption from a sub-basal to a basal or stimulated state (Fig. 5.5). NOR dose curves determined in the presence of 1 µM glucagon (Fig. 5.6) gave more consistent data and illustrate the potential of glucagon to enhance catecholamine-induced resting muscle oxygen uptake *in vitro*. Under the influence of glucagon, the concentration range of the positive phase of NOR-induced $\dot{V}O_2$ change is extended and is greater in magnitude. Consequently, higher NOR concentrations are required to induce the negative $\dot{V}O_2$ phase. Thus glucagon may be selectively opposing the onset of type B activity, resulting in a simultaneous enhancement of type A (stimulatory) $\dot{V}O_2$, given that the net effect is likely to be the combination of simultaneous type A and type B contributions. Parallel experiments conducted with insulin (Table 5.1) gave no

evidence of this hormone having a similar potential thermoregulatory role in chicken muscle.

The biphasic actions of NOR demonstrated in this *in vitro* study may explain why the administration of exogenous NOR *in vivo* is generally not found to be thermogenic in birds (Chaffee and Roberts, 1971). Paradoxically, however, the sympathetic nervous system is activated during cold exposure (Reviewed by Hissa, 1988). Fujita *et al.* (1992) have shown that the plasma values of NOR and ADR in chronically catheterised chickens after feeding are 9.9 ± 4.6 and 1.8 ± 0.2 nM respectively. These values are within the range of the concentrations which give enhanced muscle oxygen uptake in the present study. However, local concentrations near sympathetic nerve cell terminals may be significantly greater than reported plasma concentrations. Thus if catecholamine concentrations were to rise due either to external administration, or increased endogenous release from an activated sympathetic nervous system, muscle bed oxygen consumption may in fact be inhibited. Equally, reports that exogenous glucagon is thermogenic *in vivo* via an unknown mechanism of action (Barré, 1983; Duchamp *et al.*, 1993b) may be explained, at least in part, by the interaction of glucagon with the thermoregulatory effects of catecholamines resulting in a shift to the right of the inhibitory phase of the dose response curves for $\dot{V}O_2$, thereby restoring increased $\dot{V}O_2$. Thus nonshivering thermogenesis in birds could conceivably require the simultaneous presence of catecholamines and glucagon.

The results presented in this chapter suggest that the chicken (and perhaps birds generally) has the potential to effect muscle NST *in vitro*. It is therefore surprising that birds do not usually exhibit this potential for regulatory NST *in vivo* except after long-term cold-acclimation. It is plausible that the vasoconstrictive effects of catecholamines usually inhibit thermogenesis by opening functional vascular shunts or reducing muscle perfusion *in vivo*. The ability to use this potential muscle NST might therefore depend on adaptive mechanisms aimed at reducing the negative vascular effects of catecholamines possibly in conjunction with increased secretion of vasodilatory agents such as glucagon and/or with altered vascular responses. Alternatively, small contractions of skeletal muscle might also act to delay the onset of catecholamine-induced $\dot{V}O_2$ inhibition in the cold on account of the contraction-induced vasodilatation of the NOR-stimulated preparation (Table 5.1). Such an effect

could conceivably account for the potentiated shivering thermogenesis exhibited by young birds in the first stage of cold-acclimation (Barré *et al.*, 1985) and could be a link between shivering thermogenesis and catecholamine-induced NST in birds and other endotherms. These possibilities require further investigation.

Chapter 6

Vascular Control in Comparative Perfusion Models.
B. Obese Zucker Rats: Models of Impaired Vascular Metabolic Control?

6.1 Introduction

Major differences exist between the obese Zucker (*fa/fa*) rat and its lean counterpart (Fa/?). The obese animals exhibit hyperphagia (Cleary *et al.*, 1980), decreased whole body oxygen consumption at ambient temperatures of 10-30°C (Kaplan, 1979), decreased low-protein diet-induced thermogenesis (Young *et al.*, 1980), and a lower maintenance energy requirement (Mowrey and Hershberger, 1982). At the tissue level, the *fa/fa* rat has defective brown adipose tissue (Levin *et al.*, 1984), but differences in the metabolic properties of other tissues, including muscle, may also exist.

In terms of glucose homeostasis the *fa/fa* animals exhibit hyperinsulinemia (Tukenkopf *et al.*, 1982), decreased sensitivity to insulin *in vivo* (Jeanrenaud, 1979), decreased ability of various tissues to bind insulin (Kobayashi and Olefsky, 1978; Le Marchand-Brustel *et al.*, 1978), and various effects distal to the insulin receptor interaction (Assimacopoulos-Jeannet and Jeanrenaud, 1976; Crettaz *et al.*, 1980). In addition, skeletal muscle of the *fa/fa* rat is insulin resistant with decreased insulin binding (Crettaz *et al.*, 1980; Czech *et al.*, 1978), rate of glycogen synthesis (Crettaz *et al.*, 1980, 1983; Ivy *et al.*, 1986; Kemmer *et al.*, 1979), rate of glycolysis (Crettaz *et al.*, 1980, 1983), and rate of glucose transport (Sherman *et al.*, 1988). Perfusion studies (Kemmer *et al.*, 1979) indicate that the hindlimb of the *fa/fa* rat has diminished basal glucose uptake, markedly diminished insulin-mediated glucose uptake, diminished lactate oxidation, and exaggerated lactate release when compared to that of the lean counterparts. Perfused hindlimb studies (Sherman *et al.*, 1988) show that the impaired insulin-mediated glucose uptake is common to all skeletal muscle fibre types.

BRL 49653 (5-(4-[2-(N-Methyl-N-(2-pyridyl)amino)ethoxy]-benzyl)thiazolidine-2,4-dione, Fig. 6.1) is a new potent insulin sensitiser agent with the ability to improve

glycaemic control in *fa/fa* Zucker rats as well as other animal models of NIDDM. Chronic oral administration of BRL 49653 (3 μmol·kg^{-1}·day^{-1} for 21 days) normalises Zucker *fa/fa* rat glucose tolerance and reduces fasting plasma insulin concentrations by 50% (Smith *et al.*, 1993; Cawthorne *et al.*, 1993). In hyperinsulinaemic (600 μU·ml^{-1}) euglycaemic clamped *fa/fa* Zucker rats, the same BRL 49653 treatment increases glucose infusion rates, resulting in both enhanced insulin suppression of hepatic glucose output and increased glucose disposal by peripheral tissues, principally skeletal muscle (Smith *et al.*, 1993). Increased skeletal muscle glucose disposal under euglycaemic clamp conditions has also been reported in high-fat-fed insulin resistant rats (but not controls) after oral BRL 49653 administration (10 μmol·kg^{-1}·day^{-1} for 4 days, Kraegan *et al.*, 1993). It is conceivable that these reported actions of BRL 49653 to normalise glucose tolerance may be due in part to a haemodynamic effect allowing improved muscle nutrient delivery.
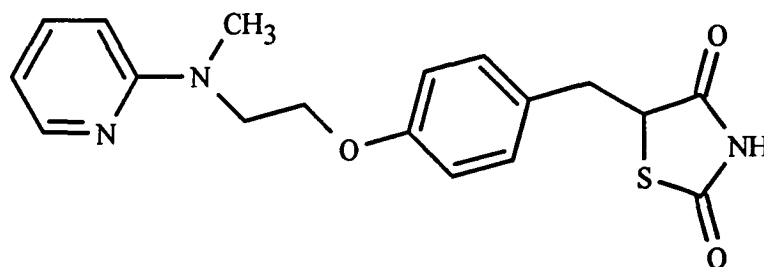
Fig. 6.1. Structure of BRL 49653. The agent is a member of the glitazone family of thiazolidinedione analogues currently being developed for the treatment of insulin resistance and non-insulin-dependent diabetes.

A systematic assessment of the thermogenic properties of perfused hindlimb from obese Zuckers has not been made, despite the marked responses to

catecholamines by perfused hindlimbs from non-obese strains demonstrated in this laboratory (Clark *et al.*, 1995) and by others (Côté *et al.*, 1985; Grubb and Folk, 1976; Richter *et al.*, 1982a). Since previous work from this laboratory on non-obese strains has led to the proposal that muscle metabolism is regulated by vasoconstrictors that act to alter the distribution of nutritive flow (discussed in Chapter 1), skeletal muscle in the obese Zucker rat was identified as being a potential model of impaired nutritive flow.

Chapter 1 proposed a link between impaired vascular control of skeletal muscle and the pathogenesis of disease states referred to as syndrome X (hyperinsulinaemia, hypertension and hyperlipidaemia; Chapter 1, Fig. 1.2) Accordingly, phenotypic differences in Zucker rat perfused hindlimb metabolism may also be linked to impaired microcirculatory function. The studies outlined in this chapter were thus governed by two primary objectives. The first was to determine if a phenotypic difference existed in perfused Zucker rat hindlimb thermogenic response to type A and type B vasoconstrictors, allowing a comparative assessment of the total nonshivering thermogenic capacity of the hindlimb controlled by the vascular system. The second intention was to confirm that perfused *fa/fa* Zucker rat hindlimb demonstrated impaired insulin-mediated glucose uptake, and to subsequently examine the effect of chronic pre-treatment with BRL 49653 on the insulin sensitivity of such preparations, and also on preparations from non-obese strains.

6.2 Methods

Experiments involving Zucker rats were performed in the laboratories of the Department of Vascular Biology, SmithKline Beecham Pharmaceuticals, The Frythe, Welwyn, Hertfordshire, United Kingdom during July and August, 1993. Experiments with hooded Wistar rats were performed in Hobart, Australia.

6.2.1 Animal care

Animals were housed and cared for in accordance with the principles of the *Guide to the Care and Use of Experimental Animals*, Vol. 1 (Canadian Council on Animal Care, 1980).

Experiments were performed using mature (20 and 27 week old) male Zucker genetically obese (*fa/fa*), 500-600 g rats, when the obesity and insulin resistance were well established, and age-matched lean male animals (Fa/?, 340-365 g). Rats were obtained from Harlan Olac Ltd, Bicester, Oxfordshire, UK and housed in groups under climate-controlled conditions (20 ± 2°C, 12 h light : 12 h dark cycle) and provided with R&M 1 (rat and mouse diet) made by SDS, Manea, Cambridgeshire, UK, and water *ad libitum*. Details concerning the non-obese hooded Wistar rats can be found in Chapter 2a.

In experiments involving BRL 49653 treatment, rats were dosed once daily by oral gavage for 7 days with either BRL 49653 (3 μmol·kg^{-1}) or vehicle (water).

6.2.2 Materials

6.2.2.1 Zucker experiments (United Kingdom)

Bovine serum albumin (fraction V), noradrenaline bitartrate, serotonin hydrochloride, (-)-isoproterenol hydrochloride, and Evans blue were obtained from the Sigma Chemical Company (UK). Heparin sodium (5000 U·ml^{-1}) was obtained from CP Pharmaceuticals Ltd (UK), sodium pentobarbitone from RMB Animal Health Ltd (UK), AR grade sodium nitroprusside from BDH chemicals (UK) and bovine soluble insulin (25 U·mg^{-1}) from Calbiochem (USA). BRL 49653 as the maleic acid salt was synthesised by SmithKline Beecham (UK).

6.2.2.2 Hooded Wistar experiments (Australia)

Both 2-Deoxy-D-[1-^{3}H]glucose (10 μCi·ml^{-1}) and [U-^{14}C] sucrose (552 mCi·mmol^{-1}) were supplied by Amersham Australia Pty Ltd, porcine insulin (Actrapid MC, 100 U·ml^{-1}) by Novo Nordisk (Denmark), and serotonin creatine sulfate by Sigma (USA). BRL 49653 was synthesised by SmithKline Beecham (UK). The remaining materials used are as listed in Chapter 2a.

6.2.3 Isolated hindlimb preparation (United Kingdom)

Animals were given an intraperitoneal injection of heparin sodium (25 U·g^{-1}) and then anaesthetized with an intraperitoneal injection of pentobarbitone sodium (60

$mg \cdot kg^{-1}$). Rats were then pithed and maintained on a respirator via a tracheal tube. Surgery was subsequently performed as described in Chapter 2a.

6.2.4 Perfusion medium

The perfusion medium for the experiments at 25°C was the same as described in Chapter 2a. For the experiments at 32°C, the amounts of bovine serum albumin and calcium were increased to 4% and 2.5 mM respectively.

The bovine serum albumin (Sigma, USA) used for the experiments with Zucker rats was used as supplied. The bovine serum albumin (Boehringer, Australia) used for experiments involving hooded Wistar rats was dialysed prior to use as outlined in Chapter 2a.

6.2.5 Perfusion procedures

The perfusion procedures using hooded Wistar rats are given in Chapter 2a. The perfusion cabinet and heat exchanger thermostat temperatures were set to 25°C or 32°C as specified.

The perfusion procedures for Zucker rats were similar to those for hooded Wistars, although there were some differences in equipment used. Perfusion medium was pumped at a fixed flow rate by a peristaltic pump (Gilson Minipuls 3 with 3.90 $cm^3 \cdot m^{-1}$ tubing) adjusted at the start of each experiment to give comparable venous PO_2 values [nominally set at a minimum of 350 mm Hg to ensure adequate O_2 supply, as established in previous studies (Ye et al., 1990b)]. The temperature of the perfusate was raised to 25°C or 32°C in a heat exchanger prior to passing through a silastic lung gassed with 95% O_2-5% CO_2. In the absence of an enclosed perfusion cabinet, homeothermic blankets (Harvard, USA) and water jackets ensured that the hindlimb preparation, the surrounding perfusate-containing tubing, and the oxygen electrode remained at 25°C or 32°C. A temperature probe positioned beneath the skin adjacent to the perfused muscle controlled the operation of the homeothermic blankets. When required, agonists were infused continuously (Gilson Minipuls 3 with microbore tubing) into a small stirred bubble trap proximal to the arterial cannula. The infusion rates gave 1 in 200 dilutions. Infusion of vehicle (0.1% ascorbic acid in

isotonic saline) had no apparent effect on $\dot{V}O_2$ or perfusion pressure in any of the experiments.

6.2.6 Oxygen uptake and perfusion pressure determinations

Oxygen tension in the venous perfusate was monitored continuously as described in Chapter 2a. Oxygen consumption was calculated (Chapter 2a) using the appropriate Bunsen coefficient for 25°C or 32°C (Christoforides *et al.*, 1969). The oxygen electrode was calibrated before and after each experiment using recirculating buffer gassed with 95% O_2 and then air. Perfusion pressure was monitored continuously using a fluid-filled transducer (CEC Instrumentation Ltd).

6.2.7 Determination of perfused hindlimb tissue in Zucker rats

In the final stages of perfusion experiments, all agonists were removed and the preparation was allowed to return to an unstimulated steady state. The flow was stopped and a 1% solution of Evans blue dye was injected into the arterial cannula at the same flow rate to that used throughout the experiment. The resultant stained tissue was then excised, blotted dry, dissected into muscle and fat and weighed to determine the amounts of tissue perfused.

6.2.8 Glucose uptake determinations

6.2.8.1 Arteriovenous glucose uptake

Arteriovenous (A-V) glucose uptake for experiments involving Zucker rats was determined using a glucose analyser (YSI 2300 STAT, Yellow Springs Instruments, USA) using inflow and outflow glucose samples. Perfusate glucose for the experiments with hooded Wistar rats was determined by manual colourimetric assay (GOD-Perid method, Boehringer Mannheim, Germany). The glucose uptake was calculated by multiplying the A-V difference in glucose concentration by the perfusate flow rate and dividing by the weight of tissue perfused.

6.2.8.2 Uptake of 2-Deoxy-D-[1-^{3}H]glucose (2DG) by individual muscles

Based on the method of Ferre *et al.*, (1985), tracer amounts of 2DG (10 μCi·ml^{-1}; 15 Ci·mmol^{-1}) and [U-^{14}C]sucrose (3.14 μCi·ml^{-1}; 552 mCi·mmol^{-1} in 2 mM sucrose/0.9% NaCl were infused at 40 μl·min^{-1} during the final 16.0 min. of the perfusion period. Since the hindlimb preparations were non-recirculating, the ratio of 2DG remained constant, allowing a quantitative determination of 2DG uptake without the need to monitor 2DG removal from the perfusate. Muscles of the perfused preparation: the soleus, plantaris, gastrocnemius red, gastrocnemius white, extensor digitorum longus, tibialis anterior, and all of the anterior and posterior muscles of the thigh were subsequently dissected apart and then frozen and powdered under liquid N$_2$. Samples of powder were taken for dry weight determination (48 h at 80°C). Separate samples were homogenised in water, centrifuged (8000 g, 15 min) and the supernatants used for radioactive counting. The muscle glucose uptake (R'g, μmol·g^{-1}·h^{-1}) was calculated as follows:

$$\frac{2 \times [^3\text{H d.p.m. in muscle} - (^{14}\text{C d.p.m. in muscle} \times {}^3\text{H d.p.m.}/^{14}\text{C d.p.m. ratio in perfusate})]}{\text{dry wt muscle (g)} \times (\mu\text{moles glucose per ml perfusate} / {}^3\text{H d.p.m. per ml perfusate})}$$

6.2.9 Statistical analysis

Data are given as means ± SE. Statistically significant differences (P ≤ 0.05 and P ≤ 0.01) were determined using Student's unpaired *t*-test or analysis of variance (ANOVA). In general, 3-6 animals were used to determine individual data points.

6.3 Results

6.3.1 Vasoconstrictor effects in perfused Zucker preparations

6.3.1.1 Differences between obese and lean Zuckers

Progeny of the Zucker strain display two phenotypes which manifest as obese homozygotes *(fa/fa)* and a mixture of lean animals which are either homozygotes for

leanness (Fa/Fa) or heterozygotes (Fa/*fa*). Significant differences for the male age-matched Zucker rats used in this study included body weight, heart weight, and the tissue composition of the hindlimb (Table 6.1). The perfused tissue of the hindlimb of the obese (*fa/fa*) animals (deduced by dye distributions) was comprised of significantly less muscle, significantly more fat and, in total, weighed significantly more than the hindlimb of the lean (Fa/?) animals (Table 6.1). The proportion of fat : muscle of the lean Zucker rat hindlimb (10.6 ± 0.7% fat) was slightly higher than that for 6-8 week old non-obese hooded Wistar rats (4.3 ± 0.1% fat). Differences in the proportion of hindlimb fat : muscle between *fa/fa* and Fa/? were taken into account in expression of the data (see below); some apparent differences were not significant when muscle was assumed to be the sole tissue responsible for hindlimb $\dot{V}O_2$.

Table 6.1. Body mass, heart mass, and perfused hindlimb analysis of obese and non-obese Zucker rats.

Phenotype	n	Body mass (g)	Heart mass (g)	Perfused hindlimb		
				Muscle (g)	Fat (g)	Total (g)
Obese	6	566.7±15.5	1.80±0.08	22.03±0.72	12.18±0.82	34.22±0.84
Lean	6	350.8±4.5[a]	1.45±0.09[c]	25.94±1.08[c]	2.79±0.25[a]	28.73±1.04[b]

Note: Values are means ± SE for 20 week old male obese and lean rats. 'Total' is defined as the sum of dye-containing perfused muscle and fat dissected from the hindlimb following perfusion with Evans blue and excludes skin and bone. [a]P < 0.0001, [b]P < 0.01, [c]P < 0.05, significantly different from obese.

Table 6.2 shows basal (pre-noradrenaline and pre-serotonin) properties of the perfused hindlimbs of the obese and lean rats. The flow rate, which was constant throughout each perfusion, determined the resting or basal parameters of perfusion pressure, venous PO_2 and thus $\dot{V}O_2$. Table 6.2 shows that $\dot{V}O_2$ for the obese hindlimb was significantly less than that for the lean hindlimb when expressed in terms of total tissue perfused, but not so when expressed in terms of the mass of muscle perfused.

Basal $\dot{V}O_2$ for the whole hindlimb ($\dot{V}O_2$ per g, Table 6.2 × total mass, Table 6.1) was also significantly less (P < 0.05; n = 6) for the obese (151.2 ± 10.3 μmol·h^{-1}·hindlimb^{-1}) than the lean (183.3 ± 7.2 μmol·h^{-1}·hindlimb^{-1}) animals.

Table 6.2. Basal perfusion pressure and rate of oxygen uptake by hindlimbs of obese and non-obese Zucker rats.

	n	Flow rate (ml·min^{-1}·g^{-1})	Pressure (mm Hg)	Venous PO$_2$ (mm Hg)	$\dot{V}O_2$ (μmol·h^{-1}·g^{-1})
Obese	6	0.200±0.012	28.3±1.6	419.1±12.6	4.42±0.30
		(0.312±0.016)			(6.80±0.34)
Lean	6	0.230±0.015	25.4±1.7	360.0±7.8[a]	6.38±0.25[b]
		(0.257±0.017)			(7.07±0.32)

Note: Values are means ± SE and have been calculated on the basis of the perfused mass of muscle plus fat of the hindlimbs as shown in Table 6.1. Values shown in parentheses are expressed in terms of the perfused mass of muscle only. Arterial PO$_2$ was 663.7 ± 3.3 (n = 12). [a]P < 0.01, [b]P < 0.05, significantly different from obese.

6.3.1.2 Effects of noradrenaline in perfused obese and lean hindlimbs

Noradrenaline caused a marked vasoconstriction in the perfused rat hindlimb of both phenotypes. Fig. 6.2 shows dose-dependent rises in pressure to greater than 200 mm Hg for perfused hindlimbs from obese and lean Zuckers. Dose curves for each hindlimb were constructed using step-wise increasing doses of infused noradrenaline. At each dose, the increase in pressure remained constant provided the dose remained constant (data not shown). Fig. 6.2 shows that at each dose of noradrenaline the pressure development by the obese hindlimb tended to be greater than that of the lean hindlimb. The difference was significant statistically at 32 nM noradrenaline.
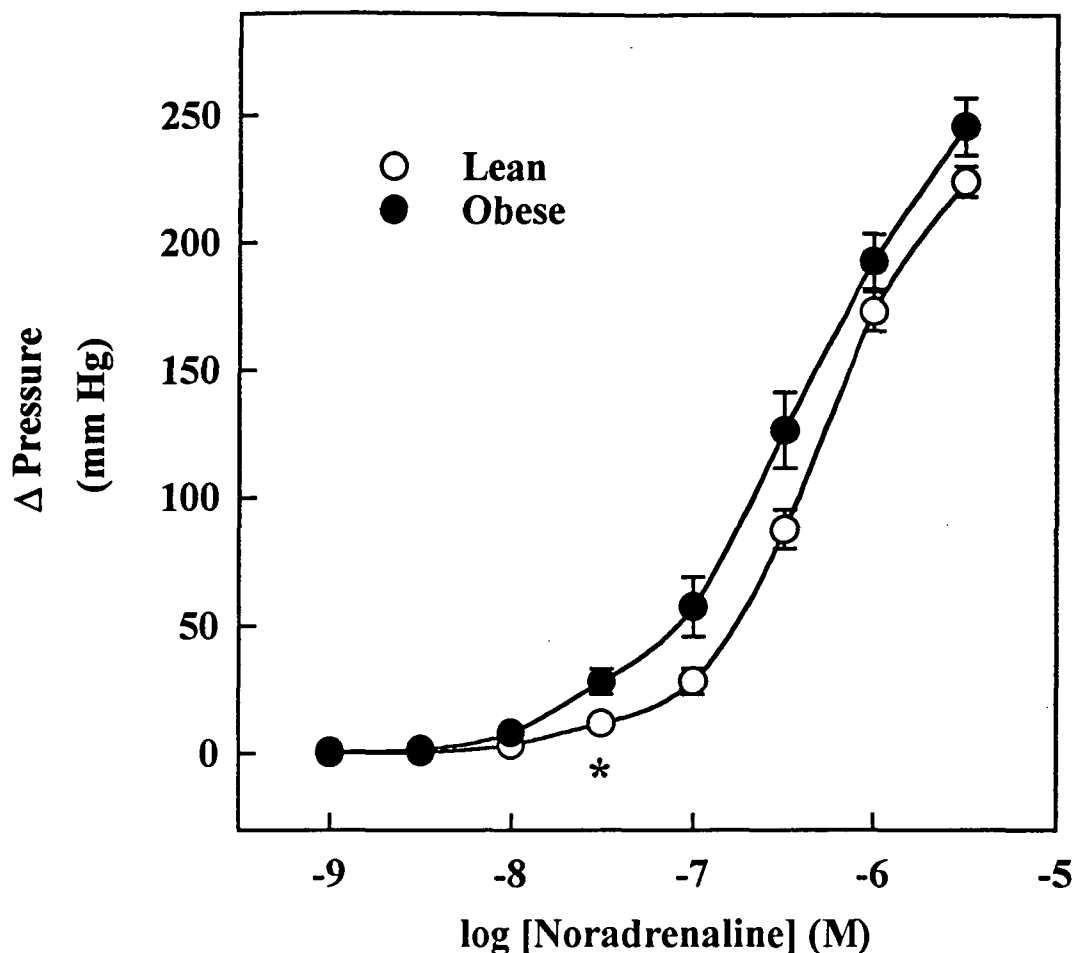
Fig. 6.2. Effect of noradrenaline on perfusion pressure of constant-flow hindlimbs of obese and lean Zucker rats (both $n = 3$). Basal (pre-noradrenaline) values for perfusion pressure were as given in Table 6.2. When not visible, SE bars are within the symbol. *P < 0.05, significantly different from obese.

The dose-dependent rise in perfusion pressure due to noradrenaline contrasts with the effect of this catecholamine on $\dot{V}O_2$. Fig. 6.3 shows that the steady-state $\dot{V}O_2$ response has essentially two components, both of which were present in the obese as well as the lean hindlimb. These components comprise a steady-state stimulatory phase evident over a concentration of 3.2 to 100 nM noradrenaline, and a steady-state inhibitory phase commencing at concentrations greater than 100 nM noradrenaline and extending to the maximum concentration used (3.2 μM). It is important to note that at concentrations greater than 1 μM noradrenaline, the value

for $\dot{V}O_2$ was less than basal (pre-noradrenaline). In Fig. 6.3A the results are expressed in terms of the total perfused tissue. The upper trace shows absolute values and significant differences between the obese and lean hindlimbs are readily apparent. Hindlimbs from obese Zuckers have lower basal values and are significantly less responsive to noradrenaline in terms of increased $\dot{V}O_2$, reaching only 72% of the absolute values for $\dot{V}O_2$ obtained by the lean hindlimbs. In addition, the inhibitory effect of noradrenaline over the range 100 nM to 3.2 μM is less pronounced with the $\dot{V}O_2$ of the obese hindlimb decreasing from a maximum of 8.03 $\pm$ 0.45 to 3.53 $\pm$ 0.21 $\mu mol \cdot h^{-1} \cdot g^{-1}$ of total perfused tissue. Over the same concentration range of noradrenaline, $\dot{V}O_2$ by the lean hindlimb decreased from 11.03 $\pm$ 0.71 to 4.20 $\pm$ 0.22 $\mu mol \cdot h^{-1} \cdot g^{-1}$ of total perfused tissue. Thus the obese hindlimb response was approximately 66% of that of the lean. Normalising the data to the basal (pre-noradrenaline) rate shows that the shape of the dose curves are indistinguishable except for the greater inhibitory effect of noradrenaline in lean hindlimb at maximal doses (Fig. 6.3A, lower trace).

When the data for $\dot{V}O_2$ were expressed in terms of the mass of muscle perfused none of the differences noted above for the hindlimbs were statistically significant (Fig. 6.3B).

6.3.1.3 Vasodilator blockade of the noradrenaline-mediated thermogenesis

A time course for the effect of isoproterenol and sodium nitroprusside on noradrenaline-mediated decrease in venous PO_2 and increase in perfusion pressure for the obese hindlimb is shown in Fig. 6.4. The vasoconstrictor action of noradrenaline was associated closely with an increase in $\dot{V}O_2$ as seen by the decrease in venous PO_2. Infusion of a maximal dose of isoproterenol partially blocked, and a maximal dose of sodium nitroprusside completely blocked, both effects mediated by noradrenaline (Fig. 6.4). The increases in $\dot{V}O_2$ and perfusion pressure due to noradrenaline were completely reversible and returned to basal values when the catecholamine was removed (data not shown).
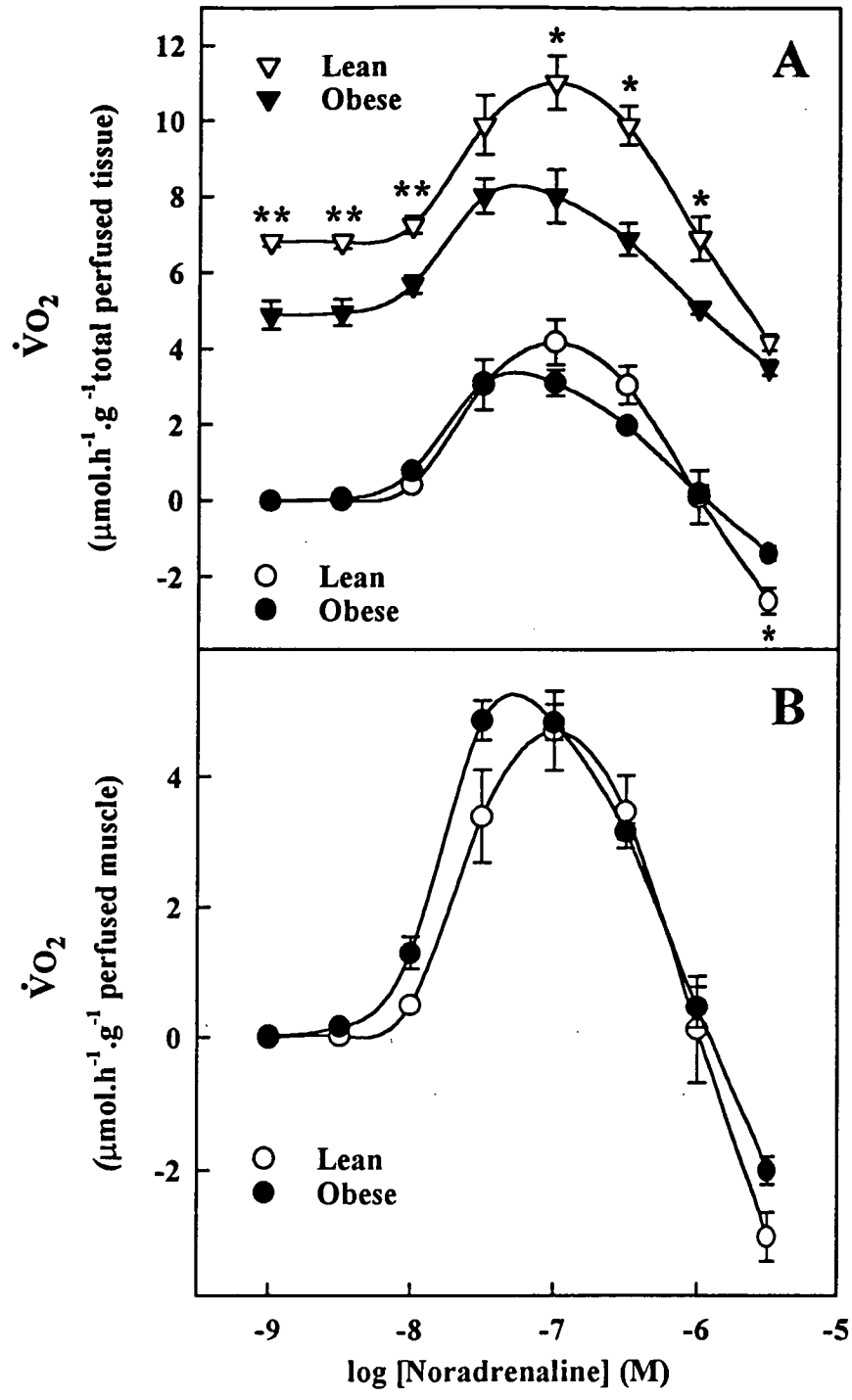
Fig. 6.3. Effect of noradrenaline on oxygen uptake by constant-flow perfused hindlimbs of obese and lean Zucker rats (both $n = 3$). (A) Absolute $\dot{V}O_2$ values ($\blacktriangledown$, ∇) and changes in $\dot{V}O_2$ ($\bullet$, O), calculated as a function of the total mass of perfused tissue (Table 6.1). (B) Changes in $\dot{V}O_2$ expressed as a function of the mass of perfused muscle. When not visible, SE bars are within the symbol. *P < 0.05, **P < 0.01, significantly different from obese.
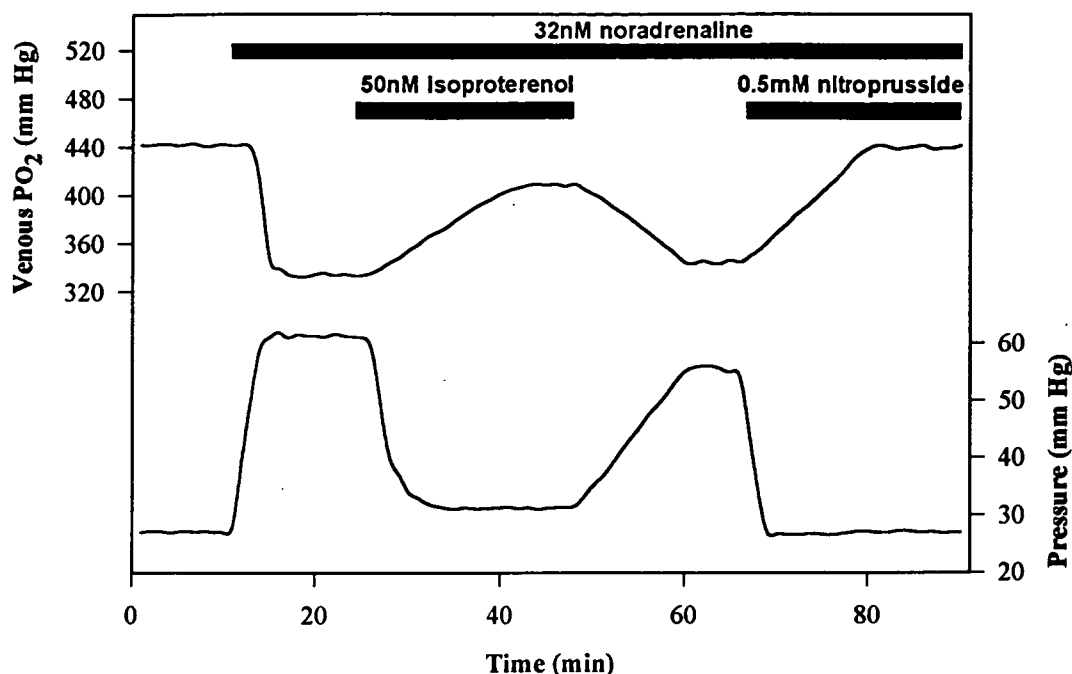
Fig. 6.4. **Effect of vasodilators, isoproterenol and sodium nitroprusside, on noradrenaline-mediated decreases in venous PO₂ and perfusion pressure by perfused hindlimbs of obese Zucker rats. The data were obtained after the dose-response curve for noradrenaline had been completed. The trace shown is a selection from three similar experiments.** ·

6.3.1.4 Effects of serotonin in perfused obese and lean hindlimbs

Fig. 6.5 shows the effect of serotonin on perfusion pressure in constant-flow hindlimbs of the obese and lean Zucker rats. The concentration-response curves were similar with each reaching a maximum pressure of approximately 190 mm Hg over basal at 3.2 μM serotonin. This contrasts with the effect of serotonin on $\dot{V}O_2$ by these hindlimbs (Fig. 6.6). For both obese and lean hindlimbs, serotonin (10 nM-3.2 μM) reduced $\dot{V}O_2$. When expressed as a function of the total mass of perfused tissue the obese hindlimb response was only 48% of that of the lean (Fig. 6.6A) decreasing from 3.93 ± 0.28 to 2.30 ± 0.37 μmol·h⁻¹·g⁻¹ of total perfused tissue. Over the same concentration of serotonin (10 nM-3.2 μM), $\dot{V}O_2$ by the lean hindlimb decreased from 5.83 ± 0.26 to 2.67 ± 0.48 μmol·h⁻¹·g⁻¹ of total perfused tissue.
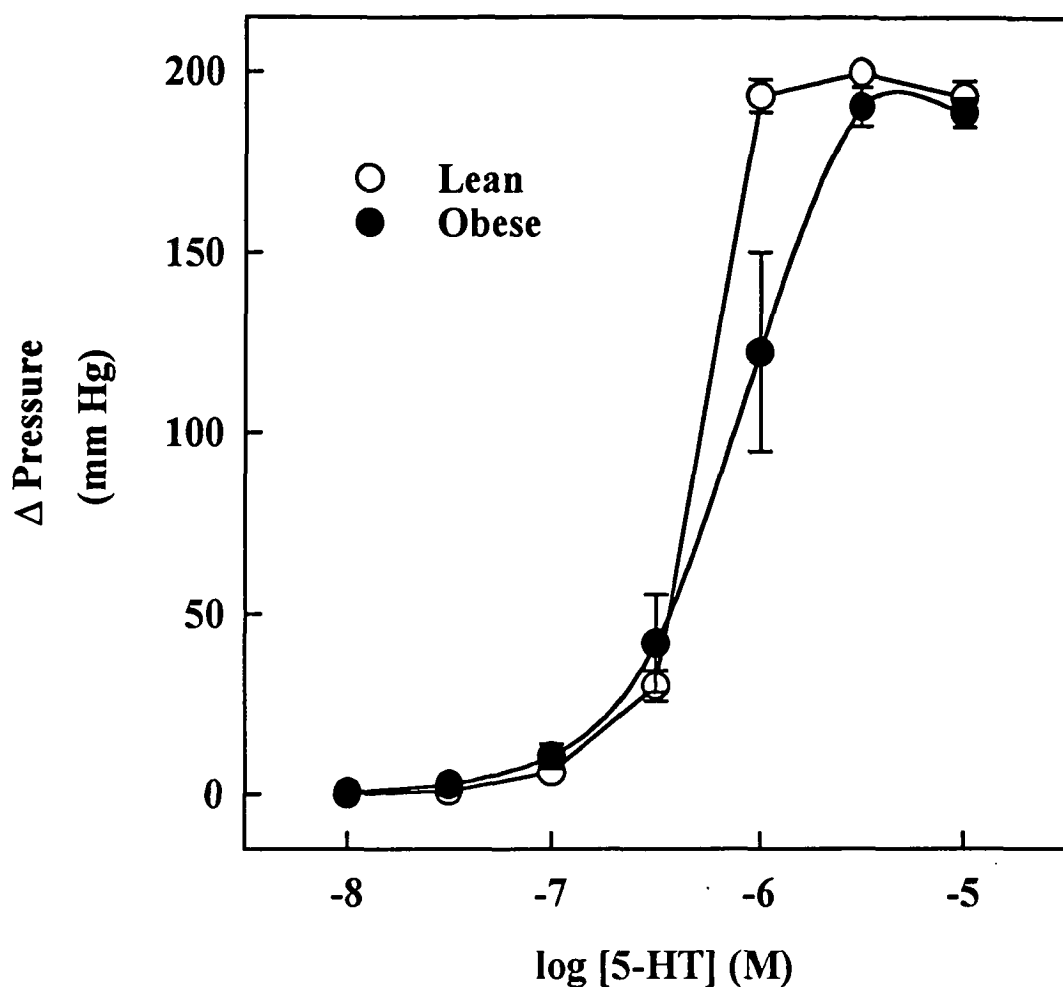
Fig. 6.5. Effect of 5-HT on perfusion pressure of constant-flow hindlimbs of obese and lean Zucker rats (both n = 3). Basal (pre-5-HT) values for perfusion pressure were as given in Table 6.2. When not visible, SE bars are within the symbol.

Fig. 6.6B shows that when expressed on the basis of the mass of perfused muscle the differences in response to serotonin remained significant. Thus, over the concentration range of 10 nM-3.2 μM serotonin, the decrease in $\dot{V}O_2$ by the obese hindlimb was approximately 70% of that of the lean.
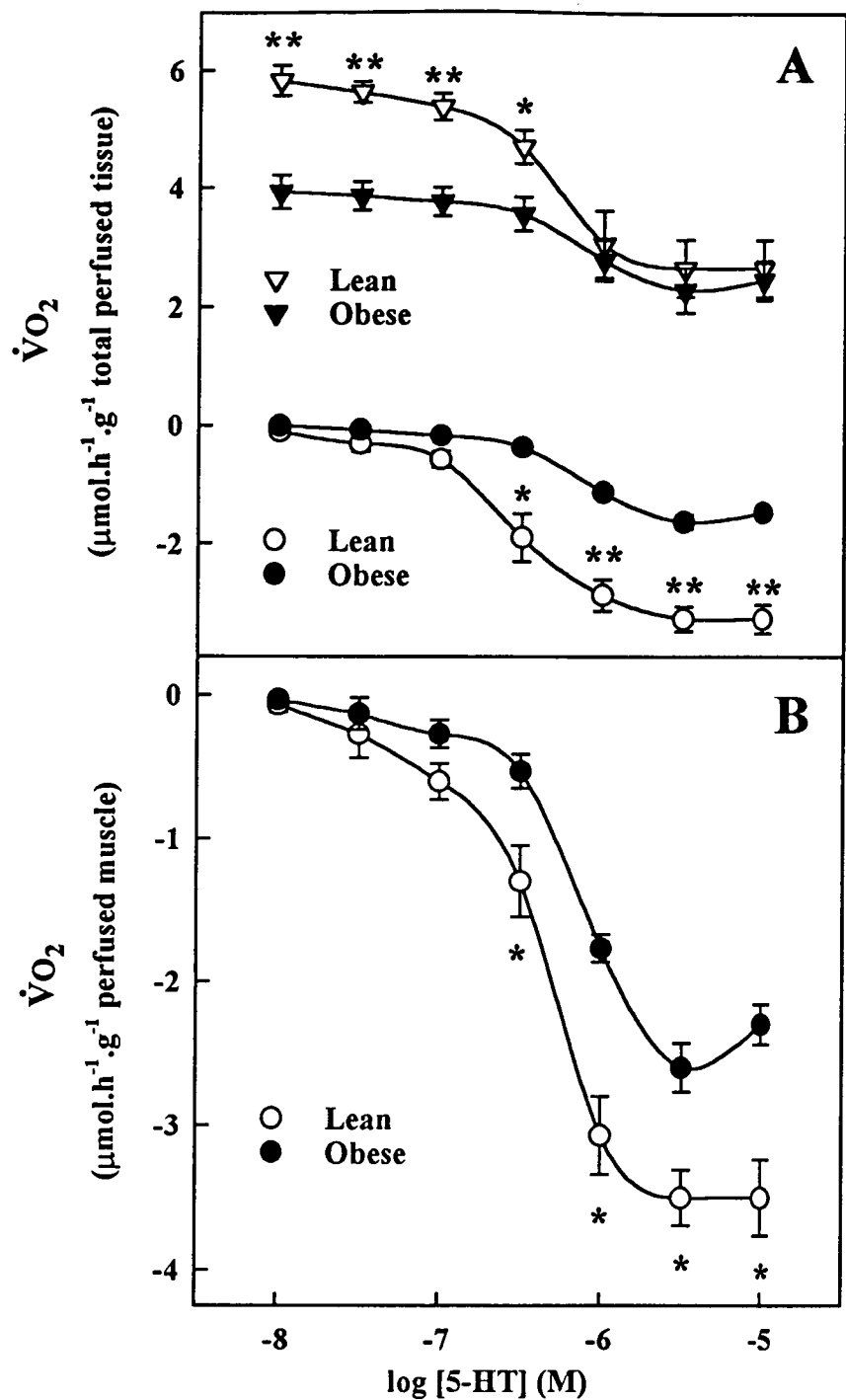
Fig. 6.6. Effect of 5-HT on $\dot{V}O_2$ by constant-flow perfused hindlimbs of obese and lean Zucker rats (both $n = 3$). (A) Absolute $\dot{V}O_2$ values ($\blacktriangledown$, $\triangledown$) and changes in $\dot{V}O_2$ ($\bullet$, O), calculated as a function of the total mass of perfused tissue (Table 6.1). (B) Changes in $\dot{V}O_2$ expressed as a function of the mass of perfused muscle. When not visible, SE bars are within the symbol. *P < 0.05, **P < 0.01, significantly different from obese.

6.3.2 Insulin-mediated glucose uptake experiments in perfused Zucker preparations

Table 6.3 shows body weight, heart weight, and perfused hindlimb muscle and fat content of lean and obese Zucker rats, and obese Zucker rats treated with BRL 49653 for 7 days. At 27 weeks of age, the obesity was pronounced and the homozygote *(fa/fa)* rats showed significant differences from the age-matched lean *(Fa/?)* rats in terms of body weight, hindlimb muscle and hindlimb fat content (Table 6.3). Treatment of obese animals with BRL 49653 for one week lowered significantly the amounts of hindlimb muscle and fat when compared with age-matched obese animals given vehicle alone, although overall body weight did not differ significantly. The proportion of muscle to fat in the hindlimb was not affected by BRL 49653 treatment.

Table 6.3. Body weight, heart weight, and perfused hindlimb analysis of non-obese and obese Zucker rats treated with BRL 49653.

Age (wk)	Phenotype and treatment	n	Body wt. (g)	Heart wt. (g)	Perfused Hindlimb Muscle (g)	Fat (g)
27	Lean	5	438.0±11.4	1.61±0.05	31.47±1.15	3.83±0.59
27	Obese	4	587.5±5.5[a]	1.70±0.09	21.62±0.57[a]	10.69±2.53[a]
20	Obese + BRL 49653	5	579.0±24.4	1.90±0.09	20.28±1.36	10.37±0.41
20	Obese + vehicle	5	584.0±12.20	2.05±0.08	24.43±0.82[b]	14.49±1.38[b]

Note: Values are means ± SE for 27 week old male lean and obese rats, and for two groups of 20 week old male obese rats that had received BRL 49653 or vehicle for 7 days. [a]$P < 0.05$, significantly different from lean. [b]$P < 0.05$, significantly different from BRL 49653-treated. Differences between groups of differing age (and therefore litter) have not been identified as being significant.

6.3.2.1 Comparison of lean and obese Zucker rats
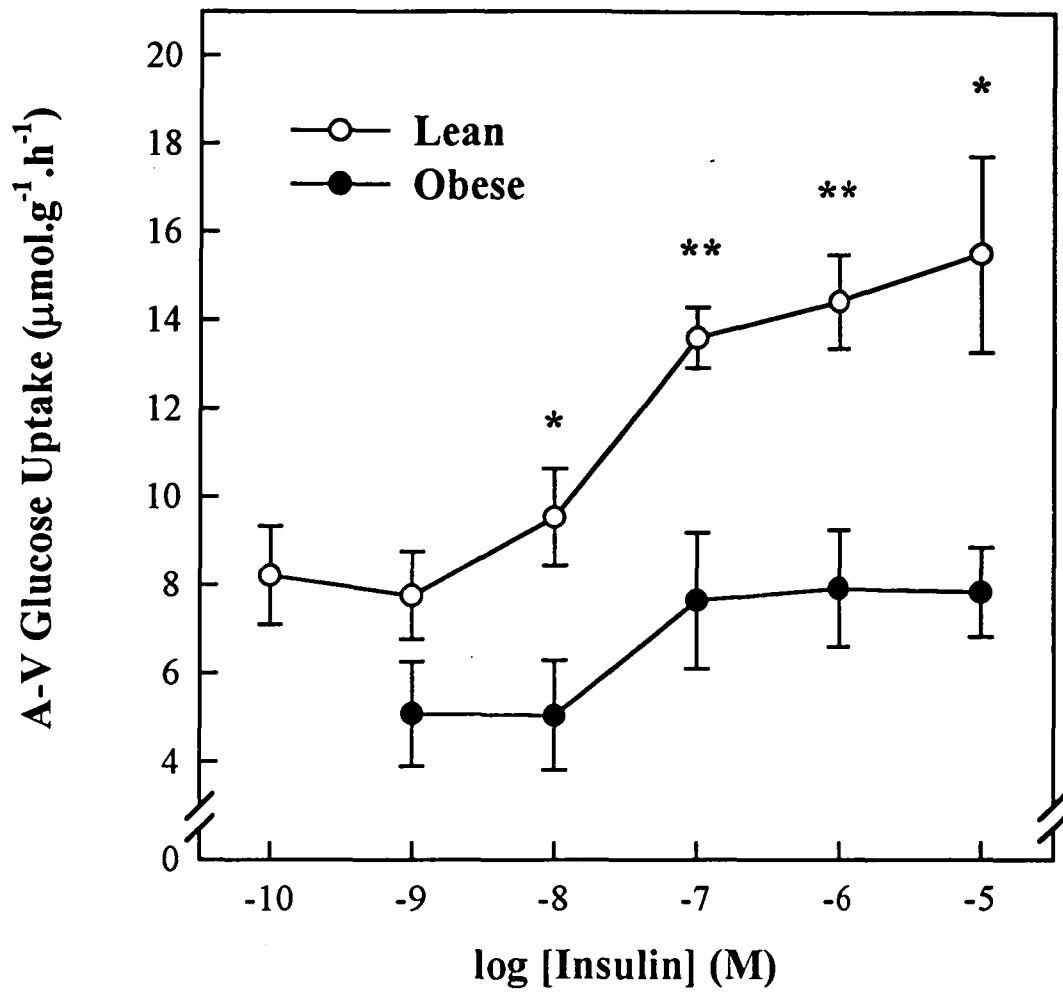
Fig. 6.7. Insulin dose-response curves for A-V glucose uptake by perfused hindlimbs from obese and lean Zucker rats. Values are means ± SE for five lean and four obese animals all of 27 weeks of age, and are expressed in terms of perfused mass of muscle + fat. *P < 0.05, **P < 0.01, significantly different from obese.

Fig. 6.7 shows the insulin dose-response curves for A-V glucose uptake at 32°C by perfused hindlimbs of 27-week old lean and obese Zucker rats. Basal (pre-insulin) values for glucose uptake tended to be greater in the lean hindlimbs relative to the obese hindlimbs, as reported by Kemmer *et al.* (1979). Hindlimbs from lean rats were more responsive than those from obese rats (consistent with the observations of

Kemmer *et al.*, 1979 and Sherman *et al.*, 1988) and the trend became significant at $\geq$ 10 nM insulin. The half maximal effect of insulin for both lean and obese hindlimbs was 40 nM insulin but the obese hindlimb showed only approx. 50% of the glucose uptake capacity of the lean hindlimb (Fig. 6.7) at all insulin concentrations.

6.3.2.2 Thiazolidinedione (BRL 49653) treatment of obese Zucker rats
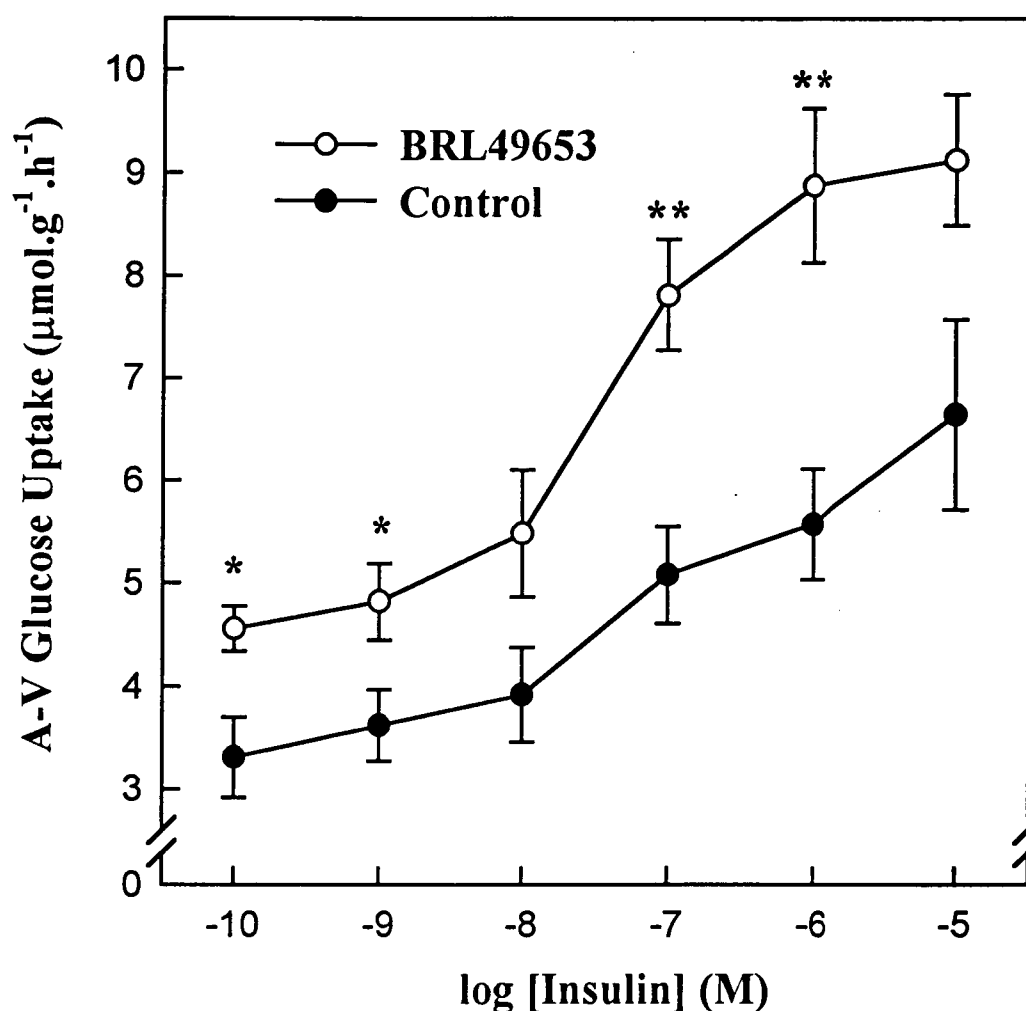
Fig. 6.8. Insulin dose-response curves for A-V glucose uptake by perfused hindlimbs from 20-week old obese Zuckers that were treated with BRL 49653 (3 μmol·kg^{-1}, *p.o.*) or vehicle for 7 days. Values are means $\pm$ SE for five animals in each group, and are expressed in terms of perfused mass of muscle + fat. *P < 0.05, **P < 0.01, significantly different from vehicle treated.

Fig. 6.8 shows the effect of 7 days of treating 20-week old obese animals with BRL 49653 on insulin-mediated A-V glucose uptake by the perfused hindlimbs. Vehicle-treated age-matched *fa/fa* rats had a lower basal (pre-insulin) glucose uptake rate, as well as a smaller insulin response (Fig. 6.8) than hindlimbs from the older obese animals (Fig. 6.7). However, treatment with BRL 49653 significantly increased the basal rate of glucose uptake and, at maximal doses of insulin (≥ 1 µM), there was a 50% increase in glucose uptake. The sensitivity to insulin remained unaltered by BRL 49653 treatment with the half maximal concentration remaining at 40 nM.

6.3.3 Thiazolidinedione (BRL 49653) treatment of hooded Wistar rats

In a further set of experiments, hooded Wistar (non-obese) rats were treated according to an identical protocol with BRL 49653 or vehicle. Hindlimbs were perfused in order to investigate the effect of treatment on glucose uptake in the presence and absence of insulin, and the effect on thermogenic responses to 5-HT vasoconstriction.

6.3.3.1 Glucose uptake in perfused hooded Wistar hindlimb preparations

Fig. 6.9 shows the basal (no insulin) and the insulin-stimulated (15 nM) A-V glucose uptake of hooded Wistar hindlimb preparations following treatment with BRL 49653 or vehicle. 15 nM insulin was chosen to give a maximal effect on glucose uptake (data not shown; Chiasson *et al.*, 1981). Treatment with BRL 49653 had no significant effect on either basal or insulin-stimulated A-V glucose uptake in this non-obese model (Fig. 6.9).
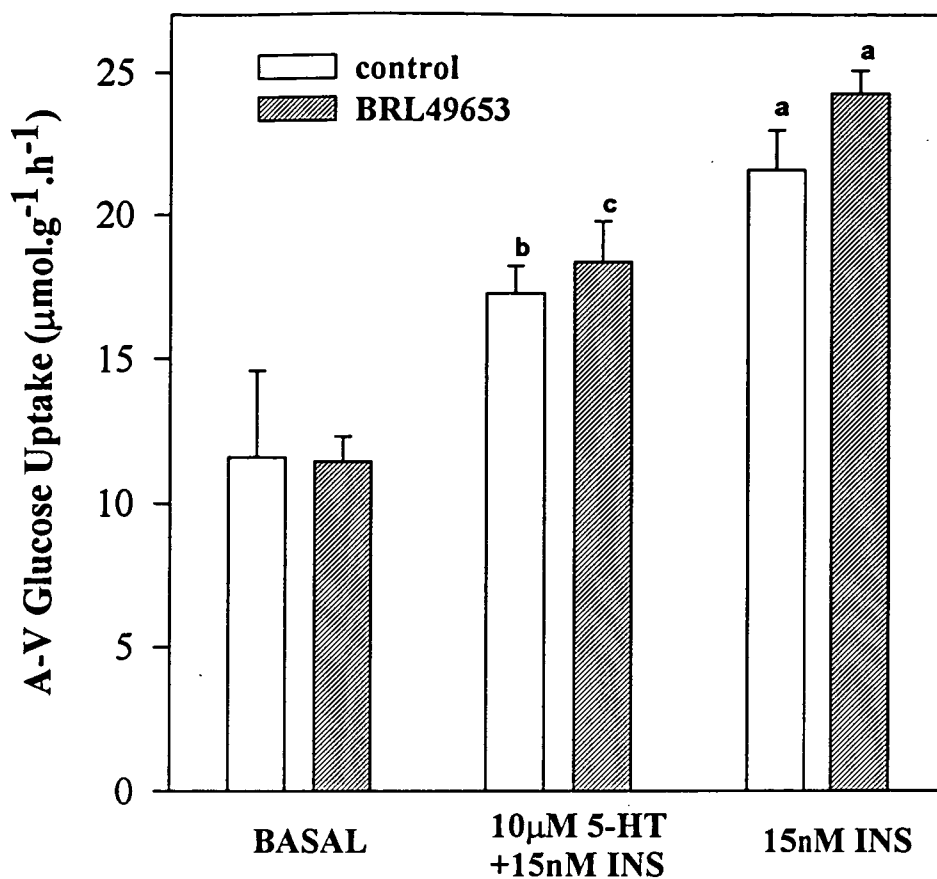
Fig. 6.9. A-V glucose uptake at 32°C in perfused hindlimbs of control (*n* = 4) and BRL 49653-treated (*n* = 4) hooded Wistar rats under basal, maximal insulin (15 nM), and acute insulin resistant conditions (10 μM 5-HT + 15 nM insulin, Rattigan *et al.*, 1993). Significant differences (all P < 0.05): [a]from control basal; [b]from control 15 nM insulin; [c]from BRL 49653 15 nM insulin.

The insulin-mediated (15 nM) 2DG uptake (R'g) into individual muscles of the lower leg and muscle groups of the thigh for the two treatment groups is given in Fig. 6.10. These data correspond to a period when the overall A-V glucose uptake was constant. Although there was a general trend for the BRL 49653 treatment group to have higher R'g values, none of these differences were found to be significant by one-way analysis of variance (ANOVA).
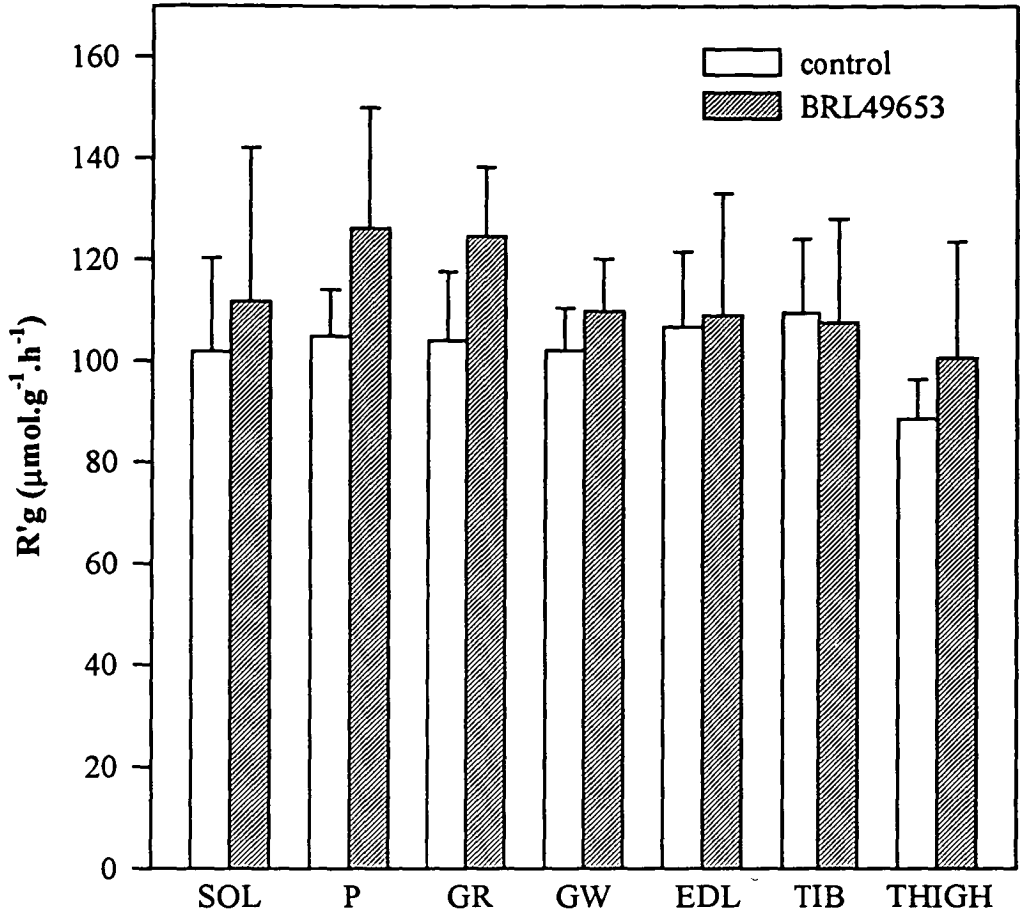
Fig. 6.10. Insulin-mediated (15 nM) 2DG uptake (R'g) in individual muscles and muscle groups of control (n = 4) and BRL 49653-treated (n = 4) hooded Wistar rats at 32°C. The muscles examined were the soleus (SOL), plantaris (PLAN), gastrocnemius red (GR), gastrocnemius white (GW), extensor digitorum longus (EDL), anterior tibialis (TIB), and the combined muscles of the thigh (THIGH). Values are means ± SE. One-way ANOVA testing indicated that there were no significant differences between control and BRL 49653 mean R'g values.

6.3.3.2 Serotonin effects in perfused hooded Wistar hindlimb preparations

Insulin-mediated glucose uptake was significantly inhibited by 10 µM 5-HT in both the control experiments (Fig. 6.9, in accordance with the findings of Rattigan *et al.*, 1993) and the experiments with BRL 49653 treated animals (Fig. 6.9). However, there was no significant difference between the two treatment groups in the magnitude of the 5-HT-mediated acute insulin resistance (Fig. 6.9).
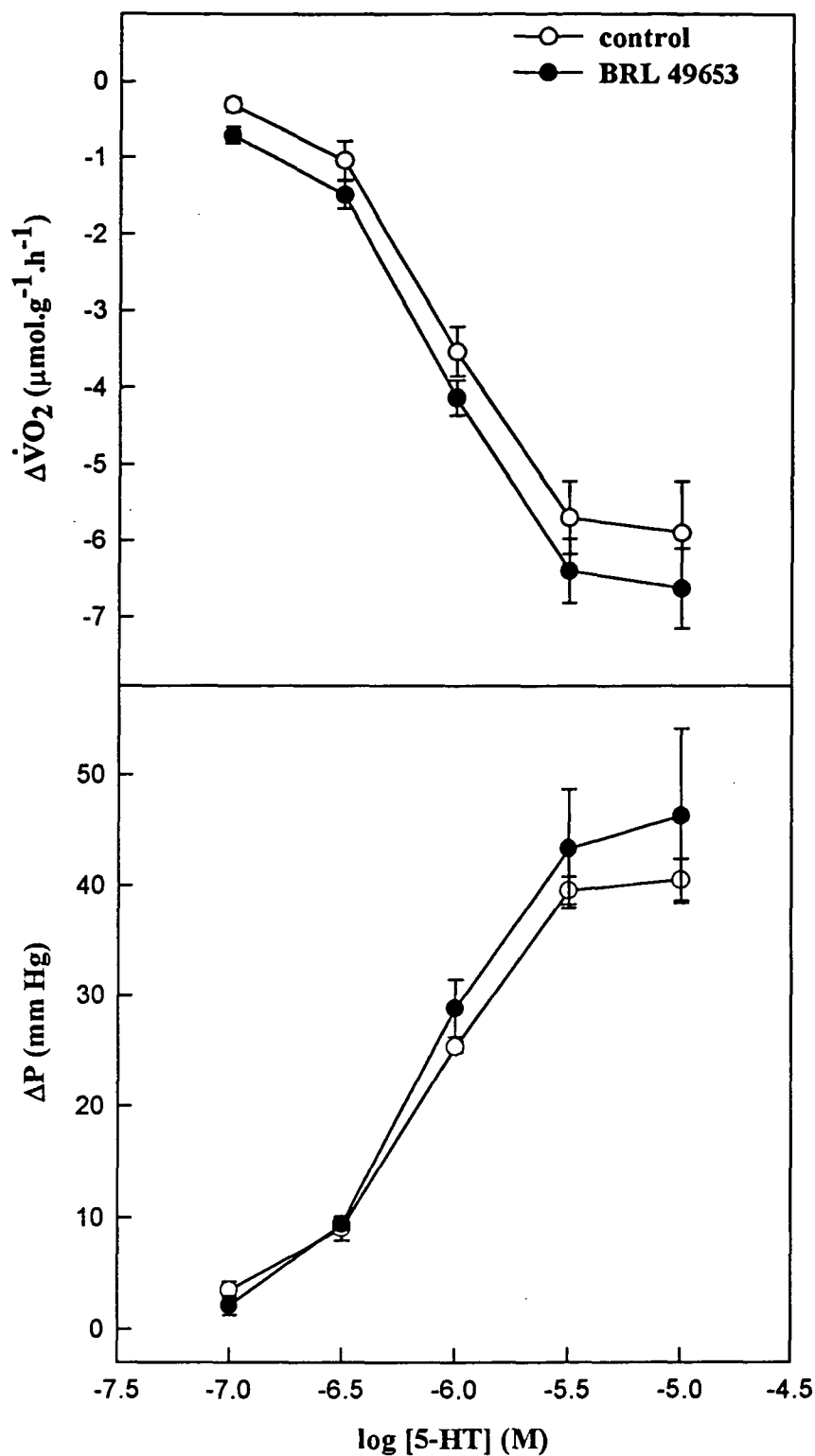
Fig. 6.11. 5-HT dose-response curves for $\Delta\dot{V}O_2$ and perfusion pressure changes (ΔP) in perfused hindlimbs of control ($n = 4$) and BRL 49653-treated ($n = 4$) hooded Wistar rats. Values are means $\pm$ SE. Where not visible, SE bars are within the symbol.

5-HT dose-response curves for $\dot{V}O_2$ and perfusion pressure in the two treatment groups are given in Fig. 6.11. Despite the responses being consistently larger in the BRL 49653 treatment group, no pairs of data points were significantly different (repeated-measures ANOVA).

6.4 Discussion

6.4.1 Vasoconstrictor regulation of thermogenesis
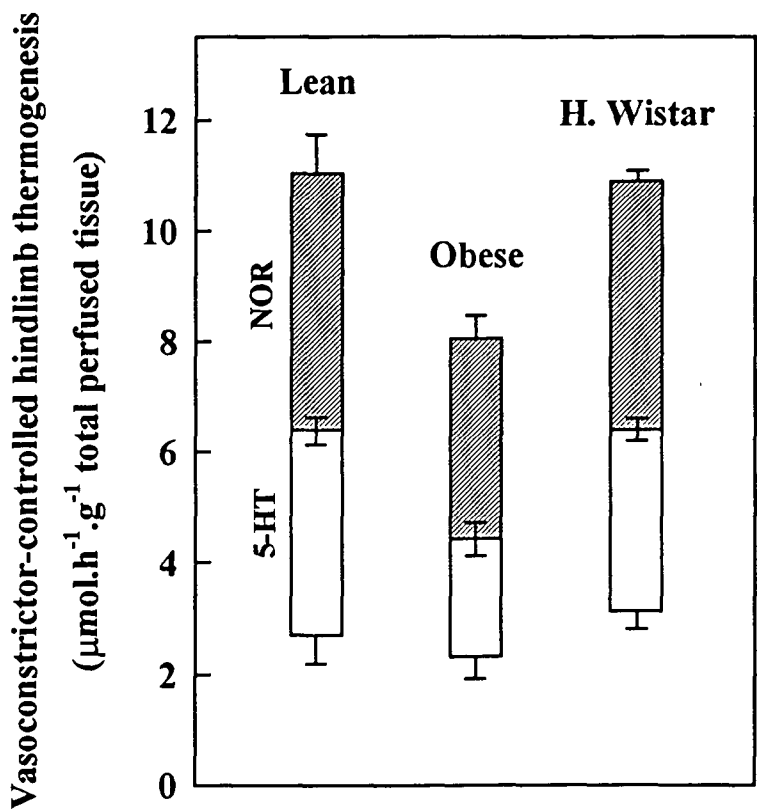
Fig. 6.12. Vasoconstrictor-controlled thermogenesis by the perfused rat hindlimb. Data are shown for lean ($n = 3$) and obese ($n = 3$) Zucker rats (Table 6.2, Figs. 6.3 and 6.6), as well as for 6-8 week old non-obese hooded (H.) Wistar rats ($n = 5$, Dora *et al.,*. 1991, 1992a). The hatched bars indicate the maximum increase in $\dot{V}O_2$ due to noradrenaline and the open bars indicate the maximum decrease in $\dot{V}O_2$ due to serotonin; the basal $\dot{V}O_2$ is indicated by the boundary. Values are means ± SE. Rates have been calculated in terms of total tissue perfused and includes muscle and fat but not skin or bone.

Two findings emerge from the present study that have implications for whole body thermogenesis of the obese Zucker rat. Firstly, the constant-flow perfused hindlimb of the obese animal - when compared to that of the lean - has a lower basal $\dot{V}O_2$ and lower maximal $\dot{V}O_2$ mediated by noradrenaline (Fig. 6.12). These differences appear to result directly from the lower content of muscle mass in the obese hindlimb and do not reflect intrinsic differences between muscle from lean and obese phenotypes. However the findings imply that for lean and obese Zuckers of equal body mass, the basal and fully stimulated thermogenic potential of muscle is less in the obese phenotype, and in proportion to the mass of muscle present. Secondly, the constant-flow perfused hindlimb of the obese animal has a diminished inhibitory response to high concentrations of noradrenaline and to serotonin in terms of $\dot{V}O_2$ and this effect appears to be intrinsic to the muscle. Taken together, the similar response to low concentrations of noradrenaline and decreased response to high dose noradrenaline and to serotonin by obese muscle when compared to that of the lean, suggests that vasoconstrictor-regulated thermogenesis in the obese Zucker is altered.

Data from Figs. 6.3 and 6.6 as well as our previous studies using various vasoconstrictors and vasodilators (reviewed by Clark *et al.*, 1995) can be used to illustrate the magnitude and significance of the altered thermogenesis of the obese hindlimb (Fig. 6.12). Thus values from Fig. 6.3 reflect the maximum thermogenic capacity of the hindlimb that can be activated by noradrenaline or other type A vasoconstrictors that increase $\dot{V}O_2$ by the rat hindlimb (Clark *et al.*, 1994). Fig. 6.12 shows that whilst the values for the lean hindlimb and that of the non-obese hooded Wistar strain are in close agreement, the value for the obese hindlimb is markedly lower. Given that the perfusion conditions were similar for the lean and obese hindlimbs, this suggests that the latter would have markedly less capacity to respond to vascular thermogenic stimuli, either in response to cold, or to over-eating. A difference in $\dot{V}O_2$ of 3-4 $\mu mol \cdot h^{-1} \cdot g^{-1}$ between the obese hindlimb and those of lean animals, would correspond to a reduction of 0.95-1.27 $W \cdot kg^{-1}$ hindlimb at 37°C assuming a standard average energy value of 4.83 $kcal \cdot L^{-1}$ O_2 at STP (1 kcal = 4.1855 kJ) (Brown and Brengelmann, 1965) and a Q_{10} of 2.5 (Paul, 1980). Previous estimates from this laboratory (Ye *et al.*, 1990b) suggest that hindlimb (which is largely muscle in non-obese strains) has the potential to contribute up to 0.28 W in a

200 g, warm-acclimated, non-obese rat. The absence of 0.95-1.27 $W \cdot kg^{-1}$ hindlimb would thus represent an absence of 0.09 W or 34% of the potential thermogenic capacity of this hindlimb tissue.

Since serotonin produced a dose-dependent inhibition of $\dot{V}O_2$ that reached a plateau, data from Fig. 6.6 can be used to define the apparent lower limit of vasoconstrictor controlled thermogenesis. Fig. 6.12 shows that the values for obese and lean Zucker hindlimbs do not differ significantly, nor do they differ from the value for the non-obese hooded Wistar strain.

Fig. 6.12 also shows that in the obese phenotype the basal $\dot{V}O_2$ (without noradrenaline or serotonin) is significantly lower (P < 0.05) than in either the lean or the non-obese strain. This finding suggests that under similar perfusion conditions the basal thermogenic output by the obese hindlimb is diminished. If this occurred *in vivo* it might reflect a decreased thermogenic need in response to the increased insulating capacity of the hindlimb fat. Alternatively the diminished thermogenesis under basal conditions might be contributory to the development of obesity by altering the energy balance.

It is important to note that the diminished response to noradrenaline of the obese hindlimb is not apparent when striated muscle is assumed to be the sole tissue of the hindlimb responsible for O_2 consumption. Thus expression of the data for obese and lean hindlimbs in terms of the mass of muscle perfused (Table 6.2) yields dose-response curves for noradrenaline (Fig. 6.3B) and basal (pre-noradrenaline) values for $\dot{V}O_2$ (Table 6.2) that are similar. Indeed such observations are consistent with those of other workers who found no difference in basal $\dot{V}O_2$ of perfused obese and lean hindlimbs when expressed on the basis of mass of muscle perfused (Kemmer *et al.*, 1979) or between $\dot{V}O_2$ of isolated incubated solei from obese and lean Zuckers (Crettaz *et al.*, 1980). Thus, taken together, our present findings and those of others (Kemmer *et al.*, 1979; Crettaz *et al.*, 1980) suggest that the change in tissue composition of the hindlimb associated with the obese phenotype (Table 6.2) plays a predominant role in the diminished response to both stimulatory as well as inhibitory effects of noradrenaline on $\dot{V}O_2$. However the fact that the diminished response to serotonin by the obese hindlimb was still evident when muscle was assumed to be the sole thermogenic tissue of the hindlimb implies that the obese phenotype is associated

also with an intrinsic defect in the hindlimb muscle or its vasculature. This defect may have additional implications for the thermogenic capacity of obese skeletal muscle. Thus the propensity to develop obesity (Zucker, 1975) and the poor response to cold exposure (Trayhurn *et al.*, 1976; Levin *et al.*, 1980; Kraul *et al.*, 1985) may derive from a lower contribution to thermogenesis from muscle as well as impaired brown adipose tissue (Levin *et al.*, 1984).

As pressor effects were similar for obese and lean hindlimbs (Fig. 6.5), an impaired response by the obese hindlimb to serotonin, in terms of inhibition of muscle $\dot{V}O_2$ (Fig. 6.6B), suggests that there is a lower proportion of nutritive vessels relative to functional shunts (discussed in Chapter 1). Thus an impaired response by the obese hindlimb to serotonin does not result from relatively fewer functional shunts, but rather is consistent with the presence of less capacity for nutritive O_2 delivery. Such a reduction in the availability of nutritive delivery may also diminish glucose and insulin access and contribute to the insulin resistance of obese Zucker hindlimbs (Fig. 6.7, discussed below). The obese phenotype is not associated with major changes in muscle specific enzyme activities including cytochrome oxidase (Wardlaw and Kaplan, 1984) nor is there a decrease in the proportion of oxidative fibres (Pujol *et al.*, 1993).

6.4.2 Insulin-mediated glucose uptake in perfused Zucker rat hindlimb

The obese Zucker hindlimb was significantly insulin resistant compared to lean Zuckers (Fig. 6.7). However, lean Zucker hindlimbs were in turn significantly insulin resistant relative to hooded Wistar hindlimb preparations (Fig. 6.9) under the same conditions (A-V glucose uptake of 14.1 ± 1.0 $\mu mol \cdot g^{-1} \cdot h^{-1}$ at 1 μM insulin in *fa/fa* Zuckers was significantly lower than 21.4 ± 1.9 $\mu mol \cdot g^{-1} \cdot h^{-1}$ at 15 nM insulin in hooded Wistars, $P < 0.01$).

Oral treatment with BRL 49653 at 3 $\mu mol \cdot kg^{-1} \cdot day^{-1}$ for 7 days was found to significantly increase insulin-mediated glucose uptake of perfused Zucker rat hindlimbs. The effect was manifest as an increased responsiveness to insulin without a change in EC_{50} (Fig. 6.8). It is also important to note that the basal (pre-insulin) was also enhanced following BRL 49653 treatment making it unlikely that the thiazolidinedione was simply enhancing the effect of insulin. It is possible that as in

adipose tissue of *ob/ob* mice, treatment with BRL 49653 increases the tissue content of glucose transporters (Young *et al.*, 1993). However, since these are the first studies with this agent in perfused hindlimb preparations, the mechanism of action and the target tissue(s) remain unresolved.

6.4.3 Hindlimb perfusions of BRL 49653-treated hooded Wistar rats

Identical chronic pre-treatment of a lean strain of rats (hooded Wistars) with BRL 49653 did not result in improved basal (pre-insulin) nor insulin-mediated perfused hindlimb A-V glucose uptake (Fig. 6.9) as noted in the obese Zucker rat. Individual muscles and muscle groups from treated rats showed no significant improvement in R'g values (Fig. 6.10). These results are consistent with the notion that BRL 49653 will only improve glycaemic control in animal models with established insulin resistance.

Given that experiments with 5-HT have identified a potential defect in obese Zucker vascular control (Fig. 6.6), there exists the possibility of the demonstrated insulin resistance being linked to such a defect, and that the chronic action of BRL 49653 is related to a reduction in its effect. 5-HT dose-response curves were determined in BRL 49653-treated hooded Wistar rats. However, as expected in a non-obese and non-insulin resistant strain, these curves were not significantly altered from the $\dot{V}O_2$ and perfusion pressure control curves (Fig. 6.11). Further exploration of any link between impaired vascular control and insulin resistance in perfused obese Zucker hindlimbs would commence with the determination of 5-HT dose-response curves in BRL 49653-treated obese Zucker rats and comparison to the curves in Fig. 6.6.

Acute 5-HT-mediated hindlimb insulin resistance was not significantly altered in treated hooded Wistar rats (Fig. 6.9). Such 5-HT-mediated insulin resistance is not apparent in unperfused incubated muscles, raising the possibility of a role for the vascular system in glycaemic control (Rattigan *et al.*, 1993; discussed in Chapter 1). If BRL 49653 were acting to restore impaired nutritive capacity in some manner, it would be unlikely to promote any improvement in glucose uptake or magnitude of responses to 5-HT in a perfusion model already possessing full nutritive capacity (Fig. 6.12). Using similar reasoning, 5-HT-induced acute insulin resistance which may be

the result of vascular shunting away from nutritive regions within muscle (Dora *et al.*, 1991, 1992a), would not be altered in magnitude by BRL 49653 in a hindlimb preparation with full nutritive capacity.

6.4.4 Evidence for thiazolidinedione vascular effects

The thiazolidinedione family of compounds (which includes BRL 49653) have been found to ameliorate hypertension, both in rodents (Buchanan *et. al.*, 1995; Kaufman *et al.*, 1995; Zhang *et al.*, 1994; Pershadsingh *et al.*, 1993) and in humans (Ogihara *et al.*, 1995). The basis of the antihypertensive activity is not fully understood. Many authors assume the effect to be related to the lowering of plasma insulin concentrations *in vivo* (Ogihara *et al.*, 1995). However, Zhang and colleagues (1994) maintain that the antihypertensive actions of thiazolidinedione agents are not specifically related to the amelioration of insulin-mediated glucose uptake. This conclusion was reached on the basis of experiments showing that pioglitazone reduced hypertension in the one-kidney, one clip rat, a hypertension model not associated with insulin resistance. Buchanan *et al.* (1995) have published evidence for a direct vascular effect of pioglitazone in rats to reduce blood pressure *in vivo*. It was argued that the extent of this reduction could not be explained by alterations in insulin concentrations, insulin sensitivity or free magnesium levels. In further experiments *in vitro*, Buchanan *et al.* (1995) demonstrated that pioglitazone reduced the vasoconstrictor-induced contractility of aortic rings, a direct effect due, at least in part, to inhibition of agonist-mediated calcium uptake by vascular smooth muscle. Similarly, Pershadsingh *et al.* (1993) have noted that ciglitazone may be modifying the cell calcium response to pressor agents.

It is therefore possible that the actions of chronic BRL 49653 reported in this chapter are related to modified vascular function. It is tempting to speculate that part of the improved glycaemic control in treated rats might be the result of restored nutritive/non-nutritive flow distribution.

Chapter 7

Final Discussion and Conclusions

7.1 Summary of Major Findings

7.1.1 Mechanisms of vanilloid activity

A series of isolated naturally-occurring vanilloid compounds were found to be capable of modulating perfused rat hindlimb $\dot{V}O_2$ and lactate efflux in close association with increases in perfusion pressure. These effects were similar to those previously reported for capsaicinoid agents (Cameron-Smith *et al.*, 1990). Although the effective concentration ranges of these compounds varied markedly, it became apparent that the vanilloid compounds could be classified as a unique group of non-adrenergic, non-endogenous vasomodulators capable of modulating perfused rat hindlimb metabolism.

The relative potencies of the naturally-occurring vanilloids in the hindlimb model were consistent with those reported in neuropharmacological studies where receptor interactions have been firmly established. Furthermore, the rapid kinetics of vanilloid effects in the hindlimb model were indicative of receptor-mediated activity. The failure of vanilloids to induce any responses in similar isolated perfusion preparations of chicken muscle suggested that direct non-specific cellular effects were unlikely. The ability of vanilloids to either stimulate or inhibit $\dot{V}O_2$ raised the possibility that two vanilloid receptor subtypes (VN_1/VN_2) may be involved. Experiments with PPAHV, a new vanilloid found to abolish positive binding cooperativity in neural preparations (Szallasi *et al.*, 1996), gave preliminary indications that such cooperative binding is not the cause of the biphasic nature of the $\dot{V}O_2$ dose-response curves. The generation of bell-shaped $\dot{V}O_2$ dose-response curves was, however, consistent with the notion of overlapping receptors with opposite actions, as reviewed by Szabadi (1977) and also Rovati and Nicosia (1994), acting in accordance with their staggered affinities to firstly stimulate and then inhibit $\dot{V}O_2$. Subsequent experiments under various states of metabolic challenge produced further evidence supporting the notion of vanilloid receptor duality in the rat hindlimb model.

Removal of external Ca^{2+} eliminated the stimulation of increased $\dot{V}O_2$ and lactate production, responses attributed to the putative VN_1 receptor. The inhibition of $\dot{V}O_2$ and lactate production, responses hypothesised to be mediated by the putative VN_2 receptor, were largely unaffected by the absence of external Ca^{2+}. The infusion of cyanide, azide, or the application of hypoxia all suggested that the putative VN_2 (inhibitory) receptor was not functionally dependent on oxygen.

The notion of vanilloid receptor heterogeneity has also emerged in studies undertaken elsewhere involving neural tissue. Indeed, studies examining the binding of [^{3}H]RTX (reviewed by Szallasi, 1994), vanilloid cooperative binding and functional potency assays (Acs *et al.*, 1995b), and differing capsaicin-induced currents in rat trigeminal cells (Liu and Simon, 1994) have all yielded evidence supporting some form of functional heterogeneity.

7.1.2 Vanilloid structure-activity relationships

The present study is the first to examine the structural requirements for vanilloid molecules acting to modulate muscle $\dot{V}O_2$ *in vitro*. Given that bell-shaped $\dot{V}O_2$ dose curves were a characteristic of the natural vanilloid agents, it was hypothesised that structural modifications might enable selectivity for putative VN_1 receptors, thus resulting in agents with potential *in vivo* thermogenic activity. Although systematic structural modifications did not achieve clear separation of the putative VN_1 and VN_2 effects on $\dot{V}O_2$, the maximal increases in $\dot{V}O_2$ were variable, suggesting some degree of selectivity. One synthetic vanilloid compound, selected on the basis of its strong $\dot{V}O_2$-stimulatory activity *in vitro*, was found to be thermogenic in preliminary studies using anaesthetised whole rats.

The structure-activity profile developed for the synthetic vanilloid molecules in the perfused rat hindlimb system was relatively similar to that which emerged from neuropharmacological studies (Walpole *et al.*, 1993a-c; Chen *et al.*, 1992; Park *et al.*, 1991). It must be concluded, therefore, that the putative VN_1 receptor is similar to those described on sensory neurones and in the CNS. Alternatively, the effects observed in the perfused rat hindlimb model may be mediated by neurokinins released by neurones associated with the vascular bed. Possible neurokinin interactions are currently under investigation in this laboratory.

7.1.3 Further evidence for dual vanilloid receptors

Very recent data emerging from this laboratory have provided additional support for the involvement of dual vanilloid receptors in the responses observed in the perfused rat hindlimb system (Griffiths *et al.*, 1996). Capsazepine, a known competitive vanilloid antagonist, has been shown to competitively inhibit capsaicin-induced $\dot{V}O_2$ and perfusion pressure changes in the perfused rat hindlimb model. Capsazepine has been previously shown to competitively inhibit the actions of capsaicin in a number of experimental systems (Urban and Dray, 1991; Dickenson and Dray, 1991; Bevan *et al.*, 1992; Maggi *et al.*, 1993). In the perfused hindlimb model (Griffiths *et al.*, 1996), capsazepine was apparently specific for vanilloid actions; capsazepine infusion had no effect on the actions of the non-vanilloid agonists. A more detailed analysis of the inhibitory actions of capsazepine in the perfused hindlimb model (Griffiths *et al.*, 1996) revealed that low concentrations of capsazepine selectively inhibited the increased $\dot{V}O_2$ attributed to the putative VN_1 receptor.

The same study (Griffiths *et al.*, 1996) found ruthenium red to be a specific but non-competitive inhibitor of the capsaicin-induced hindlimb responses. This result was similar to those described by others using isolated rat *vas deferens* and urinary bladder preparations (Maggi *et al.*, 1993). Again, ruthenium red was not effective in blocking the actions of non-vanilloid agonists in the hindlimb. At low concentrations, ruthenium red selectively inhibited the high dose capsaicin effects attributed to the putative VN_2 receptor.

The final section of this recent study (Griffiths *et al.*, 1996) involved prior and co-infusion of the neurotoxin tetrodotoxin with a view to disrupting any possible influences exerted by sensory neurones in response to capsaicin. Previous studies have suggested that the responses of capsaicin-sensitive sensory neurones are linked to dual cellular mechanisms (Lou *et al.*, 1992). The evidence for these mechanisms, one proposed to be tetrodotoxin-sensitive (low capsaicin concentrations), and their role in the release of sensory transmitters has been summarised by Maggi (1993). However, in the perfused rat hindlimb the capsaicin-mediated changes were tetrodotoxin-resistant (Griffiths *et al.*, 1996). This observation raised the possibility that the cellular mechanisms induced by capsaicin at low concentrations may differ from those of other

tissues, given that low concentrations of capsaicin result in tetrodotoxin-sensitive bronchoconstriction in the perfused guinea-pig lung (Lou *et al.*, 1992).

7.1.4 Comparative perfusion studies - perfused chicken muscle

The present study included the first report of vasoconstrictor-induced thermogenesis and dose-dependent dual effects of catecholamines in avian skeletal muscle. The perfusion preparation developed for the study was demonstrated to be viable primarily by the comparison of muscle high energy phosphate metabolite concentrations with those of *in vivo* muscle samples. In particular, the measurement of creatine phosphate concentrations proved to be a sensitive index of muscle oxygen supply.

The vasoconstrictor-induced changes in $\dot{V}O_2$ occurred in perfused chicken muscle beds with surgically divided somatic and sensory nerve connections, eliminating the possibility of motor neurone involvement. Similarly, the crushing of sympathetic nerve fibres associated with the popliteal artery effectively ruled out any sympathetic mediation. Furthermore, responses induced in the presence of tubocurarine illustrate that the thermogenic actions were independent of any form of skeletal muscle contraction and that they represented true nonshivering thermogenesis *in vitro*.

As observed in perfused rat hindlimb preparations, changes in $\dot{V}O_2$ were always associated with vasoconstriction; nitrovasodilation blocked both effects. The catecholamine-mediated increases in $\dot{V}O_2$ were blocked by α_1-adrenergic antagonism, but the role of β-adrenergic receptors in the $\dot{V}O_2$ changes was apparently minimal. The ability of serotonin to both stimulate and inhibit $\dot{V}O_2$ in the perfused chicken muscle preparation was different to the responses seen in rat hindlimb preparations, where 5-HT inhibits $\dot{V}O_2$ at all effective concentrations (Dora *et al.*, 1991, 1992a). This result was indicative of differing vasoconstrictor-mediated patterns of vascular control in chicken muscle, possibly reflecting variations in the distribution of 5-HT receptors. However, the observed $\dot{V}O_2$ responses to vasoconstrictors in the chicken preparation suggested that these agents were altering the balance of flow distribution between nutritive and non-nutritive vascular networks in a comparable fashion to that postulated in perfused rat skeletal muscle (discussed in Chapter 1).

The characteristic bell-shaped $\dot{V}O_2$ dose curves produced by catecholamine infusion may provide an explanation for the conflicting data of others concerning the thermogenic effects of exogenous noradrenaline in birds. The finding that glucagon acted to increase the stimulatory concentration range of noradrenaline in perfused chicken muscle may have implications for reports of calorigenic glucagon activity *in vivo*. The data obtained *in vitro* in the present study not only suggests that birds have the potential to enact muscular nonshivering thermogenesis *in vivo*, but raises the possibility that such NST may be due to the combined actions of catecholamines and glucagon.

7.1.5 Comparative muscle perfusion studies - perfused Zucker rat hindlimbs

Perfused obese Zucker rat hindlimbs served as a comparative model for investigating potential relationships between altered haemodynamic function and the expression of obesity and insulin resistance. Thus the initial aim was to define the upper and lower limits of $\dot{V}O_2$ in hindlimbs of obese animals using type A and type B vasoconstrictors, enabling the resultant hindlimb window of vasoconstrictor-controlled thermogenesis (Chapter 6, Fig. 6.12) to be compared to that in lean rat models: the lean Zucker littermates and the hooded Wistars.

Several of the differences found in the obese Zucker vasoconstrictor-controlled $\dot{V}O_2$ window were the direct result of a lower proportion of muscle relative to fat in the total amount of tissue perfused. Nevertheless, it was apparent that these tissue proportion differences resulted in reduced obese Zucker thermogenic potential per hindlimb. On the other hand, a diminished obese Zucker type B response was evident regardless of the calculation method. Thus the diminished type B effect was apparently due to an intrinsic defect in the obese Zucker skeletal muscle. This was interpreted in terms of a lower nutritive capacity; a lower proportion of nutritive vasculature resulting in impaired muscle metabolic responses. It was postulated that the development of obesity in the Zucker rat may derive in part from a lower muscle contribution to overall thermogenesis as well as defective brown adipose tissue (Levin *et al.*, 1984).

It is interesting to note, however, that the magnitude of the type A response of obese Zucker hindlimbs was essentially no different to those of either of the lean

hindlimb models. If the magnitude of the type A response is assumed to reflect the capacity for nutritive vascular recruitment (as discussed in Chapter 1) then the argument that a diminished type B response is indicative of emaciated nutritive capacity inevitably leads to a conundrum. An alternative explanation requires a consideration of the distribution of flow between putative nutritive and non-nutritive vascular networks under basal conditions. If one accepts the arguments supporting the presence of dual circulatory systems within skeletal muscle (Barlow *et al.*, 1961; Grant and Payling Wright, 1970; Lindbom and Arfors, 1984; Saltzman *et al.*, 1992; Newman *et al.*, 1996), it follows that a reduced capacity for lowering basal metabolism (type B response) may be the result of an altered balance of nutritive/non-nutritive flow under basal conditions. Thus it might be hypothesised that the obese Zucker rat may not have rarefied nutritive vascular networks, but rather has an increased component of non-nutritive flow under basal conditions. Should this be the case, it would be expected that basal $\dot{V}O_2$ of muscle alone would be lower in obese animals. This interpretation is not supported by the data. Thus whilst the data lend substantial support to the proposal that differences may exist in the vascular control of metabolism in perfused obese Zucker muscle, a satisfactory explanation based on the redistribution of flow between nutritive and non-nutritive vascular networks (as discussed in Chapter 1) remains elusive.

Experiments examining perfused Zucker hindlimb glucose uptake confirmed previous reports (Kemmer *et al.*, 1979; Sherman *et al.*, 1988) that obese hindlimbs were insulin resistant relative to lean controls. Chronic treatment of obese Zucker rats with the thiazolidinedione insulin sensitising agent BRL 49653 resulted in a significant increase in both basal and insulin-mediated glucose uptake of obese Zucker hindlimbs. However, similar chronic BRL 49653 treatment of hooded Wistar rats did not result in improved arteriovenous glucose uptake, nor was the uptake of 2-deoxyglucose into individual muscles increased. These results imply that thiazolidinedione treatment was effective only in models of established insulin resistance; glycaemic control was not altered in an experimental model which, based on arguments presented in Chapter 1, can be predicted to possess near-maximal nutritive flow capacity.

It is tempting to postulate that the observed insulin resistance of the perfused obese Zucker hindlimb is directly related to the haemodynamic defect identified using

vasoconstrictors. If such a link exists, chronic actions of BRL 49653 to ameliorate the impaired state of glycaemic control may, following further experimentation, be interpreted as further evidence supporting the vascular actions already attributed to this class of agents (Buchanan *et al.*, 1995; Kaufman *et al.*, 1995; Zhang *et al.*, 1994; Pershadsingh *et al.*, 1993; Ogihara *et al.*, 1995).

7.2 Relationship Between In Vitro Oxygen Consumption and Regulatory NST In Vivo

Regulatory nonshivering thermogenesis is a common adaptive response to cold found in a number of mammalian species (Chaffee and Roberts, 1971) as well as several species of birds, including chickens, ducklings, and penguins (Barré *et al.*, 1986; Duchamp *et al.*, 1989; El Halawani *et al.*, 1970). A potentially effective method of demonstrating an association between changes in $\dot{V}O_2$ observed *in vitro* and regulatory NST in intact animals is to establish that the *in vitro* effect is in fact potentiated in animals with laboratory-induced NST. A collaborative study involving this laboratory (F. Marmonier, C. Duchamp, F. Cohen-Adad, T.P.D. Eldershaw, and H. Barré, submitted) has investigated $\dot{V}O_2$ responses induced by noradrenaline in perfused muscle preparations (using a method based on that described in Chapter 5) of cold-acclimated and glucagon treated ducklings, models previously reported to exhibit muscle NST *in vivo* (Barré *et al.*, 1986, 1987b; Duchamp and Barré, 1993; Duchamp *et al.*, 1993b). These experiments were able to demonstrate that ducklings exhibiting *in vivo* NST, as a result of either cold-acclimation or chronic glucagon treatment, did in fact show significantly higher $\dot{V}O_2$ responses to noradrenaline when perfused *in vitro* relative to control thermoneutral ducklings. A similar relationship was found by Shiota and Masumi (1988) using thermoneutral and cold-acclimated rats. Grubb and Folk (1976) reported that sustained $\dot{V}O_2$ responses were markedly enhanced in perfused muscle of cold-acclimated rats, although initial responses were unchanged. These results taken together, particularly the relationship found in ducklings in view of the absence of brown adipose tissue (Barré *et al.*, 1986; Saarela

et al., 1989, 1991), suggest that a thermogenic effect at the perfused muscle level is indeed indicative of the potential for skeletal muscle NST *in vivo*.

The proposed relationship between vasoconstrictor-induced perfused muscle $\dot{V}O_2$ increases and regulatory NST *in vivo* is further underlined by descriptions of marked noradrenaline-mediated α-adrenergic thermogenic effects both *in vivo* (J.-M. Ye, PhD thesis, University of Tasmania, 1995) and in constant-flow perfused muscle (Ye *et al.*, 1995) of Tasmanian bettongs. As established in× birds, marsupials such as the bettong have no detectable BAT (Haywood and Lisson, 1992), hence skeletal muscle emerges as the most likely effector tissue for the observed regulatory NST.

It may be argued that muscle perfusion using constant pressure (rather than constant flow) experimental regimes more closely approximate physiological conditions. Despite additional technical difficulties, studies in this laboratory (Ye *et al.*, 1995) have attempted to demonstrate vasoconstrictor-induced $\dot{V}O_2$ under constant pressure perfusion conditions. The results show that noradrenaline does indeed stimulate increased $\dot{V}O_2$ via an α_1-adrenergic mechanism in rat hindlimb perfusion preparations at a constant pressure of 80 mm Hg. Although true physiological conditions are neither constant pressure nor constant flow (as muscle autoregulates flow), the fact that vasoconstrictor-mediated $\dot{V}O_2$ - a phenomenon examined at length in the present study - is observed under both sets of experimental conditions is supportive of the physiological relevance of data obtained using constant-flow hindlimb perfusion techniques.

7.3 Vascular Control of Muscle Thermogenesis : A General Biological Mechanism?

A number of species including the rat (Chapter 1), and also the chicken (Chapter 5), bettong (Ye *et al.*, 1995) and toad (J.-M.Ye, PhD thesis, University of Tasmania, 1995) have been used to demonstrate increased perfused skeletal muscle $\dot{V}O_2$ in response to infused noradrenaline (Fig. 7.1). The basal and maximal NOR-stimulated $\dot{V}O_2$ values for these species are given in Table 7.1.
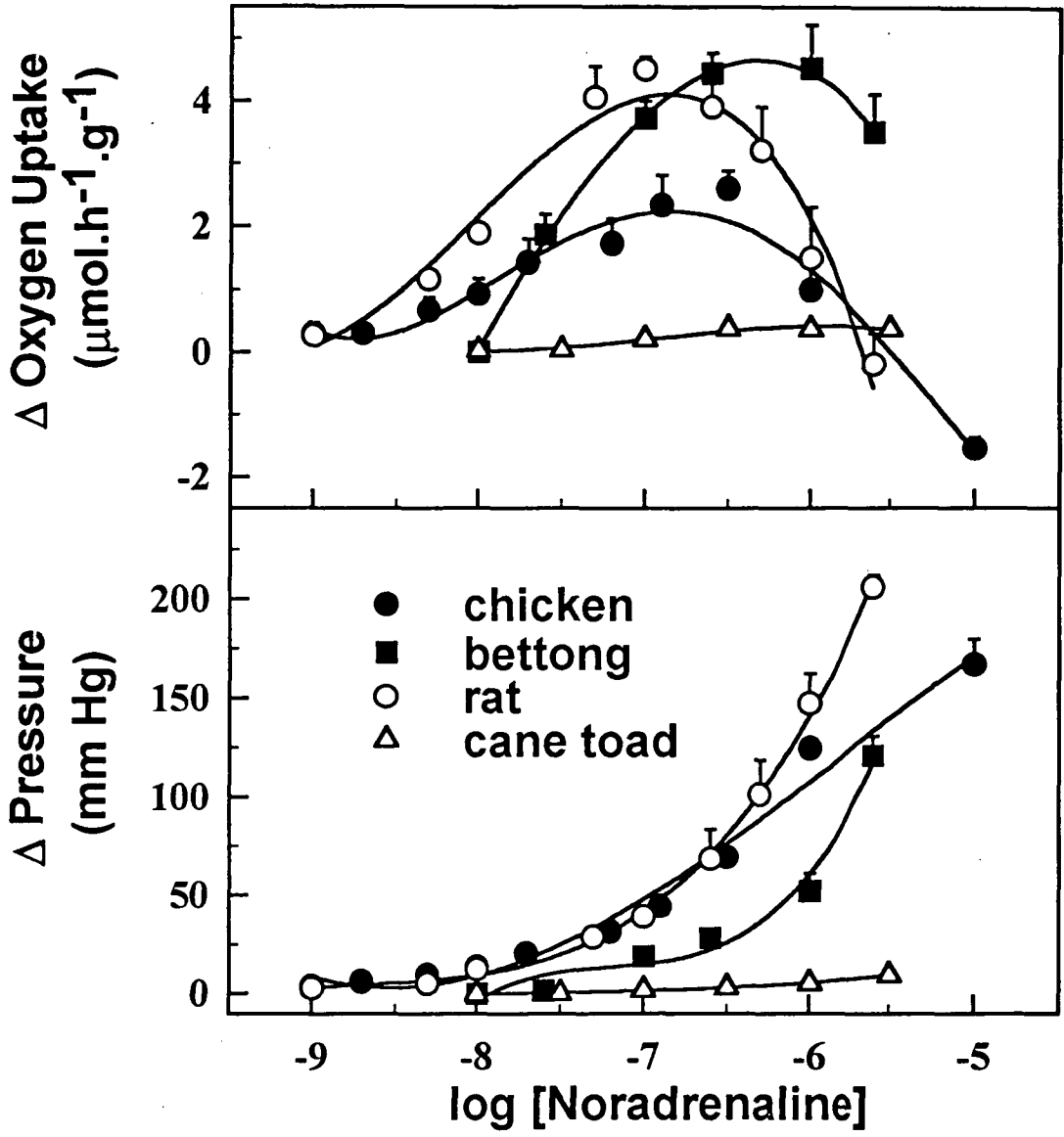
Fig. 7.1. $\Delta\dot{V}O_2$ (A) and Δ perfusion pressure (B) concentration-response curves for NOR in perfused chicken (n = 4-7), bettong (n = 5), rat (n = 5), and toad (n = 3) muscle preparations. All data points are means ± SE. Toad data points are significantly different (P < 0.05) to basal values at all NOR concentrations greater than 10^{-7}. Data sources are the same as listed in Table 7.1. Redrawn from Eldershaw *et al.* (1996).

Table 7.1. Basal and maximal NOR-stimulated V̇O₂ values for perfused muscle preparations of the chicken, rat, Tasmanian bettong, and cane toad at 25°C.

species (n)	basal V̇O₂ ($\mu mol \cdot g^{-1} \cdot h^{-1}$)	max. NOR-stimulated V̇O₂ ($\mu mol \cdot g^{-1} \cdot h^{-1}$)	percentage V̇O₂ increase	Ref.
Chicken (4-7)	7.4	9.9	35	Chapter 5, Fig. 5.3
Rat (5)	6.1	10.1	57	Dora *et al.*, 1992a
Bettong (5)	4.5	8.5	110	Ye *et al.*, 1995
Toad (3)	1.36	1.74	28	J.-M. Ye*

* Values from J.-M. Ye, PhD thesis, University of Tasmania, 1995.

The species featuring in Table 7.1 and Fig. 7.1 are representative of a range of vertebrate taxa, giving rise to the hypothesis that vascular control of resting muscle thermogenesis is an underlying nonshivering thermogenic mechanism, common to the skeletal muscle of all vertebrate species. The relatively minor V̇O₂ change observed in the toad at physiological temperature and perfusion pressure (J.-M. Ye, *opp. cit.*) is consistent with the relative inability of ectotherms to respond to thermal challenge. By contrast, the greater effects in the endothermic species - at temperatures and perfusion pressures markedly lower than considered physiological - highlight the potential contribution of skeletal muscle to NST in these species. The lack of a type B component in the toad NOR dose curve (using concentrations up to 3.2 µM) is perhaps indicative of a less complex system of vascular control. Nevertheless, the observation that the perfused amphibian preparation does possess the capacity to increase V̇O₂ is evidence that the vascular control mechanism may have existed over an extended evolutionary period (Fig. 7.2). Thus the mechanism may have been the primary means of regulatory NST prior to the evolution of brown adipose tissue in eutherian mammals. The enhanced ability, relative to the toad, of the avian (chicken) and metatherian mammal (bettong) perfusion preparations to respond to NOR engenders speculation that such augmented thermogenic capacity may have been implicated in the phylogenetic branching of the endotherms (mammals and birds) from ectothermic (reptilian and amphibian) ancestry (Fig. 7.2).
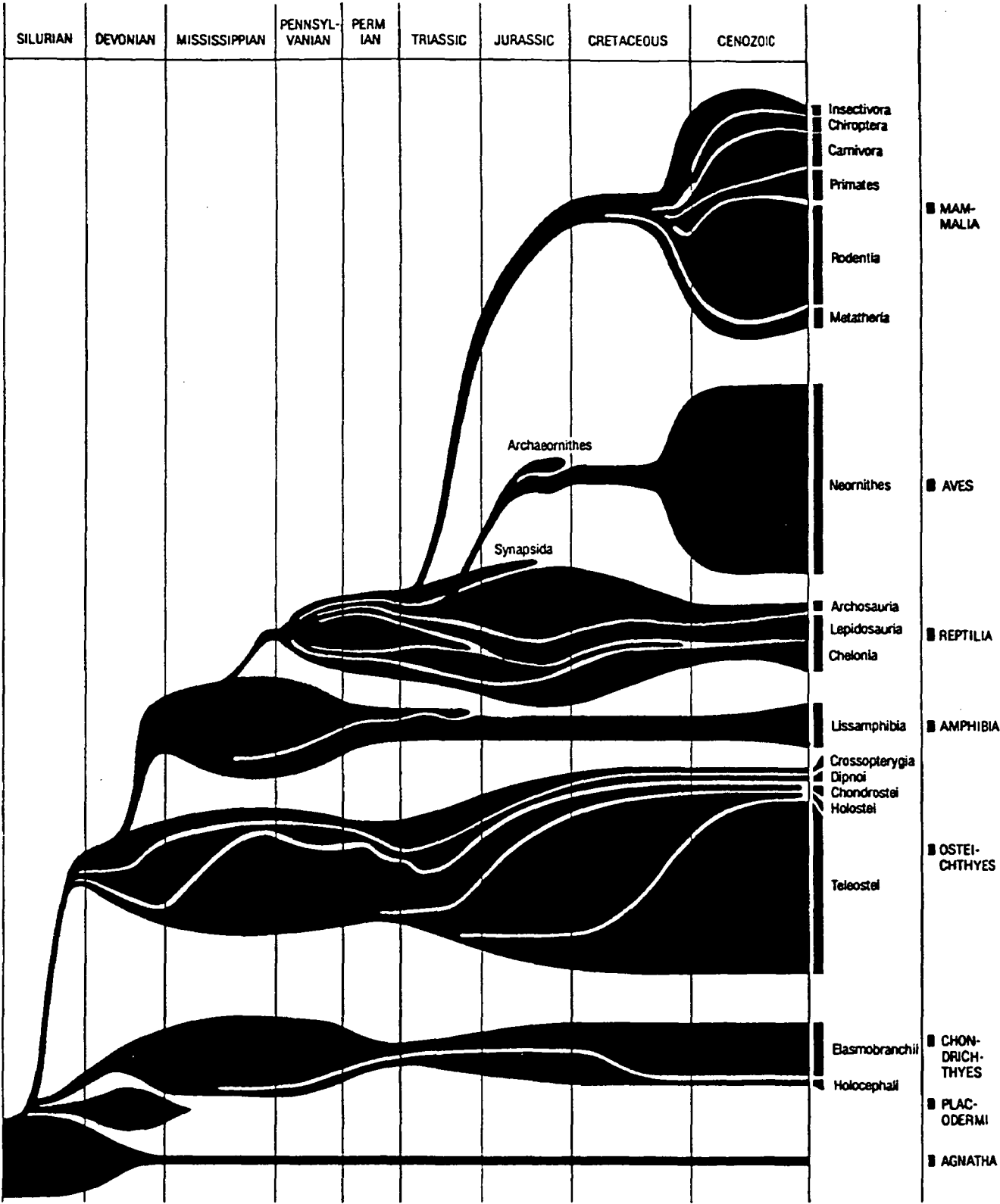
Fig. 7.2 Vertebrate phylogeny. An approximation of the evolutionary pathway taken by the vertebrates over geological time (redrawn from King and Custance, 1982). The identification of vascular control mechanisms in avian and amphibian representatives implies that the mechanism may have existed over an extended evolutionary period.

7.4 Defective Muscle Vascular Control: Pathogenic Implications

One of the stated aims of this study was to explore the possibility of an association between defective muscle vascular control and the phenotypic expression of obesity and insulin resistance in the obese Zucker rat. The use of vasoconstrictors in perfusion preparations implied that obese Zucker skeletal muscle was defective in terms of vascular control relative to lean counterparts. In terms of glucose homeostasis, skeletal muscle is regarded as being a major site of insulin-mediated glucose uptake (Baron *et al.*, 1994). Previous studies in this laboratory have demonstrated that vasoconstrictors are capable of markedly influencing IMGU in perfused, but not incubated, isolated muscle preparations (Rattigan *et al.*, 1993, 1995, 1996). Many authors now accept that insulin resistance *in vivo* may have a haemodynamic basis. In particular, the association of hypertension with poor glucose tolerance in humans (discussed in Chapter 1; Julius and Jamerson, 1994 and references therein) has been interpreted by some in terms of hypertension playing a causative role, although others argue that hyperinsulinaemia leads to hypertension (Anderson and Mark, 1993). Amongst those subscribing to the theory that hypertension is likely to precede insulin resistance, a popular hypothesis is that vascular changes associated with hypertension may ultimately lead to vascular rarefaction (Henrich *et al.*, 1988), resulting in defective distribution of insulin to target cells within skeletal muscle (Wiernsperger, 1994).

In the obese Zucker rat model, although manifestations of obesity and insulin-resistance are clearly recognisable, the existence of hypertension has been the subject of some debate. Zemel *et al.* (1992) have claimed that obese Zucker rats are hypertensive, and that the condition is independent of sympathetic input. This finding lends support to the proposal that obese Zucker rats may possess intrinsic alterations in skeletal muscle vascular architecture. The data for the basal perfusion pressures of lean and obese animals (Table 6.2) are supportive of increased peripheral resistance in obese animals; the basal perfusion pressure is 28% greater in obese hindlimbs when flow rates are normalised. The demonstration of an impaired vascular response in the present study was interpreted in terms of altered nutritive flow capacity, although there was some difficulty in rationalising all of the observed responses in terms of the

flow distribution models outlined in Chapter 1 (discussed in section 7.1.5). In view of the evidence supporting likely vascular actions of BRL 49653 (Chapter 6), it is conceivable that the ameliorated glucose uptake displayed by hindlimb preparations of BRL 49653-treated obese animals was related to haemodynamic changes.

7.5 Therapeutic Potential of Vanilloids

The ability of vanilloid molecules to modify oxygen uptake in the perfused rat hindlimb model (Chapters 2a, 2b, 3, 4) identifies these compounds as a new class of vasoactive agents apparently capable of altering haemodynamic control in perfused skeletal muscle. The experimental results support the presence of dual vanilloid receptors, one enhancing (VN_1), the other diminishing (VN_2) nutritive perfusate flow. Agents capable of putative VN_1 receptor selectivity may have potential as whole body thermogenic drugs. Preliminary investigations using a synthetic vanilloid agent with favourable *in vitro* activity (compound 6A, Chapter 4) for *in vivo* experiments support this proposal. No synthetic vanilloid agent (described in Chapter 4), was able to demonstrate exclusive selectivity for either positive or negative $\dot{V}O_2$ effects. Nevertheless, certain compounds displayed enhanced $\dot{V}O_2$ stimulatory activity, a result perhaps indicative of partial selectivity. More rigorous pharmacological analyses than the screening procedures applied in this study are required to satisfactorily assess this possibility. Recent studies in this laboratory (Griffiths *et al.*, 1996) have adopted a pharmacological approach in assessing the potential of capsazepine and ruthenium red as vanilloid antagonists in the perfused hindlimb preparation (see section 7.1.3). The apparent subtype selectivity achieved using these antagonists reinforces the possibility of achieving agonist selectivity for $\dot{V}O_2$ stimulation.

The primary mechanism of action of vasoconstrictors controlling perfused skeletal muscle metabolism is proposed to be a redistribution of perfusate flow within the perfusion preparation (discussed in Chapter 1). The agents used in experiments directly examining this proposal have been the endogenous hormones noradrenaline and serotonin (Newman *et al.*, 1996), due to their respective efficacies in inducing type A and type B responses. The actions of the vanilloids are presumed to occur as a result of similar flow redistribution, since the high and low dose alterations in perfused

hindlimb $\dot{V}O_2$, lactate efflux, perfusion pressure, and erythrocyte efflux (data not shown) are parallel to those induced by 5-HT and type A NOR respectively. It would be expected, therefore, that insulin-mediated glucose uptake would also be influenced by both high and low vanilloid concentrations, as has been observed for other vasoconstrictors (Rattigan *et al.*, 1993, 1995, 1996). Preliminary data from this laboratory (T.P.D. Eldershaw, E.Q. Colquhoun, and C.G. Griffiths) suggests that perfused rat hindlimb IMGU is acutely influenced by vanilloid infusion as predicted. The potential for type A vasoconstrictors to stimulate IMGU in humans has recently been confirmed by Jamerson *et al.* (1996). In these experiments, infused angiotensin II (type A vasoconstrictor) was observed to enhance insulin-mediated glucose uptake by human forearm. In accordance with the notion of vascular control of skeletal muscle metabolism, Jamerson *et al.* (1996) concluded that haemodynamic rather than direct angiotensin II effects were responsible for the improved glucose tolerance.

Pharmacokinetic studies indicate that capsaicinoids are able to freely cross the blood-brain barrier (Donnerer *et al.*, 1990). Consequently, vanilloid agents ultimately developed for therapeutic use in enhancing peripheral thermogenesis and glucose tolerance may need to be selective for peripheral rather than central receptors, as well as putative VN_1 receptors. Central vanilloid actions, such as the *in vivo* hypothermic response associated with capsaicin-mediated CNS stimulation (reviewed by Szolcsanyi, 1982), would be a highly undesirable property of any peripheral thermogenic agent. Taken together, a number of studies (reviewed by Szallasi, 1994) imply that differences exist between peripheral and central vanilloid recognition sites in the rat. Central (sensory ganglia and spinal cord) receptors bind cooperatively with high affinity, whereas peripheral (urinary bladder, urethra, airways, and colon) receptors bind noncooperatively with relatively low affinity. Furthermore, the order of capsaicin and capsazepine binding affinity is reversed in central and peripheral receptors. Accordingly, partial success has been reported in separating vanilloid antinociceptive and hypothermic actions (Hayes *et al.*, 1984), implying that peripheral specificity may yet be achieved given further structure-activity insights.

7.6 Pharmacokinetics of Vanilloids *In Vivo*

Current evidence suggests that vanilloid bioavailability is low following oral administration. Despite rapid absorption of capsaicinoids from the gut in rats (Kawada *et al.*, 1984; Monsereenusorn, 1980), studies suggest that capsaicin and dihydrocapsaicin (Donnerer *et al.*, 1980), as well as olvanil (a synthetic vanilloid, N-(3-methoxy-4-hydroxy-benzyl)-oleamide, Sietsema *et al.*, 1988) are markedly metabolised before entering the systemic circulation (first-pass effect) following oral administration. However, intravenous or subcutaneous doses of the same compounds resulted in higher plasma concentrations (Donnerer *et al.*, 1980; Sietsema *et al.*, 1988). Nevertheless, vanilloid clearance from the plasma is likely to be rapid given the expeditious hepatic breakdown of [^{3}H]dihydrocapsaicin noted by Donnerer *et al.* (1990) in rats. Indeed, the terminal half-life of [6]-gingerol after intravenous injection was reported to be only 7.23 minutes in rats (Ding *et al.*, 1991, Fig. 7.3).

Thus it appears likely that problems associated with poor oral and intravenous bioavailability, as well as receptor selectivity, will need to be overcome if vanilloid agents are to be successfully developed as therapeutic pharmaceuticals. The major mechanisms of metabolic degradation of capsaicinoids appear to be hydrolysis of the amide bond (Sietsema *et al.*, 1988; Kawada *et al.*, 1984), β-oxidation of the side chain (Sietsema *et al.*, 1988), and ring hydroxylation (Kawada and Iwai, 1985). Consequently, synthetic vanilloids with altered A-region substituents, no amide bond, and C-region substitution may possess greater bioavailability *in vivo*. These findings may explain the *in vivo* activity of compound 6C (Chapter 4, altered A-region and no amide bond) relative to the inactivity of capsaicinoids and ginger principles in the same system (data not shown). However, such structural modifications have thus far resulted in markedly attenuated potency *in vitro*.
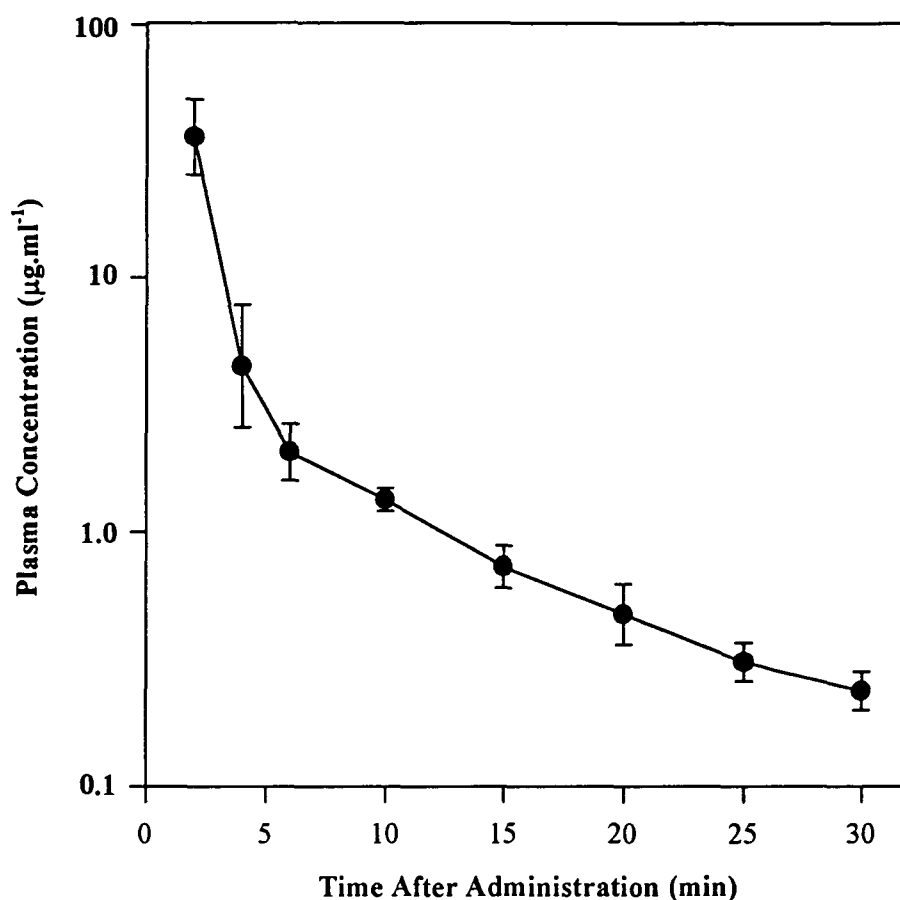
Fig. 7.3. Plasma concentration-time profile of [6]-gingerol after bolus intravenous administration (3 mg·kg⁻¹) to rats (redrawn from Ding *et al.*, 1991).

7.7 Target Areas for Future Studies

7.7.1 Vanilloid studies

The presence of vanilloid receptors in rat hindlimb preparations has not been directly established. Autoradiography studies using radiolabelled potent vanilloids such as ^{3}H[RTX] are required confirm the involvement of specific vanilloid recognition sites. However, studies with vanilloid antagonists (capsazepine and ruthenium red) have already commenced (Griffiths *et al.*, 1996). In terms of the synthesis and modelling of synthetic vanilloids, further work is required to define separate structure-activity profiles of the putative VN_1 and VN_2 receptors. Extending these studies may lead to agonists, or indeed antagonists, capable of full selectivity of

one or both of the subtypes. Agents suspected of displaying partial subtype selectivity (Chapter 4) require detailed pharmacological evaluation; such analysis may lead to divergent structure-activity profiles.

In view of the positive result obtained with preliminary whole body testing of synthetic vanilloid agents (Chapter 4, section 4.3.12), further trials *in vivo* are warranted to confirm the potential of vanilloids as pharmacological thermogenic agents. Trials in human subjects would be the logical extension of successful *in vivo* rat studies. In view of reported rapid capsaicinoid metabolic degradation *in vivo* (discussed in section 7.6) structural design with regard to maximising *in vivo* bioavailability of synthetic agents emerges as an important part of future studies. Given the triphasic $\dot{V}O_2$ effects associated with periods of constant vanilloid infusion (followed by removal) *in vitro* (Chapter 3, Fig. 3.2A), hindlimb perfusion experiments designed to simulate the pharmacokinetics of bolus vanilloid doses *in vivo* may yield useful data, particularly in terms of likely net effects of vanilloids on metabolic parameters such as $\dot{V}O_2$ and glucose uptake. Since the perfusion experimental design used in the present study incorporates a single-pass flow regime, bolus doses are not appropriate for simulating *in vivo* drug exposure. Instead, variable infusion rates designed to match predicted plasma concentration-time profiles (e.g. Fig. 7.3) are required.

It is proposed that the vanilloid effects are the result of microvascular flow redistribution, as appears to be the case for other type A and type B agents (Clark *et al.*, 1995; Newman *et al.*, 1996). Confirmation of this hypothesis necessitates the specific analysis of muscle flow distribution under vanilloid influence. Previous studies in this laboratory have used the results of vascular corrosion casting, fluorescein-labelled dextran entrapment, and post equilibration erythrocyte efflux as evidence for vasoconstrictor control of differing vascular flow routes (Newman *et al.*, 1996).

7.7.2 Comparative investigations

The proposal that vascular control of skeletal muscle metabolism is potentially a general biological mechanism warrants an extended investigation. Perfusion studies of further species, particularly those representing phylogenetic groups such as fish and reptiles, would be a logical extension of the current findings. A further area of interest

is the potential differences in muscle vascular control of species endemic to regions with markedly differing mean ambient temperatures. It might be predicted that perfused muscle preparations of species adapted to cold environments would display enhanced vasoconstrictor-induced oxygen consumption. A collaborative study based on the effects of cold-acclimation on vascular control in ducklings (discussed in section 7.2) has recently been completed (F. Marmonier, C. Duchamp, F. Cohen-Adad, T.P.D. Eldershaw, and H. Barré, submitted).

The notion of nonshivering and shivering thermogenic processes being complementary in birds was raised in Chapter 5. Electrical stimulation of muscle contraction was found to induce marked vasodilation in the present study (Chapter 5, Table 5.1). Vasodilatory effects accompanying the muscular contractions of shivering may result in potentiated noradrenaline-mediated increases in $\dot{V}O_2$ in a manner similar to that observed with glucagon. Preliminary perfusion experiments examining the effects of simultaneous muscle contraction and catecholamine infusion are currently in progress.

Perfusion studies using alternative models of obesity and insulin-resistance could conceivably strengthen the proposed link to impaired haemodynamic control. Disease models such as spontaneously hypertensive (SH) rats, genetically diabetic (*db/db*) and genetically obese (*ob/ob*) mice, high fat-fed rats, and chronically denervated rat hindlimbs (insulin-resistance) emerge as suitable subjects for such experiments. Studies using chronically denervated hindlimbs and SH rats are currently in progress.

The proposal that the actions of BRL 49653 arguably have a haemodynamic basis remains unresolved. In view of the data obtained in the present study, it might be predicted that thiazolidinedione agents act to alter the vasoconstrictor-controlled window of metabolism (Chapter 6, Fig. 6.12). Thus comparison of vasoconstrictor-mediated responses in hindlimbs of BRL 49653-treated obese Zuckers with the results obtained in non-treated obese animals (Chapter 6) may clarify the suggestion that the differences in vascular control noted in perfused obese hindlimbs were related to the impaired glycaemic control.

The ability to diagnose the relative proportions of nutritive and non-nutritive flow *in vivo* remains a longer-term objective if such microvascular phenomena are to

, be associated with obese, diabetic, and hypertensive disease states in the human. Methods based on vasculature-specific enzymatic substrate conversion are currently being developed in this laboratory.

7.8 Conclusion

Vascular control of skeletal muscle metabolism has been identified in vertebrate taxa other than mammals. This mechanism may have implications for avian NST, since the data are consistent with claims that muscle is the major site of *in vivo* regulatory NST in birds. Defective vascular control in the muscle of a rodent disease model may be related to impaired energy balance and glucose tolerance manifestations. Vanilloid molecules have emerged as a new group of agents capable of influencing microvascular haemodynamics in perfused rat muscle. These actions, apparently mediated by specific peripheral vanilloid receptors, identify vanilloids as lead compounds for pharmacological enhancement of muscle metabolic performance *in vivo*. The studies underline the potential significance of altering the distribution of nutrient and hormone access in regulating skeletal muscle metabolism, and in particular support fundamental vascular involvement in the control of skeletal muscle thermogenesis.

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Appendix 1

Thermogenesis Definitions

Nonshivering thermogenesis (NST) is defined as "heat production due to metabolic energy transformation by processes that do not involve the contraction of skeletal muscles, i.e., tone, microvibrations tremor (shivering), or tonic or voluntary contractions" (Simon, 1987). Janský (1995) has expanded this definition with the statement that NST is an effector thermoregulatory mechanism based on the thermogenic action of neuronally-released noradrenaline. Janský (1995) goes on to point out that NST may have a non-uniform physiological background, and that various mechanisms and effectors may be involved.

NST may be subdivided into obligatory and facultative (regulatory, adaptive) categories. The obligatory component is defined as the basal heat production at thermoneutrality; the energy required to maintain the integrity of the cell and the steady-state condition of the animal (Janský, 1995). Obligatory NST is thus common to both endotherms and ectotherms. The facultative component is additive to obligatory NST and represents further heat production by processes not involving skeletal muscle contraction in response to ambient temperatures falling below the thermoneutral zone of homeothermic animals (Rothwell and Stock, 1980; Trayhurn, 1994).

Other subdivisions of thermogenesis include diet-induced thermogenesis (DIT), a form of facultative NST which occurs in some animals (especially rodents) when transferred from standard food to a highly palatable 'cafeteria' diet. The extra caloric intake is dissipated in part by enhanced heat production (Rothwell and Stock, 1979; 1980). Postprandial (extra) heat production (obligatory diet-induced thermogenesis or heat increment of feeding) is defined as "an increase in metabolic heat production, relative to the postabsorptive resting level, in the hours following food intake" (Janský, 1995).

Total facultative thermogenesis represents the combined heat production of nonshivering and shivering mechanisms. However this study was primarily concerned with the nonshivering component of facultative thermogenesis. The use of the term

thermogenesis in the present work refers to facultative nonshivering thermogenesis unless otherwise stated.

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Pungent principles of ginger (*Zingiber officinale*) are thermogenic in the perfused rat hindlimb

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Summary

Crude extracts of both fresh and dry ginger induced the perfused rat hindlimb to consume oxygen in association with increases in perfusion pressure and lactate production. The principles responsible for these observations, the gingerols and shogaols, were isolated and tested for relative thermogenic activity. The gingerol homologues possessed greater molar potency than their shogaol counterparts. (6)-Gingerol was the most potent principle isolated, causing a mean maximal increase in oxygen consumption of $1.4 \pm 0.1\,\mu mol/g/h$ (21%), an increase in lactate efflux of $4.7 \pm 0.6\,\mu mol/g/h$ (87%) with a perfusion pressure increase of $7.7 \pm 0.7\,mmHg$ (30%). Increases in alkyl chain length within each homologous series led to decreased molar potency. Specific nitro-vasodilation using glyceryl trinitrate demonstrated that thermogenesis was at least partly associated with vasoconstriction. Concurrent infusion of α or β antagonists showed that neither adrenergic receptors nor secondary catecholamine release were responsible for the observed effects. Increasing doses of the ginger principles ultimately led to inhibition of steady state oxygen consumption, although perfusion pressure continued to increase. Removal of high ginger principle doses was followed by apparent increases in oxygen uptake unaccompanied by elevated perfusion pressure. As a consequence, the effective concentration ranges of the ginger principles were relatively narrow. The cause of high dose effects is as yet undetermined but may have been due in part to disruption of mitochondrial function.

Keywords: gingerols, shogaols, spices, oxygen consumption, vasoconstriction, metabolic rate

Introduction

Ginger, the rhizome of *Zingiber officinale* Roscoe, is extensively used as a flavouring additive in foods, beverages and confectionery. A herbaceous perennial belonging to the family Zingiberaceae, it has medicinal qualities of importance in traditional Chinese medicine. Legendary Chinese herbalist Shen Nung (3000 BC) recommended ginger for colds, fever, chills, tetanus and leprosy.[1] The crude drug continues to be widely used for the treatment of a number of ailments, including colds and flu, motion sickness, digestive problems and irregular menstruation.[1] Ginger is noted for its apparent ability to subjectively warm the body.[2]

We have previously demonstrated[3] that capsaicin and dihydrocapsaicin, the capsaicinoid spice principles present in hot chillies and capsicums, are thermogenic in the isolated perfused rat hindlimb; the increase in oxygen uptake being associated with vasoconstriction. These findings may help to explain those of

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Henry and Emery[4] who reported that human consumption of a meal containing chilli and mustard sauces resulted in a 25% greater increase in diet-induced thermogenesis over a three hour period than a similar meal without spices. In addition, the response of the hindlimb to the capsaicinoids was consistent with the hypothesis that vascular smooth muscle directly consumed oxygen during sustained vasoconstriction.[5-8]

Diet-induced thermogenesis may contribute significantly to the regulation of body temperature and energy balance.[9,10] The magnitude of this phenomenon is influenced both by caloric intake[11] and dietary composition.[12-14]

Pungency, a feature of chillies and capsicums, is also an important characteristic of ginger. The pungent principles of ginger are present as two phenylalanine-derived[15] homologous series, the gingerols and shogaols.[16] The shogaols are formed via an alkyl chain dehydration reaction from gingerols (Figure 1), hence they are usually present in dried rather than fresh rhizomes. Gingerols and shogaols primarily consist of the (6)-, (8)-, and (10)-homologues[17] (Figure 1), although trace amounts of other homologues have been reported following gas chromatographic studies.[18,19] Trace amounts of gingerols

Figure 1 Structures of vanillyl-containing compounds discussed in the text.

with methyl side chains have been described.[19] Zingerone. a pungent hydrolysis product of gingerols and shogaols.[16] is present in many heat treated or roasted ginger preparations.

Recent studies have reported that both ginger and its isolated pungent principles exhibit a range of pharmacological effects. Suekawa et al.[20,21] have found (6)-shogaol to have a tri-phasic effect on blood pressure in rats in vivo. It was suggested that this response was a complex phenomenon involving both CNS and peripheral activity. Other studies have found ginger principles to exhibit cardiac effects,[22,23] mutagenicity,[24,25] gastro-intestinal and analgesic activity,[23] inhibition of human neutrophil 5-lipoxygenase activity,[26] and inhibition of serotonin-induced hypothermia and diarrhoea.[27]

The gingerols and shogaols bear some similarities to the capsaicinoids in terms of both structure and function. All contain the 4-methoxy, 3-hydroxy phenyl (vanillyl) moiety, as well as a carbonyl-containing alkyl side chain. Each group of homologues is responsible for the pungent taste of the parent plant.[16] As part of our continuing search for thermogenic dietary components, we have investigated the actions of ginger and its pungent principles in the isolated perfused rat hindlimb.

Methods

Rat hindlimb perfusions

Male hooded-Wistar rats (180–200 g) were raised on a commercial diet containing 21.4% protein, 4.6% lipid, 68% carbohydrate, and 6% crude fibre with added vitamins and minerals (Gibsons, Hobart, Australia) together with water ad libitum, at a temperature of 21 ± 1°C. The surgical and perfusion procedures were performed as described previously.[5] One hindlimb was perfused at 25°C with constant flow (4 ml/min) of a modified Krebs-Ringer bicarbonate buffer containing 2% dialysed bovine serum albumin. 8.3 mM glucose and 1.27 mM calcium chloride. The methods of oxygen consumption calculation have been previously described.[5] Oxygen uptakes and perfusion pressures were calculated from steady state values. usually attained within 5 min after applying the agent. Bovine serum albumin (fraction V, Boehringer Mannheim. North Ryde. Australia) was dialysed five times against distilled water before use. The lactate assay using neutralized perchlorate samples was based on the method of Gutmann and Wahlefeld.[28] Samples for lactate analysis were taken at times corresponding to steady state oxygen consumption and perfusion pressure
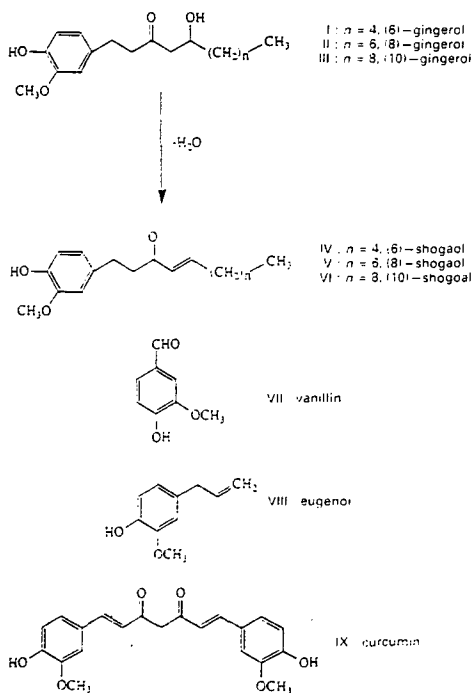
(usually 5 min after applying the agent). NAD$^+$ (free acid) and lactate dehydrogenase were purchased from Boehringer Mannheim. Prazosin hydrochloride and D,L-propranolol hydrochloride were obtained from Sigma (St Louis, Missouri, USA) whilst glyceryl trinitrate (GTN) was from G Pohl-Boskamp GmbH (Hohenlockstedt, Germany).

Crude extracts, fractions and pure ginger principles were infused as 25% ethanol (AR grade) solutions using a glass syringe with teflon tubing in a Sage Instruments Syringe pump (model 355). Other compounds were infused using LKB peristaltic pumps in water or saline solutions. Infusion rates were between 10 and 40 μl/min. Vehicle infusions were shown not to perturb basal conditions. Solutions were infused into a bubble trap prior to the arterial cannula. The perfusate in this trap was subject to continual stirring.

The data are expressed as means ± standard errors. Curves were fitted using the Sigma-Plot program (Jandel Scientific, Sausalito, California, USA). Significance of differences was assessed using the unpaired two-sided Student's t test.

Preparation of extracts and isolation of ginger principles
Crude extracts of both fresh ginger (purchased locally) and ground dried ginger (Buderim

Ginger, Yandina, Queensland, Australia and Superior Rate Corporation, Taipei, Taiwan) were prepared by percolation (×4) in methanol (HPLC grade Ajax). Fresh ginger was chopped and blended with methanol to a slurry prior to extraction. Percolation periods were generally 8–12 h after each fresh addition of methanol. Following extraction the methanol was removed under vacuum at temperatures no greater than 30°C. A typical fractionation procedure used to isolate principles from fresh ginger is shown in Figure 2.

The fractionation procedure for dry ginger was similar to that outlined in Figure 2 except that the active material following testing at stage 5 (Rf 0.65–0.8) was subjected to radial chromatography using silica gel of layer thickness 4 mm (5:2 ether:hexane). The combined active fractions were subjected to preparative TLC (Figure 2, step 6) and the resultant active band (Rf 0.75–0.80) was subjected to HPLC (Figure 2, step 8) to yield (6)- and (8)-shogaol.

The most abundant principle isolated from fresh ginger, (6)-gingerol, was identified by gas chromatography–mass spectroscopy (GC–MS) and proton nuclear magnetic resonance spectroscopy. Subsequent principles were identified by GC–MS. The purity of isolated principles was confirmed by TLC, HPLC, GC–MS and direct insertion mass spectroscopy.

Preparative TLC plates were prepared using a moving hopper slurry spreader over glass backing (Merck silica gel 60G). Short column chromatography was performed using Fluka silica gel H for TLC (dry packed). A Chromatotron apparatus (TC Research, Norwich, UK) was used for radial chromatography. Plates were prepared using Merck silica gel 60 PF254 on a glass backing.

HPLC was performed using a Waters system, incorporating 6000A pumps, a U6K injector, a model 440 u.v. absorbance detector, and a differential refractometer model R401. A Dynamax-60A C18 column (model no. 83–221C, 21.4 mm i.d. × 25 cm) was used for preparative separations whilst analytical work was carried out using a Waters Radial-Pack C18 column (8NVC184). The isocratic mobile phase was 80:20 methanol:water.

Fresh ginger (bulk)
↓ 1. Extraction
Crude MeOH extract
↓ 2. Solvent removal
Oleoresin
↓ 3. Extraction : ether/hexane 1:1
Solute   Residue
↓ 4. Short column fractionation (Si gel, 2:1 ether/hexane)
n fractions (monitored by TLC) │ 5. Hindlimb perfusions
Active fractions
↓ 6. Preparative TLC (Si gel, 5:2 ether/hexane) multi-development – 3.
Discrete bands (u.v. active)
7. Recrystallizations (n-hexane)   8. Preparative HPLC (reverse phase)
6-gingerol   6-, 8-, 10-gingerols

Figure 2 Typical extraction, isolation and testing procedure for active principles of fresh ginger (gingerols). Dried ginger, the source of shogaols, was treated in a similar fashion except that an additional radial chromatography stage was used between stages 5 and 6 and step 7 was omitted.

Results

The perfused hindlimb was initially allowed to reach steady state perfusion pressure and venous PO_2.[5] The mean arterial and venous PO_2 values were 688 ± 4 mmHg ($n = 22$) and 413 ± 8 mmHg ($n = 22$) respectively. The mean basal oxygen uptake was therefore 6.6 ± 0.2 μmol/g/h ($n =$

22).[5] The mean basal perfusion pressure was 26.0 ± 0.6 mmHg, whilst the mean lactate efflux was 5.4 ± 0.3 μmol/g/h (*n* = 19). These values are similar to those obtained during other studies from this laboratory.[3,5–8]

Exhaustive methanolic extracts of both fresh rhizomes and commercially peeled, dried and ground rhizomes were found to cause both an increase in oxygen uptake and perfusion pressure when infused into the perfused rat hindlimb. Hindlimb oxygen uptake was stimulated over the final concentration ranges of 0.05–0.15 mg/ml (fresh ginger extract) and 0.005–0.01 mg/ml (dried ginger extract). Similar extracts of other spices including garlic, gin seng, horseradish and yellow mustard had no effect when infused at final concentrations of up to 25 mg/ml.

Fractionation of the crude methanolic extracts from both fresh and dried ginger resulted in the isolation of the principles responsible for the observed thermogenic activity (Figure 1, structures I–V). Figure 3 shows typical oxygen and pressure traces produced by a series of increasing (6)-shogaol doses. The order of dose infusion did not affect the observed dose responses. Figure 4 illustrates the increases in steady state oxygen uptake, perfusion pressure and corresponding lactate production of the perfused hindlimb as a function of (6)-, (8)-and (10)-gingerol, and (6)-shogaol concentration. Increasing alkyl chain length leads to decreased potency, although maximal stimulated oxygen consumption appears to increase. In addition, Figure 4 shows the effect of alkyl chain dehydration on the potency and maximal oxygen stimulation of (6)-gingerol, the major principle of fresh ginger, by comparison with responses obtained using (6)-shogaol, the major principle of dried ginger. Experimental results using (8)-shogaol (data not shown) found the half-maximal dose to be around 20 μM, indicating that the molar potency relationship
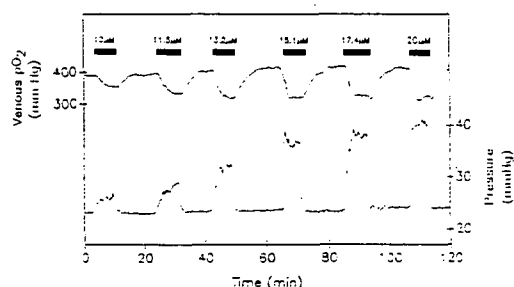
Figure 3 Typical dose response tracing of changes in venous PO_2 and perfusion pressure in perfused rat hindlimb preparations subjected to increasing concentrations of (6)-shogaol (10–20 μM, *n* = 5).
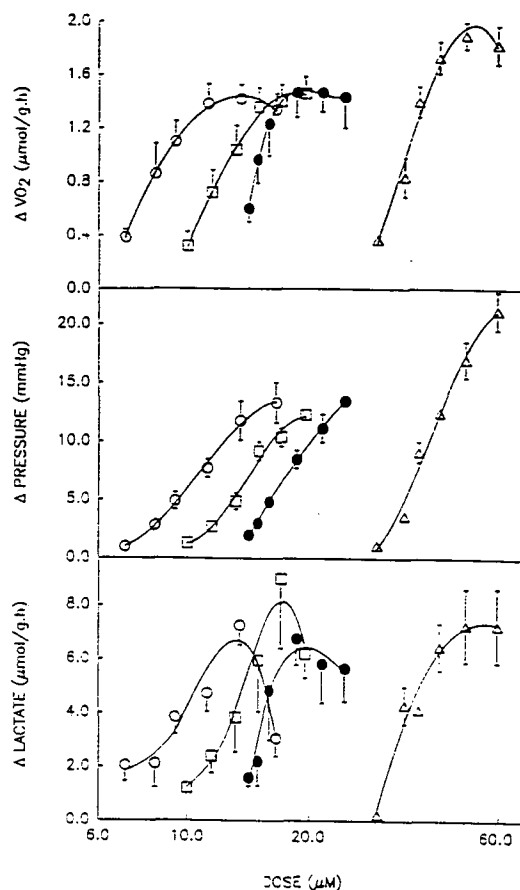
Figure 4 Dose response curves for changes in oxygen consumption, perfusion pressure and lactate efflux in response to (6)-gingerol (○, *n* = 7), (6)-shogaol (□, *n* = 5), (8)-gingerol (●, *n* = 3) and (10)-gingerol (△, *n* = 3). Basal VO_2 was 6.6 ± 0.2 μmol/g/h (*n* = 22) and basal perfusion pressure was 26.0 ± 0.6 mmHg (*n* = 22). Basal lactate efflux rate was 5.4 ± 0.3 μmol/g/h (*n* = 19). Each value is the mean ± s.e. Where error bars are not visible they are within the symbol.

between the shogaol homologues is similar to that existing between the gingerol homologues.

Increasing the doses of all the ginger principles beyond the levels shown in Figure 4 led to a progressive inhibition of steady state oxygen consumption (Figure 5). In the extreme case (Figure 5, 45 μM (6)-gingerol), oxygen consumption was inhibited to sub-basal levels following initial transient stimulation. Perfusion pressure continued to increase towards a plateau. Removal of ginger principles during high dose treatment resulted in large dose-dependent transient increases in oxygen consumption (Figure 5), whilst

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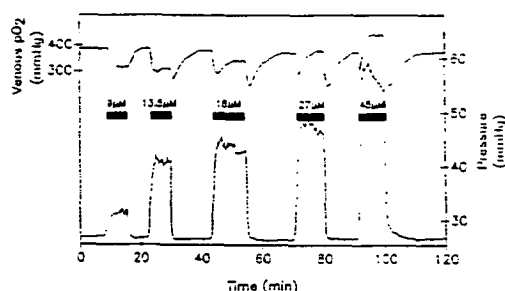
Figure 5 Typical dose response tracing of changes in venous PO_2 and perfusion pressure in perfused rat hindlimb preparations subjected to increasing high concentrations of (6)-gingerol (9–45 μM).
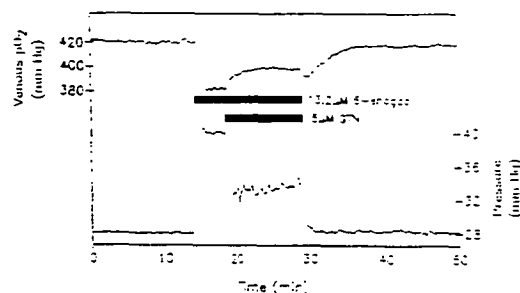
Figure 7 Typical tracing of the effect of glyceryl trinitrate (GTN, 5 μM) on changes in venous PO_2 and perfusion pressure in perfused rat hindlimb preparations stimulated with 13.2 μM (6)-shogaol.

perfusion pressure (Figure 5) and lactate production levels (data not shown) returned to basal.

A half-maximal dose of (6)-shogaol (13.2 μM) was chosen to investigate the effects of specific nitro-vasodilation, α blockade, and β blockade on the stimulated hindlimb (Figure 6). Neither prazosin (α blocker) nor propranolol (β blocker) inhibited the (6)-shogaol induced response. The slight potentiation effects induced by each antagonist were not statistically significant. GTN significantly ($P < 0.05$) inhibited the increases in oxygen uptake (56%) and perfusion pressure (72%) induced by (6)-shogaol (Figure 6). Typical oxygen and pressure traces shown in Figure 7 illustrate the effect of direct addition of 5 μM GTN to a half-maximal dose of (6)-shogaol.

Since the gingerols, shogaols and capsaicinoids

Figure 6 Effects of propranolol (PROP, 5 μM), prazosin (PRAZ, 5 μM), and glyceryl trinitrate (GTN, 5 μM) on changes in venous PO_2 (□) and perfusion pressure (▨) in perfused rat hindlimb preparations stimulated with 13.2 μM (6)-shogaol. Results are shown as the mean percentages (±s.e.) of the responses relative to those using (6)-shogaol alone. *Statistically significant effects ($P < 0.05$).

all bear the 4-methoxy, 3-hydroxy phenyl moiety, experiments were conducted to evaluate the thermogenic potential of other compounds bearing this vanillyl group. The structures selected (Figure 1, VII–IX) were vanillin, curcumin (from turmeric) and eugenol (cloves). Infusion of each over the range 0–10 mg/ml had no effect on perfusion pressure nor oxygen consumption.

Discussion

In general, perfused hindlimb tissue has proved to be responsive to thermogenic agents and hormones.[5–8,29–34] For the perfused rat hindlimb the use of a non-erythrocyte perfusate at 25°C[5,34] is comparable to constant perfusion with erythrocytes at 37°C[30,32,33] for assessing noradrenaline-induced oxygen uptake and has allowed the identification of vasopressin,[5] angiotensin[5] and the capsaicinoids[3] as potential thermogenic substances.

In the present study, the gingerols and shogaols were found to stimulate the hindlimb in a manner similar to that reported for the capsaicinoids.[3] The observed responses were not related to the order of dose infusion (data not shown). Although the ginger principles do not possess an acylamide linkage, they bear two major structural similarities to the capsaicinoids, a 4-hydroxy, 3-methoxy phenyl (vanillyl) 'head' and a carbonyl-containing alkyl 'tail'. The failure of eugenol and vanillin, structures possessing only the vanillyl moiety (Figure 1), to induce a response in the perfused hindlimb suggests that both features are necessary for thermogenic activity. Curcumin, containing two vanillyl groups with a bridging, rather than a tailing alkyl section, was also thermogenically inactive. Szolcsányi and Jancsó-Gabor[35] investigated the effect of altering the aromatic ring substituents, as well

as the length and nature of the alkyl chain on relative pungency of a range of vanillyl-derived compounds. The aromatic substituents, particularly the hydroxyl group, were of critical importance, whilst the chain length affected pungency in a more subtle manner. The overall trend was that relative pungency increased with chain length to a maximum at around 8–10 carbon atoms. Subsequent increases in chain length led to progressive pungency decreases. In the present study, the latter trend was expressed by the gingerol homologues with (6)-gingerol (10 carbon chain) having the highest molar potency. Maximal oxygen uptake, however, increased with alkyl chain length (Figure 4). (4)-Gingerol (8 carbon chain) may have still greater molar potency, but its effect is of little consequence to the overall activity of ginger due to its trace presence.[18]

Although the reported ratios of the principles in ginger are somewhat variable, the (6)-homologue is consistently found to be the major compound. Connell and Sutherland[16] found the (6)-, (8)-, (10)-gingerol ratio to be 53:17:30, whilst Chen et al.[19] reported the ratio to be 119:17:24. The (6)-gingerol content in ginger varies as a function of growth time, location and storage period, a typical value being 1.5% of dry weight.[36]

Dehydration of the alkyl chain (conversion of gingerol to shogaol) resulted in a small decrease in molar potency whilst maximal oxygen uptake was not significantly affected. Relatively few studies have directly compared the magnitude of the physiological effects induced by gingerols with those induced by shogaols. Suekawa et al. found (6)-gingerol to have a lower LD_{50} in rats, yet (6)-shogaol was reported to be more active on both the CNS and the digestive system.[23]

It has previously been demonstrated that noradrenaline-induced oxygen uptake and perfusion pressure increases in the rat hindlimb are blocked both by phentolamine and high dose propranolol.[37] In the present study the effects of (6)-shogaol were not significantly altered by either of these antagonists (Figure 6), suggesting that the ginger principles were not acting directly via adrenergic receptors, nor by secondary catecholamine release. This latter phenomenon has been reported to be activated in vivo by a number of pungent principles including capsaicin[38] and, to a lesser extent, zingerone.[39]

In the present study, GTN (a specific nitrovasodilator) was used to inhibit both the oxygen and pressure responses to (6)-shogaol. This implies that the mechanism of action is closely related to the vascular system. Szallasi and

Blumberg[40,41] have proposed the existence of a putative 'vanilloid' receptor following studies using cultured nerve cells. The possibility of the presence of such a receptor on vascular smooth muscle cannot be overlooked since work from this laboratory has provided strong evidence of a link between the vascular system and hindlimb thermogenesis. Increases in perfusion pressure and oxygen uptake induced by vasopressin, angiotensin II and noradrenaline were inhibited by sodium nitroprusside,[5] another specific nitrovasodilator. In addition, oxygen consumption by electrically stimulated skeletal muscle was found to be additive to that associated with vasoconstriction,[6] whilst variable flow experiments showed that all flow-induced increases in oxygen uptake were enhanced by noradrenaline infusion but blocked by sodium nitroprusside.[7]

Previous studies from this laboratory have reported that the vasoconstrictors norephedrine,[42] vasopressin, angiotensin II and methoxamine (unpublished results) increase lactate release from perfused hindlimb preparations in association with increases in oxygen consumption and perfusion pressure. It was concluded that the lactate was released from working vascular smooth muscle during active vasoconstriction, and was not associated with hypoxia.[7,42–43] The present study has found that ginger principles induce similar dose-related lactate release concomitant with vasoconstriction and oxygen uptake. If such lactate production occurs in vivo, it could be part of a significant long-loop thermogenic mechanism due to the high energy phosphates required for the resynthesis of lactate back to glucose in the liver (Cori cycle). In the case of (6)-gingerol, for example, the mean increase in lactate release associated with the mean maximal VO_2 is $4.7\,\mu mol/g/h$. This rate of production would require an increase of $2.4\,\mu mol/g/h$ in oxygen consumption for full conversion to glucose in the liver.

Experiments using both (6)-gingerol and (6)-shogaol found that there was no increase in lactate release associated with the large transient increases in oxygen consumption following removal of high doses of ginger principles (data not shown). This indicated that these transient periods of oxygen consumption were not associated with the reperfusion of hypoxic tissue.

Studies from this laboratory have found that serotonin, an endogenous vasoconstrictor, inhibited perfused hindlimb oxygen uptake in a dose-dependent manner, but stimulated isolated mesenteric artery oxygen consumption.[43] It was proposed that serotonin-induced vascular shunting was masking vascular thermogenesis.[43] In the

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present study high doses of ginger principles, after initial stimulation, led to sub-basal oxygen consumption (Figure 5), regardless of the order of dose infusion (data not shown). This phenomenon may also be due to vascular shunting. However, unlike serotonin, the removal of high doses of ginger principles is followed by pronounced (but temporary) periods of low venous PO_2 (i.e. increased oxygen consumption) not associated with vasoconstriction (Figure 5).

Chudapongse and Janthasoot[44] studied the effects of the analogous principle capsaicin on the energy-linked functions of isolated rat liver mitochondria. At lower doses with glutamate as substrate, capsaicin inhibited oxidative phosphorylation. At higher doses with succinate as substrate, capsaicin uncoupled mitochondrial respiration. High doses of capsaicinoids and ginger principles may be inducing similar effects within the hindlimb. Experiments performed in this laboratory have shown that hindlimb oxygen consumption induced by the known metabolic uncoupler sodium azide was inhibited by serotonin.[45] High doses of ginger principles may have caused perfusate to be shunted away from uncoupled tissue associated with microvasculature, thereby masking net oxygen uptake by the hindlimb as a whole. Removal of the shunting might allow the oxygen-depleted perfusate to depart the microvascular beds, resulting in the observed apparent increase in oxygen uptake. Testing these hypotheses will require further experimentation.

Henry and Piggott[46] have examined the effect of consuming a ginger sauce (containing unspecified amounts of ginger principles) with a meal on human subjects and found that metabolic rate was not significantly enhanced relative to subjects who consumed a control meal. Although little is known about the passage of ginger principles across the gut wall, the gingerol analogues, capsaicin and dihydrocapsaicin, are rapidly absorbed from the rat stomach and small intestine, both in vivo and in vitro.[47] Results of pharmacokinetic studies[48] have reported the half life of (6)-gingerol in rat plasma to be relatively fast (7.2 min). The hindlimb perfusion results of this study found that the final concentration range of (6)-gingerol required for thermogenic responses was relatively narrow (7–15 μM). Concentrations below this range had no effect, whilst concentrations above this range gave either a reduced effect, or a negative thermogenic effect (Figure 5). In comparison, the concentration range of noradrenaline required to increase oxygen consumption in this system is approximately 1–100 nM.[5] Thus the ginger dose used by

Henry and Piggott[46] may have resulted in final in vivo concentrations outside any thermogenic range.

If ginger principles are subsequently shown to have whole body thermogenic effects, the mechanism of action will be a moot point. The pressor response to (6)-shogaol in the whole rat body has been related to both central and autonomic (sympathetic) nervous system activity.[20,21,23] Similarly, postprandial thermogenesis in dogs has been decisively linked to autonomic activation due to oropharangeal sensory inputs.[49,50] In the rat, similar sensory input increased brown adipose tissue thermogenesis except when sympathetic innervation was deactivated.[51] Thus any thermogenic effect of ginger principles in vivo may be attributable to central or autonomic nervous system activity, or a combination of both.

The present study, however, has shown that the perfused hindlimb thermogenic responses were intimately involved with the vasculature, and were not significantly altered by α and β adrenergic receptor blockade. These findings therefore warrant future investigations of the actions of ginger and its principles in alternative vascular beds and ultimately in vivo with rats and humans in order to assess the potential of ginger as a dietary anti-obesity agent.

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RESINIFERATOXIN AND PIPERINE: CAPSAICIN-LIKE STIMULATORS OF OXYGEN UPTAKE IN THE PERFUSED RAT HINDLIMB

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Summary

The naturally occurring capsaicin-like molecules, resiniferatoxin (RTX, *Euphorbia* spp.) and piperine (*Piper nigrum*), each stimulated oxygen uptake ($\dot{V}O_2$) in association with increased vascular resistance in a concentration-dependent manner when infused into the perfused rat hindlimb. 5 μM glyceryl trinitrate (GTN, a nitro-vasodilator) significantly blocked the oxygen and pressure responses to both RTX and piperine, indicating a close relationship between changes in $\dot{V}O_2$ and the vasoconstriction. Concentrations greater than those required for maximal $\dot{V}O_2$ resulted in an inhibition of $\dot{V}O_2$, although perfusion pressure continued to increase. Time course studies showed that both RTX and piperine at high doses resulted in a tri-phasic response. An initial phase of transient $\dot{V}O_2$ stimulation was followed by a second phase of inhibition. A third phase involving an often larger but transient stimulation of $\dot{V}O_2$ followed removal of the agents and continued after the pressure returned to basal. The actions of RTX and piperine were similar to those of other active capsaicin-like molecules tested previously in this system, including capsaicinoids (*Capsicum* spp.), gingerols (*Zingiber officinale*), and shogaols (*Zingiber officinale*). RTX was the most potent, and piperine the least potent of this series. Although receptor involvement has yet to be unequivocally established, the data are consistent with the presence of a functional capsaicin-like (vanilloid) receptor in the vasculature of the rat hindlimb that mediates vasoconstriction and oxygen uptake. These findings may have implications for the future development of thermogenic agents.

Key Words: resiniferatoxin, piperine, thermogenesis, capsaicin-like vasoconstriction

Resiniferatoxin (RTX) and piperine belong to a family of capsaicin-like compounds which have attracted escalating interest in recent years (1-5). Such compounds are sometimes known as 'vanilloids' as they are distinguished by the presence of a group based on the structure of vanillin (Fig. 1, I). Capsaicin (Fig. 1, II), the pungent principle of members of the genus *Capsicum* (chillies and capsicums), has been the subject of the majority of investigations involving this class of molecules. Recent work with capsaicin-like molecules has increasingly focused on the neuropharmacological aspects of their activity (1-3).

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RTX (Fig. 1, V) is a diterpene present in the latex of some members of the genus *Euphorbia* (*E. resinifera, E. poissonii* and *E. unispina*) (6,7). The compound was first isolated after plant extracts were found to have unusually high activity in a mouse ear irritant assay (8). RTX has structural similarities to the phorbol esters, a group of compounds which act chiefly via their ability to stimulate protein kinase C (9). However, the mechanism of RTX-induced irritation has subsequently been shown to differ from that of the phorbol esters, such as phorbol 12-myristate 13-acetate (PMA) (10). Structurally, RTX is distinguished by the presence of a 4-hydroxy 3-methoxy phenyl acetate moiety in the 20 position. This homo vanillyl group has been shown to be essential for the potent, yet transient (6), irritant activity exhibited by RTX (11,12). A number of studies have reported that RTX acts as an ultra potent capsaicin analogue (reviewed in 1 and 2), its potency generally ranging from 10 to 10,000 times that of capsaicin for responses such as induced hypothermia, neurogenic inflammation, and stimulation (followed by desensitisation) of specific sub populations of sensory neurons.

Piperine (Fig. 1, IV) is best known as the pungent principle of black pepper (*Piper nigrum*). Both piperine and RTX have been found to stimulate some capsaicin-sensitive afferent neurons (13-15). In a relatively wide spectrum of pharmacological activity, other noteworthy actions of piperine include the stimulation of serotonin synthesis in the rat brain (16), inhibition of smooth muscle nerve stimulation (17), anticonvulsant activity (18), and modulation of glucuronidation activity (19).

Previous studies using neural tissue (reviewed in 2) have reported cross-tolerance of RTX and capsaicin, as well as piperine and capsaicin (20). Such findings are consistent with a common mechanism of action. In addition, receptor-binding experiments using [³H]-RTX have provided direct evidence of specific binding by sensory ganglion membranes not associated with protein kinase C (21). Furthermore, alternative capsaicin-like compounds have been shown to inhibit the binding of [³H]-RTX (see review 2). The putative vanilloid receptor, found on specific subsets of afferent neurons, is thought to be a ligand-gated non-specific cation channel (22).

In this laboratory, we have shown that capsaicinoids (23) and also the ginger (*Zingiber officinale*) capsaicin-like principles: gingerols (24, Fig. 1) and shogaols (24), stimulate oxygen consumption ($\dot{V}O_2$) in association with vasoconstriction in the isolated perfused rat hindlimb. The effects were similar to, but of less magnitude, than those mediated by infusion of catecholamines, angiotensin (I-III) or vasopressin in the same system (25). The responses to the capsaicin-like principles were not due to stimulation of adrenoreceptors, since full doses of prazosin (α_1 blocker) and propranolol (β_1/β_2 blocker) had no inhibitory actions (23,24). However, the responses were significantly impeded by nitro-vasodilators (23,24). These findings highlighted the possibility of direct stimulus of vascular smooth muscle via vanilloid receptors on the smooth muscle itself, or alternatively via the release of non-adrenergic vasoactive agents by autonomic neurons embedded in the vessel walls.

In the present study, we have extended our investigation of thermogenesis mediated by capsaicin-like agents by examining the effects of both RTX and piperine in the perfused rat hindlimb model.

Methods

Male hooded-Wistar rats (180-200 g) were raised on a commercial diet (Gibsons, Hobart, Australia) containing 21.4% protein, 4.6% lipid. 68% carbohydrate, and 6% crude fibre with added vitamins and minerals together with water *ad libitum*. The environmental temperature was maintained at 21 ± 1°C. The surgical and perfusion procedures were performed as described previously (25). One hindlimb was perfused (25°C) at constant flow (4.0 ml/min) with a modified Krebs-Ringer bicarbonate buffer containing 2% dialysed bovine serum albumin, 8.3 mM glucose, and 1.27 mM

calcium chloride. The perfusion medium was continuously gassed with carbogen (O$_2$:CO$_2$ 95:5). The methods for calculating $\dot{V}O_2$ have been previously described (25). Viability of the hindlimb preparation as used in this study, has also been documented previously (25-28).
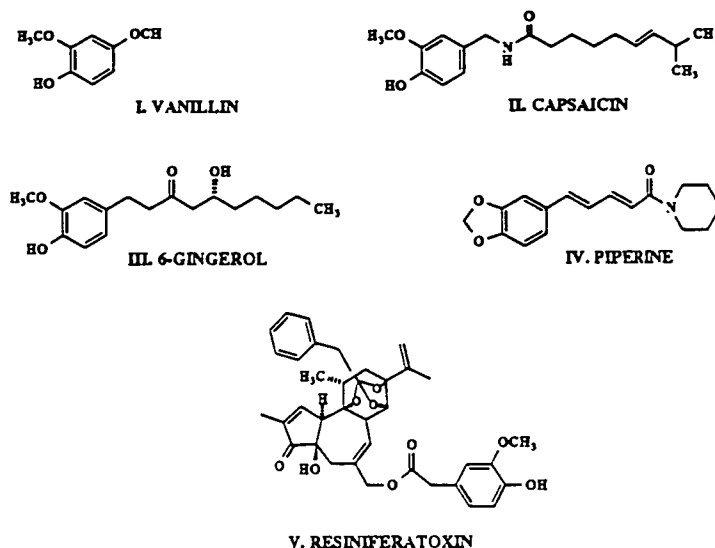
Fig. 1.

Structures of vanillin and the capsaicin-like (vanilloid) agents discussed in the text.

Bovine serum albumin (fraction V Boehringer Mannheim, North Ryde, Australia) was dialysed five times against distilled water before use. Glyceryl trinitrate (GTN) was purchased from G Pohl-Boskamp GmbH & Co. (Hohenlockstedt, Germany), piperine was purchased from Sigma (St. Louis, Missouri, USA), whilst RTX was a generous gift from Dr. D.J. de Vries, Australian Institute of Marine Science, Townsville, Australia.

Agents were infused using glass syringes with teflon tubing in a Sage Instruments syringe pump (model 355). Ethanolic piperine solutions were infused at 5 μl/min. RTX, in 20% ethanol solutions, was infused at rates between 10 μl/min and 40 μl/min. Stock ethanolic GTN solutions were diluted with saline and infused at 5 μl/min. Vehicle infusions had no effect on basal conditions. Solutions were infused into a stirred bubble trap immediately prior to the arterial cannula.

The data are expressed as means ± standard errors. Regression curves were fitted using the Sigma-Plot program (Jandel Scientific, Sausalito, California, USA). Significance of differences was assessed using the unpaired two-sided Student's t-test.

Results

The isolated perfused rat hindlimb was initially allowed to reach steady state perfusion pressure and venous $pO2$. The mean arterial and venous $pO2$ values were 666 ± 11 mmHg (n=24) and 375 ± 19 mmHg (n=24) respectively. The mean basal $\dot{V}O_2$ was therefore 7.1 ± 0.4 μmol/g/h (n=24). The mean basal perfusion pressure was 23.6 ± 0.9 mmHg (n=24). These values are consistent with those observed during other studies performed in this laboratory (23-28).

Figure 2 shows typical perfusion tracings of the effects of $\dot{V}O_2$-stimulatory doses of RTX (1 nM) and piperine (75 µM). The stimulation of perfusion pressure and $\dot{V}O_2$ was rapid in both cases with steady state conditions being attained within 5 minutes of commencing infusion of each agent. Similarly, basal conditions were re-established rapidly, within 10 minutes of agent removal. As noted previously for other capsaicin-like molecules (23,24), blockade of α_1 and β_1/β_2 adrenoreceptors using 5 µM prazosin and 5 µM propranolol, respectively, had no inhibitory effects on the increased $\dot{V}O_2$ caused by either RTX or piperine (data not shown).
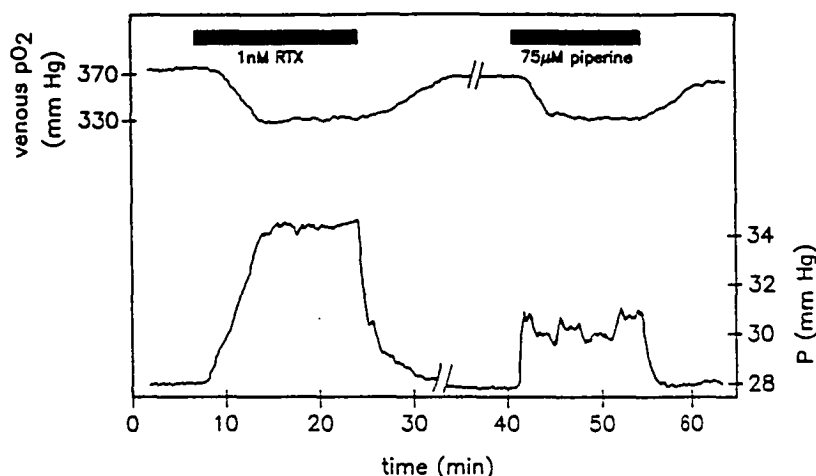
Fig. 2.

Typical time courses resulting from the infusion of RTX (1 nM) or piperine (75 µM). Tracings for venous pO_2 and perfusion pressure (P) are shown.

The effects of RTX and piperine on steady state $\dot{V}O_2$ and perfusion pressure as a function of concentration are shown in Figure 3. Dose curves for capsaicin (23) and [6]-gingerol (24) are also included. RTX was clearly the most potent, and piperine the least potent, agent used in this study. The molar potency of RTX was approximately 500-fold that of capsaicin, which in turn was 150-fold more potent than piperine (Fig. 3).

The effects of glyceryl trinitrate (GTN, a nitro-vasodilator) on stimulation of oxygen uptake and perfusion pressure at steady state are shown using time course plots (all $n=3$) in Figure 4. Values immediately prior to GTN infusion were significantly different ($P<0.05$) from those at the end of the GTN infusion period in which $\dot{V}O_2$-stimulatory concentrations of either RTX or piperine were used. Higher concentrations of GTN resulted in no further inhibitory effects (data not shown).

Typical traces of the tri-phasic oxygen responses of the vanilloids at high concentrations are shown in Figure 5. Both piperine (150 µM) and RTX (5 nM) caused inhibition of $\dot{V}O_2$ to sub-basal levels following initial transient stimulation. Removal of either agent during high concentration infusions resulted in large transient increases in $\dot{V}O_2$, whilst perfusion pressure returned to basal (Fig. 5).The magnitude of the inhibition and of the final transient $\dot{V}O_2$ were concentration-dependent (data not shown) in a fashion similar to that observed for other capsaicin-like molecules (24).
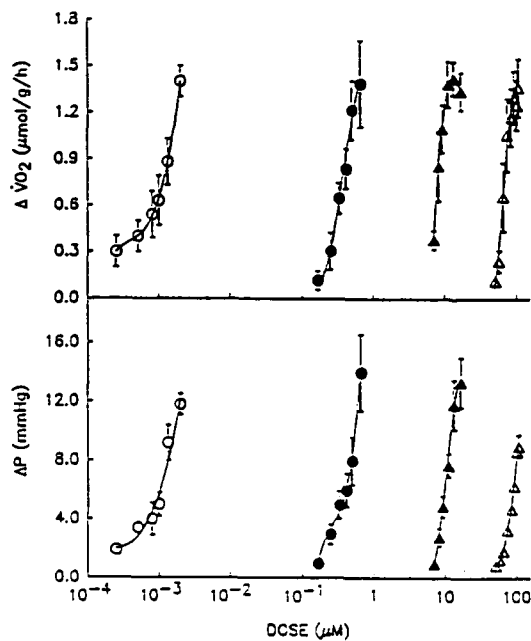
Fig. 3.

Dose response curves for changes in oxygen uptake ($\Delta\dot{V}O_2$) and perfusion pressure in response to RTX (O, n=8), capsaicin ($\bullet$, n=5-13, data taken from 23), (6)-gingerol ($\blacktriangle$, n=7, data taken from 24), and piperine ($\triangle$, n=8). The basal $\dot{V}O_2$ was 7.1 ± 0.4 μmol/g/h (n=24) and the basal perfusion pressure was 23.6 ± 0.9 mmHg (n=24). Each value represents the mean ± S.E. Where error bars are not visible they are within the symbol.
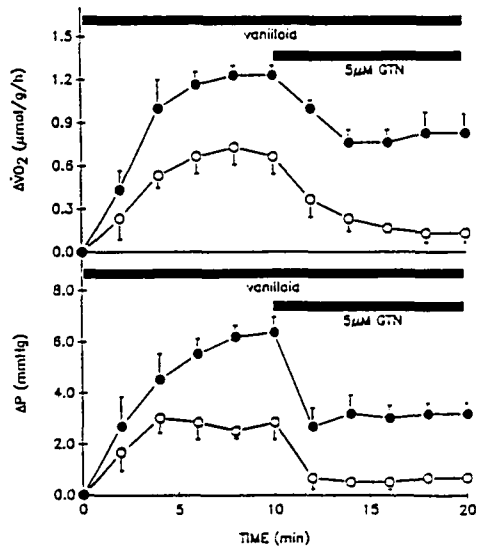
Fig. 4

Time course plots showing the effect of 5 μM GTN on changes in oxygen uptake (Δ $\dot{V}O_2$) and perfusion pressure (ΔP) induced by $\dot{V}O_2$-stimulatory concentrations of RTX ($\bullet$, 1 nM, n=3) and piperine (O, 75 μM, n=3).
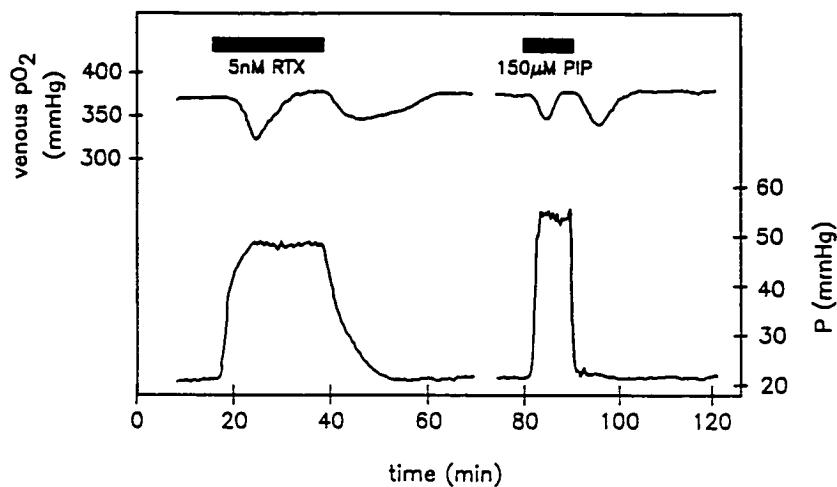
Fig. 5.

Typical time course tracings for the tri-phasic changes in venous pO_2 and perfusion pressure (P) induced by supra-maximal concentrations of RTX (5 nM) and piperine (150 µM, PIP).

The effects of a series of prolonged infusions with $\dot{V}O_2$-stimulatory concentrations of piperine are shown in Figure 6. There were no apparent changes in magnitude of the stimulated $\dot{V}O_2$ nor the increase in perfusion pressure throughout the series of infusions.
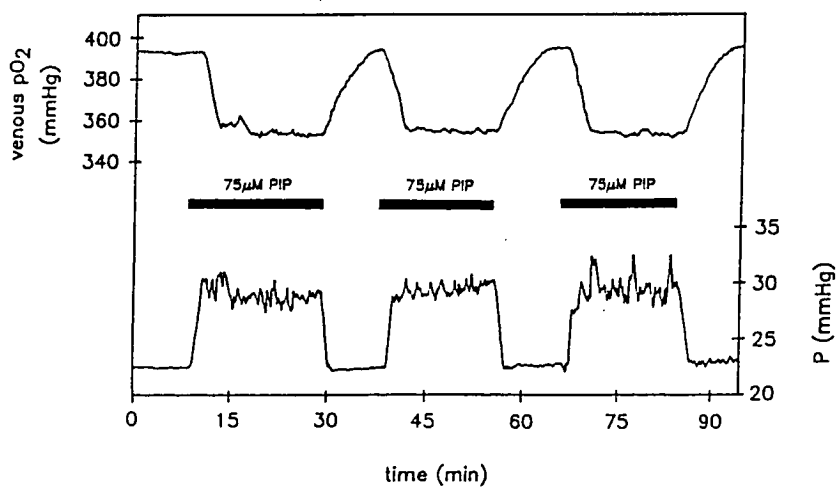
Fig. 6.

Time course tracings showing the effects of a series of prolonged infusions of piperine (75µM) on venous pO_2 and perfusion pressure.

Discussion

Both RTX and piperine stimulated the hindlimb to consume oxygen and the vascular bed to constrict in a manner similar to that of other active capsaicin-like compounds in this system (23,24). However, the molar potencies of RTX and piperine were vastly different. RTX, containing a classical vanillyl (3-methoxy 4-hydroxy phenyl) group (Fig. 1, V), was around 500-fold more potent than capsaicin, a result consistent with other studies which report RTX to be an ultra potent capsaicin analogue (reviewed in 2). Piperine, on the other hand, contains a vanillyl-like moiety in which the substituent groups form a secondary ring, giving a benzodioxolane fused ring system (Fig. 1, IV). The lower potency of piperine (approximately 150-fold less potent than capsaicin) may reflect the altered structure of this group.

High concentrations of both agents resulted in a steady-state inhibition of $\dot{V}O_2$ associated with increased perfusion pressure. We have previously found that the endogenous vasoconstrictors serotonin (29) and high concentrations of norepinephrine (30) also cause inhibited $\dot{V}O_2$ with elevated perfusion pressure in the same perfusion model. It has been proposed that these effects are the result of a redistribution of vascular flow. Studies are currently in progress to elucidate the mechanism by which capsaicin-like agents cause $\dot{V}O_2$ inhibition.

Concurrent infusion of GTN significantly blocked the $\dot{V}O_2$ and pressure increases induced by low doses of both RTX and piperine, indicating that the oxygen uptake is associated with the vasoconstriction. This result is consistent with previous work in this laboratory which has repeatedly demonstrated a relationship between the vascular system and increases in hindlimb $\dot{V}O_2$ (25-28).

RTX and capsaicin are known to stimulate a specific group of primary afferent neurons to release the vasodilatory neuropeptides substance P and calcitonin gene-related peptide (CGRP) from their peripheral endings (31). However, in the perfused rat hindlimb active capsaicin-like compounds cause net vasoconstriction. Other studies have found capsaicin to have either contractile or relaxing endothelium-independent effects on vascular smooth muscle (32,33). The likely explanation is that constriction and relaxation are both components of acute *in vitro* capsaicin treatment (33) Chronic pre treatment with capsaicin *in vivo* (thereby ablating capsaicin-sensitive sensory neurons) has resulted in isolated guinea pig vessels constricting rather than dilating when subsequently challenged with capsaicin *in vitro* (33). This suggested that contraction was the result of a direct action on vascular smooth muscle, whilst relaxation was due to the release of neuropeptides (32). The stimulatory responses induced by RTX and piperine in the present study are not subject to changes in magnitude following prolonged stimulations nor by repeated stimulations (Fig. 6). Such changes might be expected if afferent nerve fibres in the vessel walls were being subject to progressive depletion of vasodilatory neuropeptides.

Although the participation of receptors has yet to be unequivocally established, the relative potencies of the capsaicin-like compounds in the perfused rat hindlimb are consistent with those reported in neuropharmacological studies (reviewed in 2) where direct evidence of a vanilloid receptor has been obtained. Furthermore, the rapid kinetics of the responses (Fig. 2) is consistent with a receptor-mediated mechanism in the hindlimb vasculature. Previous work using the perfused rat hindlimb model has shown that noradrenaline-induced increases in oxygen uptake and perfusion pressure are blocked by adrenoreceptor antagonists (34). The use of specific α_1 (prazosin) and β_1/β_2 (propranolol) adrenoreceptor antagonists did not result in any diminution of the responses to the agents in this study (data not shown) nor in previous work with other capsaicin-like molecules

(23,24). These findings suggest that they were not acting directly on adrenoreceptors, nor were the actions due to secondary release of catecholamines.

Despite the thermogenic activity of capsaicin-like compounds *in vitro*, many such agents are reported to cause hypothermia *in vivo* in a variety of species (reviewed in 35). The hypothermic response is thought to be the due to stimulation of the warm-sensors of the preoptic/anterior hypothalamic area, thus impairing body temperature regulation (reviewed in 35). However, desensitization of these warm-sensors is readily achieved; a single subcutaneous dose of capsaicin (50-75mg/kg) in the rat results in the absence of capsaicin-induced hypothermia for several months. Furthermore, such desensitization results in pyrexia at room temperature and long periods of hyperthermia at high ambient temperatures. In addition, desensitized rats showed a pyrogen-induced increase in core temperature associated with increased $\dot{V}O_2$ and vasoconstriction. Attempts to synthesize analogs with antinociceptive but not hypothermic properties have met with some success (36), suggesting that hypothermia need not necessarily be a feature of all active capsaicin analogs.

Studies in the human in the absence of any prior desensitization procedures have found that meals containing capsaicin caused elevated body temperature during the first sleep cycle (37) and an increase in metabolic rate relative to a non-spicy control meal (38).

The notion of a vanilloid-sensitive thermogenic mechanism in the rat hindlimb raises the intriguing possibility of developing capsaicin analogs without hypothermic actions as anti-obesity agents *in vivo*. Such selectivity may be possible given that the afore-mentioned hypothermic effects are both readily desensitized and separable from other actions by means of structural manipulations.

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RESTING MUSCLE: A SOURCE OF THERMOGENESIS CONTROLLED BY VASOMODULATORS

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Summary

Perfused, but neither incubated nor perifused, hindlimb muscle responds to a variety of vasomodulators, including noradrenaline, by rapidly altering the rate of oxygen consumption and metabolite release. The vascular tissue of muscle is identified as highly energetic and may be the major contributor to hindlimb thermogenesis. In addition, vasomodulators may control the delivery of nutrients to specialized skeletal muscle mitochondria by altering the microvascular distribution of flow. We propose that resting skeletal muscle contributes to whole body thermogenesis of endotherms and that it is controlled by total, as well as zonal (within muscle), nutrient delivery.

Introduction

Endothermic animals invoke heat producing mechanisms often referred to as facultative thermogenesis, in response to either cold or (over)eating. The mechanisms appear separate from shivering and involve, in many cases, an increase in sympathoadrenal activity. A starting point for unravelling the processes of facultative thermogenesis has been the observation that noradrenaline when injected *in vivo*, rapidly (within seconds or minutes) stimulates whole body oxygen uptake. In rats, oxygen uptake (and therefore thermogenesis) increases by up to 100 per cent when noradrenaline is injected [1]. In addition it has been assumed that the effect of noradrenaline *in vivo* can be effectively mimicked *in vitro* by exposing individual tissues to noradrenaline. For brown adipose tissue this is certainly the case and all preparations (tissue fragments, isolated cells and slices) respond markedly to the addition of catecholamine with values for oxygen uptake and heat production consistent with estimates for this tissue *in vivo* [2]. Other tissues such as liver [3] also respond positively to noradrenaline but in this case the hormone has a general effect to increase a diverse range of metabolic interconversions.

Skeletal muscle has been an enigma to researchers who study thermogenesis. It constitutes over 40 per cent of the body's mass and when working has the potential to be markedly thermogenic. Unlike brown adipose tissue, isolated muscles when incubated or perifused *in vitro* with noradrenaline do not respond by showing an increase in oxygen uptake or heat flux [4,5]. However several research groups have reported that infused sympathomimetic substances increased oxygen uptake in non-contracting skeletal muscle receiving its nutrient supply by the normal vascular route. These groups included Lundholm and Svedmar in 1965; Sutherland and Robison in 1966; and Schmitt, Meunier, Rochas, and Chatonnet in 1973 Mejsnar and Jansky in 1973; Grubb and Folk in 1977; Chapler, Stainsby, and Gladden in 1980; Richter, Ruderman, and Galbo in 1982 and Côté, Thibault and Vallières in 1985 (cited in Ref. 6, or references therein). For the perfused rat hindlimb the effects produced by noradrenaline were marked, showing rapid increases of 39 to 111% over basal oxygen uptake. Calculations from these figures indicate that oxygen uptake from the skeletal muscle would be at least 40% of the total oxygen uptake that could be contributed by brown fat. In addition data of Foster and Frydman in 1979 (cited in Ref. 6), which focused particular attention in the whole animal on BAT,also indicated a substantial role for skeletal muscle especially in warm-adapted and normal rats. These animals showed a 30% increase in skeletal muscle blood flow and a 60% increase in muscle oxygen consumption after noradrenaline administration, suggesting that muscle could produce an equal amount of heat to BAT in the whole animal, even though oxygen consumption was much less per gram of tissue.

Since the effects of noradrenaline and adrenaline on oxygen uptake by perfused rat hindlimb were mediated by α-adrenergic receptor mechanisms, and were associated with increased perfusion pressure (Grubb and Folk 1977; Richter *et al.* 1982a; Richter *et al.* 1982b; Côté *et al.* 1985, cited in Ref. 6, or references therein) it appeared possible that the increased oxygen uptake was controlled by the vascular system. Thus this communication presents our findings on the effects of various vasomodulators on perfused hindlimb, not only from the rat but from the chicken and a small Australian marsupial *Bettongia gaimardi* (bettong).

Materials and Methods

The rat hindlimb was perfused as described previously (see Ref. 6 and references therein). The lower hindlimb (16.8 $\pm$ 0.6 g) of 5-8 week old chickens (598 $\pm$ 26 g body wt) was perfused via the popliteal artery using conditions identical to that used for the rat. Similar procedures were used for the perfusion of the lower hindlimb (24.7 $\pm$ 2.8 g) of bettongs (1,130 $\pm$ 0.13 g body wt). For each preparation, perfusion pressure and venous PO_2 were continuously monitored using in-line arterial pressure transducer and venous O_2 electrode, respectively. Details for stimulation of the lower calf muscles in the perfused rat hindlimb are given elsewhere [7]. Venous samples were collected for lactate and glycerol assays. Lactate was determined

Table 1. Vasomodulator effects on perfused rat hindlimb[a]

Parameter:	Vasoconstrictors	
	Type A[b]	Type B[c]
Perfusion pressure	↑	↑
Oxygen uptake	↑	↓
Lactate efflux	↑	↓
Glycerol efflux	↑	↓
Urate efflux	↑	↓
Uracil efflux	↑	↓
Insulin mediated glucose uptake	n.t.[d]	↓
Skeletal muscle contraction	n.t.[d]	↓
Perfusate distribution volume	↑	↓
Effect of the following on vasoconstriction and associated changes:		
Removal of external Ca^{2+}	B[e]	NB[e]
Replacement of O_2 by N_2	B	NB
Addition of N_3^-, CN^-	B	NB
Addition of vasodilators	B[f]	B[g]

a. Hindlimb perfused at 25°C with constant flow. Data is from references 6-10 or references therein, or is unpublished.

b. Includes α adrenergic agonists: noradrenaline, adrenaline, phenylephrine, methoxamine, amidephrine, norephedrine, ephedrine; peptides: vasopressin, angiotensins I, II, III, oxytocin, neuropeptide Y; "vanilloid" agonists: capsaicin, dihydrocapsaicin, gingerols, shogaols, piperine, resiniferatoxin. Also low frequency sympathetic nerve stimulation.

c. Includes serotonin (≥0.1 μM), noradrenaline at high doses (≥1 μM), high dose vanilloids and high frequency sympathetic nerve stimulation.

d. Not tested.

e. B = blocked; NB = not blocked.

f. Includes nitroprusside, nifedipine, isoprenaline, adenosine, AMP, ADP, ATP and UTP.

g. Includes nitroprusside, carbamyl choline and isoprenaline (partial blockade).

Figure 1. α-Adrenergic stimulation of glycerol production by the perfused rat hindlimb. Perfusions were conducted at 25°C and contained 20 μM DL-propranolol.
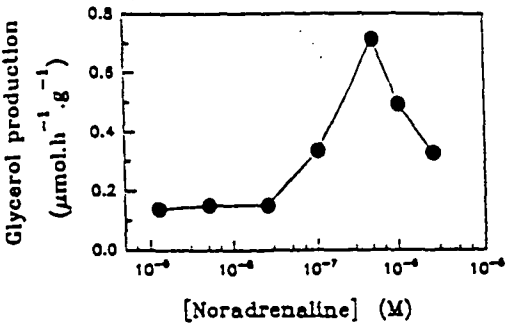
spectrophotometrically and glycerol spectrofluorometrically using standard enzymatic procedures.

Results and Discussion

Table 1 summarizes our findings and shows that the perfused rat hindlimb responds to many different vasoconstrictors that fall into two categories which we have called Type A and B. Type A vasoconstrictors stimulate a marked increase in oxygen consumption simultaneously with a rise in perfusion pressure in the rat hindlimb perfused at constant flow [6,10 and references therein]. The Type A category is associated with other changes including increased lactate [5], glycerol (Figure 1), urate [7], and uracil [7] efflux as well as increased perfusate distribution volume (unpublished). The vascular sites responsible for Type A vasoconstriction require external Ca^{2+} and oxygen; in the absence of either moiety or with respiratory poisons present, the pressor effect of Type A vasoconstrictors does not occur [9]. Table 1 also shows that vasodilators with differing modes of action block the vasoconstrictors, inhibiting the increases in perfusion pressure and oxygen uptake as well as the metabolic changes. It is important to note that the effect of the Type A vasoconstrictors to increase oxygen uptake is additive to the oxygen uptake due to skeletal muscle contraction and that the nitrovasodilators are selective, having no effect on the latter. Alpha and α_1-antagonists block sympathomimetic stimulation of oxygen and pressure but not the actions of other vasoconstrictors such as angiotensin II or capsaicin. Beta antagonists augment the action of most sympathomimetic vasoconstrictors especially those recognized as having significant beta actions. The increased glycerol release mediated by noradrenaline in the presence of propranolol (Figure 1) relates closely to the α_1-adrenergic effect of this catecholamine to cause vasoconstriction and oxygen uptake and implies that both of these may be supported in part by fatty acid oxidation.

Recently our hindlimb experiments have included species where the presence of brown adipose tissue is doubtful. Table 2 shows that constant-flow perfused hindlimbs from either the chicken or the bettong, respond positively to noradrenaline with increased pressure and oxygen uptake. This implies that resting muscle thermogenesis may be widely used amongst the endotherms regardless of the presence, or absence, of brown fat. The data of Tables 1 and 2 and Figure 1, together with rat hindlimb perfusions at varying but fixed flows and fixed pressures (Ye et al. 1990, cited in Ref. 6) suggests that working vascular tissue may be responsible for the Type A vasoconstrictor-induced increase in oxygen uptake. However for this to be so, the vascular smooth muscle cells responsible must be capable of high rates of oxygen consumption under load and be present in sufficient quantity in the hindlimb to account for the rates of oxygen consumption noted.

Finally Table 1 also identifies a group of vasoconstrictors (Type B) that lead to decreased oxygen consumption with increased vascular resistance in the constant-flow perfused rat hindlimb. This group includes serotonin, noradrenaline at high doses (similar to predicted concentrations at vascular smooth muscle synapses), high frequency sympathetic nerve stimulation and high dose vanilloids. In all respects the metabolic effects of the Type B vasoconstrictors are the opposite to those of Type A and are therefore potentially negatively thermogenic. We have proposed that Type B vasoconstrictors result in functional vascular shunting which coincides with reduced nutritive flow even though overall flow through the hindlimb remains unaltered [9]. The sites (vessels) controlling vascular shunting appear to be distinguishable in terms of their fuel and Ca^{2+} requirements (Table 1). Neither serotonin nor high dose noradrenaline require oxygen for vasoconstriction if glucose is present [9] and vasoconstriction is reduced but still present if Ca^{2+} is omitted from the buffer [9].

Table 2. Effects of noradrenaline on perfused hindlimbs[a]

Species	Perfusion pressure (mm Hg)		Oxygen uptake (μmol/g per h)	
	Basal	Noradrenaline[b]	Basal	Noradrenaline[b]
Rat	29±1	45±2[c]	6.4±0.2	9.6±0.3[c]
(n)	(19)	(3)	(19)	(3)
Chicken	39±4	56±3[c]	7.2±0.3	8.9±0.3[c]
(n)	(6)	(6)	(6)	(6)
Bettong	31±2	83±10[c]	4.6±0.4	9.5±0.8[c]
(n)	(5)	(5)	(5)	(5)

a. All perfusions were conducted with constant-flow of approx. 0.28 ml/g per min at 25°C with medium containing 2% serum albumin and 1.27 mM $CaCl_2$ (Ref 6. and references therein). Data for rat from Colquhoun *et al*. 1988 in Ref. 6.

b. Maximum oxygen uptake occurred at 50, 20 and 1000 nM noradrenaline for rat, chicken and bettong hindlimbs, respectively.

c. Significantly greater ($P<0.05$) than companion "basal" values.

We propose that resting muscle has the potential to contribute to whole body thermogenesis in endotherms and its contribution, in either a positive or negative manner, is controlled by the vascular system and cannot be observed with isolated incubated preparations. In addition to the effects of noradrenaline to increase oxygen uptake by constant-flow perfused muscle mediated by α_1-adrenergic receptors, increases in total flow to muscle *in vivo* e.g.

resulting from increased cardiac output (β-adrenergic receptor-mediated) have the potential to increase the thermogenic contribution by skeletal muscle.

Overall the results are consistent with our earlier proposals that work performed during constriction and resting flow [6] by the vascular smooth muscle ("hot pipes") of the hindlimb may account for the increase in oxygen consumption. The release of lactate, and to a lesser extent glycerol, during vasoconstriction have the potential to add further to thermogenesis *in vivo*. If vascular tissue does not consume sufficient oxygen, the data still suggest that it plays a key role in controlling thermogenesis by neighbouring skeletal muscle mitochondria. Thus increased oxygen uptake may be the result of vasoconstrictor-induced change in the distribution of flow so as to supply oxygen to previously unaccessed regions of muscle that contain specialized mitochondria adapted for thermogenesis. Vasoconstrictors that act to inhibit muscle oxygen uptake might do so by opening functional vascular shunts diverting flow away from thermogenic vasculature on thermogenic skeletal muscle mitochondria.

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Potential defect in the vascular control of nonshivering thermogenesis in the obese Zucker rat hind limb

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Vascular control of nonshivering thermogenesis in the perfused hind limb of obese and lean Zucker rats was compared using two vasoconstrictors, norepinephrine and serotonin. For hind limbs of both phenotypes, norepinephrine infusions resulted in a dose-dependent uninterrupted increase in perfusion pressure and a biphasic change in oxygen uptake ($\dot{V}O_2$), characterized by a stimulation at low concentrations, and an increasing inhibition at higher concentrations that gradually overcame the stimulation in a dose-dependent manner. At concentrations of norepinephrine greater than 1 μM, the inhibitory effect predominated and gave rise to values for $\dot{V}O_2$ less than basal. The obese hind limb had a lower basal $\dot{V}O_2$ and a lower maximal $\dot{V}O_2$ mediated by norepinephrine than the lean rat, but these differences appeared to relate largely to the lower muscle mass and higher content of fat of the obese hind limb. Serotonin infusions resulted in a dose-dependent increase in perfusion pressure and an accompanying decrease in $\dot{V}O_2$. Pressure changes were identical for the obese and lean hind limbs, but the decrease in $\dot{V}O_2$ due to serotonin was greater in the hind limbs from the lean rats, and this difference remained when the data were expressed in terms of muscle mass perfused. It is concluded that the relatively lower muscle content of the obese hind limb accounts for its lower basal and lower maximal norepinephrine-mediated thermogenesis. In addition, an intrinsic defect in obese hind limb muscle response to serotonin is present, which may be indicative of a decrease in the potential for vasoconstrictor-regulated thermogenesis that could have implications for whole-body energy balance by the obese phenotype.

Key words: muscle thermogenesis, muscle oxygen uptake, genetically obese rat, norepinephrine, serotonin.

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On a comparé le contrôle vasculaire de la thermogenèse sans frisson dans le membre postérieur perfusé de rats Zucker maigres et obèses en utilisant deux vasoconstricteurs, la norépinéphrine et la sérotonine. Dans les membres postérieurs des deux phénotypes, la perfusion de norépinéphrine a provoqué une augmentation continue dose-dépendante de la pression de perfusion et une variation biphasique de la capture d'oxygène ($\dot{V}O_2$), caractérisées par une stimulation à faibles concentrations, en plus de provoquer une inhibition croissante aux concentrations plus élevées, qui a éliminé graduellement la stimulation en fonction de la dose utilisée. Aux concentrations de norépinéphrine supérieures à 1 μM, l'effet inhibiteur a prédominé, donnant lieu à une augmentation aux valeurs de $\dot{V}O_2$ inférieures aux valeurs de base. La norépinéphrine a induit une $\dot{V}O_2$ basale et une $\dot{V}O_2$ maximale plus faibles dans les membres postérieurs obèses que dans les membres postérieurs maigres, mais ces différences ont semblé être reliées en grande partie à la plus faible masse musculaire et à la plus haute teneur en gras des membres postérieurs obèses. La perfusion de sérotonine a provoqué une augmentation dose-dépendante de la pression de perfusion et une diminution concomitante de la $\dot{V}O_2$. Les variations de pression ont été identiques pour les membres postérieurs maigres et obèses, mais la diminution de $\dot{V}O_2$ induite par la sérotonine a été supérieure dans les membres postérieurs des rats maigres; de plus, cette différence s'est maintenue lorsque les données ont été exprimées en termes de la masse musculaire perfusée. On conclut que la teneur musculaire relativement plus faible du membre postérieur obèse explique sa thermogenèse maximale et basale plus faible en présence de norépinéphrine. De plus, la présence d'une anomalie intrinsèque de la réponse du muscle du membre postérieur obèse à la sérotonine pourrait être un indice de diminution du pouvoir de régulation de la thermogenèse par les vasoconstricteurs, ce qui pourrait avoir des répercussions sur l'équilibre énergétique total du phénotype obèse.

Mots clés : thermogenèse musculaire, capture d'oxygène par le muscle, rats génétiquement obèses, norépinéphrine, sérotonine.

[Traduit par la Rédaction]

Introduction

Major differences exist between the obese Zucker (*fa/fa*) rat and its lean counterpart (*Fa/?*). The obese animals exhibit hyperphagia (Cleary et al. 1980), hyperinsulinemia (Tukenkopf et al. 1982), decreased whole-body $\dot{V}O_2$ at ambient tempera-

tures of 10–30°C (Kaplan 1979), decreased low protein diet induced thermogenesis (Young et al. 1980), and a lower energy requirement (Mowrey and Hershberger 1982).

At the tissue level, the *fa/fa* rat has defective brown adipose tissue (Levin et al. 1984), but differences in the metabolic properties of other tissues, including muscle, may also exist. Perfusion studies (Kemmer et al. 1979) indicate that the hind

limb of the *fa/fa* rat has diminished basal glucose uptake.
markedly diminished insulin-mediated glucose uptake. dimin-
ished lactate oxidation. and exaggerated lactate release com-
pared with that of the lean counterparts. However, the
thermogenic properties of perfused hind limb from obese
Zucker rats have not been systematically assessed. even
though perfused hind limbs from nonobese strains show
marked responses to norepinephrine in terms of increased oxy-
gen uptake (Côté et al. 1985; Grubb and Folk 1976; Richter
et al. 1982; Colquhoun et al. 1988; Dora et al. 1992). Previous
work from this laboratory on nonobese strains has led to the
proposal that muscle thermogenesis is regulated by vasocon-
strictors that act to alter the distribution of nutritive flow
(Clark et al. 1994). Vasoconstrictors were of either of two
types. Type A vasoconstrictors, which included norepineph-
rine at low dose, vasopressin, angiotensin II, and several
others, increased oxygen consumption of the constant flow
perfused rat hind limb of a nonobese strain (Clark et al. 1994).
Type B vasoconstrictors, which included norepinephrine at
high dose (≥ 1 μM), serotonin, and others, decreased oxygen
consumption in the same preparation. Thus type A or type B
vasoconstrictors were proposed to increase or decrease.
respectively, the extent of nutritive flow within muscle (Clark
et al. 1994).

The objectives of the present study were thus twofold. The
first objective was to determine whether a phenotypic differ-
ence existed in perfused muscle thermogenic response to
norepinephrine. The second was to determine the response to
serotonin by perfused hind limbs, which in conjunction with
the data from norepinephrine perfusions would allow a com-
parative assesssment of the total nonshivering thermogenic
capacity of the hind limb controlled by the vascular system.

Methods

Animal care

Animals were housed and cared for in accordance with the princi-
ples of the *Guide to the Care and Use of Experimental Animals*.
Vol. 1 (Canadian Council on Animal Care 1980). Experiments were
performed using 20-week-old male Zucker genetically obese (*fa/fa*).
500- to 600-g rats. when the obesity and insulin resistance were well
established. and age-matched lean male animals (*Fa/?*. 340–365 g).
Rats were obtained from Harlan Olac Ltd.. Bicester. Oxfordshire.
U.K.. and housed in groups under climate-controlled conditions
(20 ± 2°C. 12 h light : 12 h dark cycle) and provided with R&M 1
(rat and mouse diet). made by SDS. Manea. Cambridgeshire. U.K..
and water ad libitum. Details concerning the nonobese hooded Wistar
rats can be found elsewhere (Dora et al. 1991. 1992).

Materials

Bovine serum albumin (fraction V). norepinephrine bitartrate.
serotonin hydrochloride. and Evans blue were obtained from the
Sigma Chemical Company (U.K.). Heparin sodium (5000 U/mL)
was obtained from CP Pharmaceuticals Ltd. (U.K.). and sodium pen-
tobarbitone was obtained from RMB Animal Health Ltd. (U.K.).

Isolated hind limb preparation

Animals were given an intraperitoneal injection of heparin sodium
(250 U/100 g) and then anesthetized with an intraperitoneal injection
of pentobarbitone sodium (6 mg/100 g body weight). Rats were then
pithed and maintained on a respirator via a tracheal tube. Surgery was
performed as described previously (Colquhoun et al. 1988). Flow
was restricted to one hind limb by ligating the contralateral common
iliac artery.

Perfusion medium

The perfusion medium was an erythrocyte-free modified Krebs–
Ringer bicarbonate buffer. essentially as described previously

(Colquhoun et al. 1988). containing 1.27 mM $CaCl_2$. 8.3 mM glu-
cose. and 2% undialysed bovine serum albumin.

Perfusion procedures

The perfusion buffer reservoir was kept on ice and gassed with
95% O_2 – 5% CO_2 while stirring. Perfusate was pumped at a fixed
flow rate by a peristaltic pump adjusted at the start of each experiment
to give comparable venous Po_2 values (nominally set at a minimum
of 350 mmHg (1 mmHg = 133.3 Pa) to ensure adequate O_2 supply.
as established in previous studies (Ye et al. 1990)). The temperature
of the perfusate was raised to 25°C in a heat exchanger prior to pass-
ing through a silastic lung gassed with 95% O_2 – 5% CO_2.
Homeothermic blankets (Harvard. South Natick. Mass.) and water
jackets ensured that the hind-limb preparation. the surrounding
perfusate-containing tubing. and the oxygen electrode remained at
25°C. A temperature probe positioned beneath the skin adjacent to
the perfused muscle controlled the operation of the homeothermic
blankets. When required. agonists were infused continuously (Gilson
Minipuls 3 with microbore tubing) into a small stirred bubble trap
proximal to the arterial cannula. The infusion rates gave 1 in 200 dilu-
tions. Infusion of vehicle (0.1% ascorbic acid in isotonic saline) had
no apparent effect on Vo_2 or perfusion pressure.

$\dot{V}o_2$ and perfusion pressure determinations

Oxygen tension in the venous perfusate was monitored continu-
ously using an in-line Clark-type oxygen electrode of 0.5-mL capac-
ity. The oxygen electrode was maintained at 25°C. The arterial Po_2
remained constant throughout each experiment. The oxygen electrode
was calibrated before and after each experiment. using recirculating
buffer gassed with 95% O_2 and then air. Calculation of Vo_2 was per-
formed as previously described (Colquhoun et al. 1988). Perfusion
pressure was monitored continuously at the bubble trap proximal to
the arterial cannula using a fluid-filled transducer (CEC Instrumenta-
tion Ltd.. U.K.).

Determination of perfused hind limb tissue

Upon completion of a perfusion. all agonists were removed. and
after conditions returned to basal. a 1% solution of Evans blue was
injected into the arterial cannula at a flow rate similar to that used
throughout the experiment. The resultant stained tissue was then
excised. blotted dry. dissected into muscle and fat. and weighed to
determine the amounts of tissue perfused.

Statistics

Significance of difference between lean and obese perfused hind
limbs was assessed by using the unpaired Student's *t* test. Values are
means ± SE for *n* = 3 for each group of lean + norepinephrine. lean
+ serotonin. obese + norepinephrine. and obese + serotonin. For
the nonobese hooded Wistar rats *n* = 5 for + norepinephrine. *n* = 4
for + serotonin. and *n* = 24 for control (basal conditions).

Results

Differences between obese and lean Zucker rats

Progeny of the Zucker strain display two phenotypes. which
manifest as obese homozygotes (*fa/fa*) and a mixture of lean
animals. which are either homozygotes for leanness (*Fa/FA*)
or heterozygotes (*Fa/fa*). Significant differences for the male
age-matched Zucker rats used in this study included body
weight. heart weight. and the tissue composition of the hind
limb (Table 1). The perfused tissue of the hind limb of the
obese (*fa/fa*) animals (deduced by dye filling) was composed
of significantly less muscle and significantly more fat and. in
total. weighed significantly more than the hind limb of the lean
(*Fa/?*) animals (Table 1). The proportion of fat to muscle of
the lean Zucker rat hind limb (10.6 ± 0.7% fat) was slightly
higher than that for 6- to 8-week-old nonobese hooded Wistar
rats (4.3 ± 0.1% fat). Differences in the proportion of hind-
limb fat to muscle between *fa/fa* and *Fa/?* were taken into

TABLE 1. Body mass, heart mass, and perfused hind limb analysis of obese and nonobese Zucker rats

Phenotype	n	Body mass (g)	Heart mass (g)	Perfused hind limb		
				Muscle (g)	Fat (g)	Total (g)
Obese	6	566.7 ± 15.5	1.80 ± 0.08	22.03 ± 0.72	12.18 ± 0.82	34.22 ± 0.84
Lean	6	350.8 ± 4.5^a	1.45 ± 0.09^c	25.94 ± 1.08^c	2.79 ± 0.25^a	28.73 ± 1.04^b

NOTE: Values are means $\pm$ SE for 20-week-old male obese and lean rats. Total is defined as the sum of dye-containing perfused muscle and fat dissected from the hind limb following perfusion with Evans blue, and excludes skin and bone. $^a p < 0.0001$. $^b p < 0.01$. $^c p < 0.05$, significantly different from obese.

TABLE 2. Basal perfusion pressure and rate of oxygen uptake by hind limbs of obese and nonobese Zucker rats

Phenotype	n	Flow rate ($mL \cdot min^{-1}$)	Pressure (mmHg)	Venous P_{O_2} (mmHg)	$\dot{V}_{O_2}$ ($\mu mol \cdot h^{-1} \cdot g^{-1}$)
Obese	6	0.200 ± 0.012	28.3 ± 1.6	419.1 ± 12.6	4.42 ± 0.30
		(0.312 ± 0.016)			(6.80 ± 0.34)
Lean	6	0.230 ± 0.015	25.4 ± 1.7	360.0 ± 7.8^a	6.38 ± 0.25^b
		(0.257 ± 0.017)			(7.07 ± 0.32)

NOTE: Values are means $\pm$ SE and have been calculated on the basis of the perfused mass of muscle plus fat of the hind limbs, as shown in Table 1. Values in parentheses are expressed in terms of the perfused mass of muscle only. Arterial P_{O_2} was 663.7 ± 3.3 ($n = 12$). $^a p < 0.01$. $^b p < 0.05$, significantly different from obese.

account in expression of the data (see below); some apparent differences were not significant when muscle was assumed to be the sole tissue responsible for hind-limb $\dot{V}_{O_2}$.

Table 2 gives basal (pre-norepinephrine and pre-serotonin) properties of the perfused hind limibs of the obese and lean rats. The flow rate, which was constant throughout each perfusion, determined the resting or basal parameters of perfusion pressure, venous P_{O_2}, and thus $\dot{V}_{O_2}$. Table 2 shows that $\dot{V}_{O_2}$ for the obese hind limb was significantly less than that for the lean hind limb when expressed in terms of total tissue perfused, but not so when expressed in terms of the mass of muscle perfused. Basal $\dot{V}_{O_2}$ for the whole hind limb was also significantly less ($p < 0.05$; $n = 6$) for the obese (151.2 ± 10.3 $\mu mol \cdot h^{-1}$ per hind limb) than the lean (183.3 ± 7.2 $\mu mol \cdot h^{-1}$ per hind limb) animals.

Effects of norepinephrine in perfused obese and lean hind limbs

Norepinephrine caused a marked vasoconstriction in the perfused rat hind limb of both phenotypes. Figure 1 shows dose-dependent rises in pressure to greater than 200 mmHg for perfused hind limbs from obese and lean Zucker rats. Dose – response curves for each hind limb were constructed using stepwise increasing doses of infused norepinephrine. At each dose, the increase in pressure remained constant provided the dose remained constant (data not shown). Figure 1 shows that at each dose of norepinephrine the pressure development by the obese hind limb tended to be greater than that of the lean hind limb. The difference was statistically significant at 32 nM norepinephrine.

The dose-dependent rise in perfusion pressure due to norepinephrine contrasts with the effect of this catecholamine on $\dot{V}_{O_2}$. Figure 2 shows that the steady-state $\dot{V}_{O_2}$ response has essentially two components, both of which were present in the obese as well as the lean hind limb. These components comprise a steady-state stimulatory phase evident over a concen-

tration of $3.2 - 100$ nM norepinephrine, and a steady-state inhibitory phase commencing at concentrations greater than 100 nM norepinephrine and extending to the maximum concentration used ($3.2 \mu M$). It is important to note that at concentrations greater than 1 μM norepinephrine, the value for $\dot{V}_{O_2}$ was less than basal (pre-norepinephrine). In Fig. 2A the results are expressed in terms of the total perfused tissue. The upper trace shows absolute values, and significant differences between the obese and lean hind limbs are readily apparent. Hind limbs from obese Zucker rats have lower basal values and are significantly less responsive to norepinephrine in terms of increased $\dot{V}_{O_2}$, reaching only 72% of the absolute values for $\dot{V}_{O_2}$ obtained by the lean hind limbs. In addition, the inhibitory effect of norepinephrine over the range 100 nM to 3.2 μM is less pronounced with the $\dot{V}_{O_2}$ of the obese hind limb, decreasing from a maximum of 8.03 ± 0.45 to 3.53 ± 0.21 $\mu mol \cdot h^{-1} \cdot g^{-1}$ of total perfused tissue. Over the same concentration range of norepinephrine, $\dot{V}_{O_2}$ by the lean hind limb decreased from 11.03 ± 0.71 to 4.20 ± 0.22 $\mu mol \cdot h^{-1} \cdot g^{-1}$ of total perfused tissue. Thus the obese hind limb response was approximately 66% of that of the lean. Normalizing the data to the basal (pre-norepinephrine) rate shows that the shape of the dose – response curves are indistinguishable except for the greater inhibitory effect of norepinephrine in lean hind limb at maximal doses (Fig. 2A, lower trace). When the data for $\dot{V}_{O_2}$ were expressed in terms of the mass of muscle perfused none of the differences noted above for the hind limbs was statistically significant (Fig. 2B).

Vasodilator blockade of the norepinephrine-mediated thermogenesis

A time course for the effect of isoproterenol and sodium nitroprusside on norepinephrine-mediated decrease in venous P_{O_2} and increase in perfusion pressure for the obese hind limb is shown in Fig. 3. The vasoconstrictor action of norepinephrine was associated closely with an increase in $\dot{V}_{O_2}$ as

FIG. 1. Effect of norepinephrine on perfusion pressure of constant-flow hind limbs of obese and lean Zucker rats. Basal (pre-norepinephrine) values for perfusion pressure were as given in Table 2. When not visible, error bars are within symbol. *$p < 0.05$, significantly different from obese.

seen by the decrease in venous Po_2. Infusion of a maximal dose of isoproterenol partially blocked, and a maximal dose of sodium nitroprusside completely blocked, both effects mediated by norepinephrine (Fig. 3). The increases in Vo_2 and perfusion pressure due to norepinephrine were completely reversible and returned to basal values when the catecholamine was removed (data not shown).

Effects of serotonin in perfused obese and lean hind limbs

Figure 4 shows the effect of serotonin on perfusion pressure in constant-flow hind limbs of the obese and lean Zucker rats. The concentration–response curves were similar, with each reaching a maximum pressure of approximately 190 mmHg over basal at 3.2 μM serotonin. This contrasts with the effect of serotonin on Vo_2 in these hind limbs (Fig. 5). For both obese and lean hind limbs, serotonin (10 nM – 3.2 μM) reduced Vo_2. When expressed as a function of the total mass of perfused tissue, the obese hind limb response was only 48% of that of the lean (Fig. 5A), decreasing from 3.93 ± 0.28 to 2.30 ± 0.37 μmol · h^{-1} · g^{-1} of total perfused tissue. Over the same concentration of serotonin (10 nM – 3.2 μM), Vo_2 by the lean hind limb decreased from 5.83 ± 0.26 to 2.67 ± 0.48 μmol · h^{-1} · g^{-1} of total perfused tissue.

Figure 5B shows that when expressed on the basis of the mass of perfused muscle, the differences in response to serotonin remained significant. Thus, over the concentration range of 10 nM – 3.2 μM serotonin, the decrease in Vo_2 by the obese hind limb was approximately 70% of that of the lean.

Discussion

Two findings emerge from the present study that have implications for whole-body thermogenesis of the obese Zucker rat. Firstly, the constant flow perfused hind limb of the obese animal, when compared with that of the lean, has a lower basal Vo_2 and lower maximal Vo_2 mediated by norepinephrine (Fig. 6). These differences appear to result directly from the
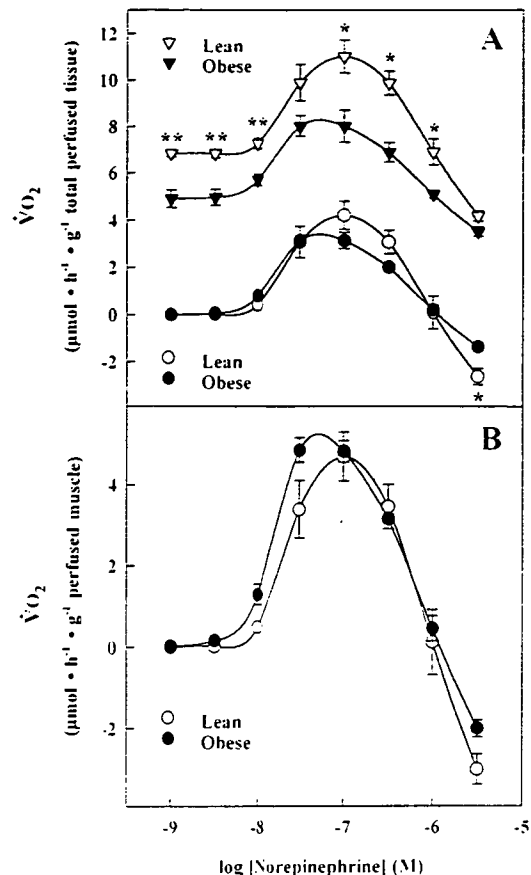
FIG. 2. Effect of norepinephrine on oxygen uptake by constant flow perfused hind limbs of obese and lean Zucker rats. (A) Absolute Vo_2 values (▼, ▽) and changes in Vo_2 (●, ○), calculated as a function of the total mass of perfused tissue (Table 1). (B) Rates expressed as a function of the mass of perfused muscle. When not visible, error bars are within symbol. *$p < 0.05$, **$p < 0.01$, significantly different from obese.

lower content of muscle mass in the obese hind limb and do not reflect intrinsic differences between muscle from lean and obese phenotypes. However, the findings imply that for lean and obese Zucker rats of equal body mass the basal and fully stimulated thermogenic potential of muscle is less in the obese phenotype and in proportion to the mass of muscle present. Secondly, the constant flow perfused hind limb of the obese animal has a diminished inhibitory response to high concentrations of norepinephrine and to serotonin in terms of Vo_2, and this effect appears to be intrinsic to the muscle. Taken together, the similar response to low concentrations of norepinephrine and decreased response to a high dose of norepinephrine and to serotonin by obese muscle, compared with that of the lean, suggests that vasoconstrictor-regulated thermogenesis in the obese Zucker rat is altered.

Data from Figs. 2 and 5 as well as our previous studies using various vasoconstrictors and vasodilators (Clark et al. 1994)
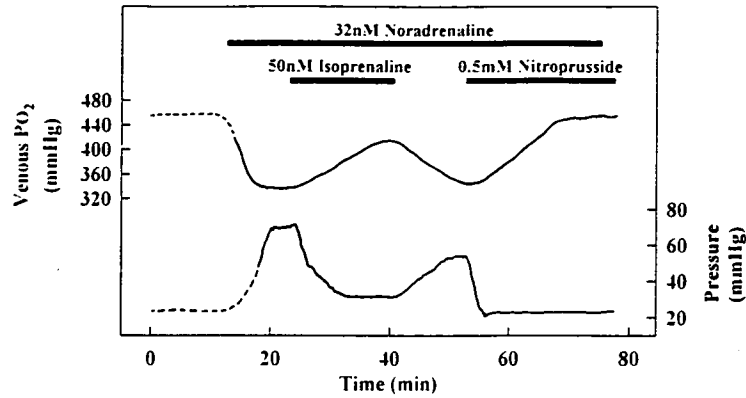
FIG. 3. Effect of vasodilators, isoproterenol, and sodium nitroprusside on norepinephrine-mediated decreases in venous Po_2 and perfusion pressure by perfused hind limbs of obese Zucker rats. The data were obtained after the dose–response curve for norepinephrine had been completed. The trace shown is a selection from three similar experiments.

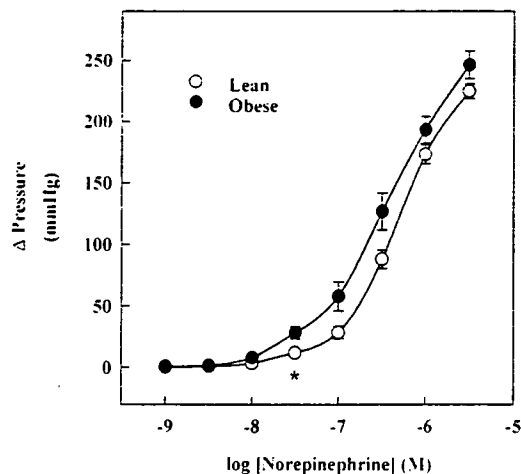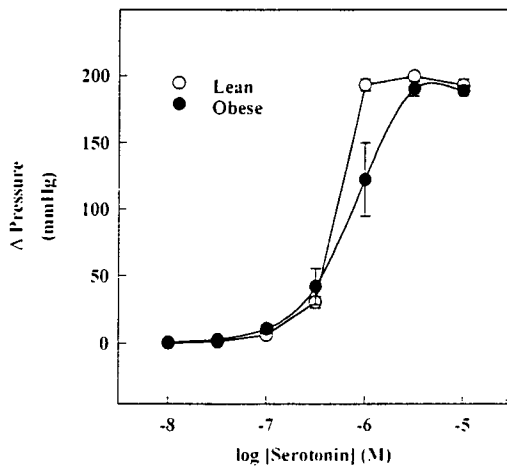
FIG. 4. Effect of serotonin on perfusion pressure of constant-flow hind limbs of obese and lean Zucker rats. Basal (pre-serotonin) values for perfusion pressure were as given in Table 2. When not visible, error bars are within symbol.

FIG. 5. Effect of serotonin on oxygen uptake by constant flow perfused hind limbs of obese and lean Zucker rats. Absolute Vo_2 values (▼, ▽) and changes in Vo_2 (●, ○) calculated as a function of the total mass of perfused tissue (Table 1). (B) Changes in Vo_2 expressed as a function of the mass of perfused muscle. When not visible, error bars are within symbol. $*p < 0.05$, $**p < 0.01$, significantly different from obese.

can be used to illustrate the magnitude and significance of the altered thermogenesis of the obese hind limb (Fig. 6). Thus values from Fig. 2 reflect the maximum thermogenic capacity of the hind limb that can be activated by norepinephrine or other membes of the type A group of vasoconstrictors that increase Vo_2 by the rat hind limb (Clark et al. 1994). Figure 6 shows that although the values for the lean hind limb and that of the nonobese hooded Wistar strain are in close agreement. the value for the obese hind limb is markedly lower. Given that the perfusion conditions were similar for the lean and obese hind limbs, this suggests that the latter would have markedly less capacity to respond to vascular thermogenic stimuli. either in response to cold or to overeating. A difference in Vo_2 of $3-4$ $\mu mol \cdot h^{-1} \cdot g^{-1}$ between the obese hind limb and those of lean animals would correspond to a loss of $0.95-1.27$ W/kg hind limb at $37°C$. assuming a standard average energy value of 4.83 kcal/L O_2 at standard tempera-

FIG. 6. Vasoconstrictor-controlled thermogenesis by the perfused rat hind limb. Data are shown for lean and obese Zucker rats (Table 2, Figs. 2 and 3), as well as for 6- to 8-week-old nonobese hooded (H.) Wistar rats (Dora et al. 1991, 1992). The closed bar indicates the maximum increase in $\dot{V}O_2$ due to norepinephrine, and the open bar indicates the maximum decrease in $\dot{V}O_2$ due to serotonin; the basal $\dot{V}O_2$ is indicated by the boundary. Values are means ± SE. Rates have been calculated in terms of total tissue perfused and includes muscle and fat but not skin or bone.

ture and pressure (1 kcal = 4.1855 kJ) (Brown and Brengelmann 1965) and a Q_{10} of 2.5 (Paul 1980). Our previous estimates (Ye et al. 1990) suggest that hind limb (which is largely muscle in nonobese strains) has the potential to contribute up to 0.28 W in a 200-g, warm-acclimated, nonobese rat. The absence of 0.95 – 1.27 W/kg hind limb would thus represent an absence of 0.09 W or 34% of the potential thermogenic capacity of this hind-limb tissue.

Since serotonin produced a dose-dependent inhibition of $\dot{V}O_2$ that reached a plateau, data from Fig. 5 can be used to define the apparent lower limit of vasoconstrictor-controlled thermogenesis. Figure 6 shows that the values for obese and lean Zucker hind limbs do not differ significantly, nor do they differ from the value for the nonobese hooded Wistar strain.

Figure 6 also shows that in the obese phenotype the basal $\dot{V}O_2$ (without norepinephrine or serotonin) is significantly lower ($p < 0.05$) than in either the lean or the nonobese strain. This finding suggests that under similar perfusion conditions the basal thermogenic output by the obese hind limb is diminished. If this occurred in vivo, it might reflect a decreased thermogenic need in response to the increased insulating capacity of the hind-limb fat. Alternatively, the diminished thermogenesis under basal conditions might be contributory to the development of obesity by altering the energy balance.

It is important to note that the diminished response to norepinephrine of the obese hind limb is not apparent when striated muscle is assumed to be the sole tissue of the hind limb responsible for O_2 consumption. Thus expression of the data for obese and lean hind limbs in terms of the mass of muscle perfused (Table 2) yields dose–response curves for norepinephrine (Fig. 2B) and basal (pre-norepinephrine) values for $\dot{V}O_2$ (Table 2) that are similar. Indeed, such observations are consistent with those of other workers, who found no difference in basal $\dot{V}O_2$ of perfused obese and lean hind limbs when
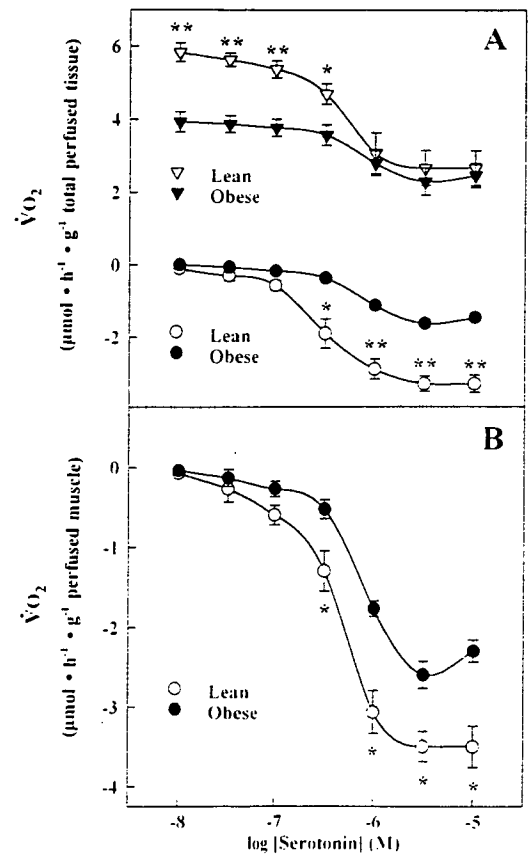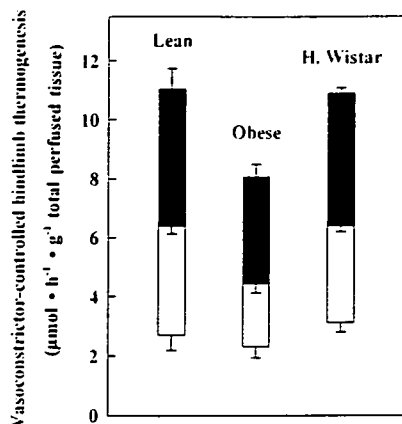
expressed on the basis of mass of muscle perfused (Kemmer et al. 1979) or between $\dot{V}O_2$ of isolated incubated solei from obese and lean Zucker rats (Crettaz et al. 1980). Thus, taken together, our present findings and those of others (Kemmer et al. 1979; Crettaz et al. 1980) suggest that the change in tissue composition of the hind limb associated with the obese phenotype (Table 2) plays a predominant role in the diminished response to both stimulatory as well as inhibitory effects of norepinephrine on $\dot{V}O_2$. However, the fact that the diminished response to serotonin by the obese hind limb was still evident when muscle was assumed to be the sole thermogenic tissue of the hind limb implies that the obese phenotype is associated also with an intrinsic defect in the hind-limb muscle or its vasculature. This defect may have additional implications for the thermogenic capacity of obese skeletal muscle.

Previous studies using constant flow perfused hind limbs from a nonobese strain have shown that vasoconstrictor concentrations of serotonin, similar to those used here, inhibited $\dot{V}O_2$ (Dora et al. 1991; 1992), decreased perfused space (Dora et al. 1991), impaired insulin-mediated glucose uptake (Rattigan et al. 1993), and impaired skeletal muscle contractility (Dora et al. 1994). Based on those findings and the knowledge that serotonin has no direct effect on isolated incubated skeletal muscle (Rattigan et al. 1993; Dora et al. 1994; Sasson 1990), we have proposed that serotonin acts to constrict relatively large vessels, diverting flow away from nutritive vessels (supplying O_2-consuming tissue) to functional vascular shunts (non-nutritive) (Dora et al. 1991, 1992, 1994; Rattigan et al. 1993), while maintaining constant flow. Similar proposals have been made by others (Rippe and Folkow 1980), and there is evidence that the action of serotonin has been located predominantly on large, rather than small, arteries (Hollenberg 1985). Since pressor effects were similar for obese and lean hind limbs (Fig. 4), an impaired response by the obese hind limb to serotonin, in terms of inhibition of muscle $\dot{V}O_2$ (Fig. 5B), suggests that there is a lower proportion of nutritive vessels relative to functional shunts. Thus an impaired response by the obese hind limb to serotonin does not result from relatively fewer functional shunts but, rather, is consistent with the presence of less capacity for nutritive O_2 delivery. Such a reduction in the availability of nutritive delivery may also diminish glucose and insulin access and contribute to the insulin resistance of obese Zucker hind limbs (Kemmer et al. 1979). The obese phenotype is not associated with major changes in muscle specific enzyme activities, including cytochrome oxidase (Wardlaw and Kaplan 1984), nor is there a decrease in the proportion of oxidative fibres (Pujol et al. 1993).

In conclusion, altered regulation of thermogenesis by the obese Zucker hind limb has been identified. Lower muscle content may be the major cause, although there is some evidence supporting the concept of diminished nutritive delivery. Calculations suggest that the implications for whole-body thermogenesis are considerable. Thus the propensity to develop obesity (Zucker 1975) and the poor response to cold exposure (Trayhurn et al. 1976; Levin et al. 1980; Kraul et al. 1985) may derive from a lower contribution to thermogenesis from muscle as well as impaired brown adipose tissue (Levin et al. 1984).

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Hormone and Metabolic Research

Treatment with the Thiazolidinedione (BRL 49653) Decreases Insulin Resistance in Obese Zucker Hindlimb

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Hindlimbs of mature age obese *fa/fa* Zucker rats were perfused and found to be markedly insulin-resistant when compared to the hindlimbs of age-matched lean Fa/? animals. Hindlimb analysis also showed a greater content of fat and a lower content of muscle in the obese. Treatment of the obese animals for 7 days with the thiazolidinedione, BRL 49653 (3 μmol/kg/day) significantly decreased the insulin resistance of the hindlimb and significantly increased the rate of weight gain in the whole rat. However, the decreased insulin resistance due to BRL 49653 could not be accounted for by an increase in the proportion of hindlimb muscle to fat or by an increase in the hindlimb muscle mass perfused.

Key words: Hindlimb Perfusion – Muscle Insulin-Resistance – NIDDM – Genetic Obesity

Introduction

Genetically obese Zucker (*fa/fa*) rats exhibit major metabolic differences compared with their lean (Fa/?) counterparts. In terms of glucose homeostasis the *fa/fa* animals exhibit hyperinsulinemia (*Turkenkopf, Johnson* and *Greenwood* 1982), decreased sensitivity to insulin *in vivo* (*Jeanrenaud* 1979), decreased ability of various tissues to bind insulin (*Kobayashi* and *Olefsky* 1978; *Le Marchand-Brustel, Jeanrenaud* and *Freychet* 1978) and various defects distal to the insulin receptor interaction (*Assimacopoulos-Jeannet* and *Jeanrenaud* 1976; *Crettaz, Prentki, Zaninetti* and *Jeanrenaud* 1980). In addition, skeletal muscle of the *fa/fa* rat is insulin resistant with decreased insulin binding (*Crettaz* et al. 1980; *Czech, Richardson, Becker, Walters, Gitomer* and *Heinrich* 1978), rate of glycogen synthesis (*Crettaz* et al. 1980; *Crettaz, Horton. Wardzala, Horton* and *Jeanrenaud* 1983; *Ivy, Sherman, Cutler* and *Katz* 1986; *Kemmer, Berger, Herberg, Gries, Wirdeier* and *Becker* 1979), rate of glycolysis (*Crettaz* et al. 1980; *Crettaz* et al. 1983), and rate of glucose transport (*Sherman, Katz, Cutler, Withers* and *Ivy* 1988). Perfused hindlimb studies (*Sherman* et al. 1988) show that the rate of glucose uptake by the obese hindlimb is reduced to around 60% of the rate of the lean over the range 0 to 15 mU·ml⁻¹ insulin and the impairment is common to all skeletal muscle fibre types.

BRL 49653 (5-(4-[2-(N-Methyl-N-(2-pyridyl)amino)ethoxy]-benzyl)thiazolidine-2,4-dione), is a new, potent insulin sensitizer agent that when administered chronically to animal models of NIDDM improves glycaemic control. In the Zucker *fa/fa* rat, oral administration of BRL 49653 (3 μmol/kg body weight for 21 days) normalises glucose tolerance and produces a 50% reduction in fasting plasma insulin concentrations (*Smith, Cawthorne, Coyle, Holder, Kirkham, Lister, Murphy* and *Young* 1993). Under hyperinsulinaemic (600 μU/ml) euglycaemic clamp conditions, BRL 49653-treatment increases glucose infusion rates resulting in both an enhanced insulin suppression of hepatic glucose output and an increased glucose disposal by peripheral tissues, principally skeletal muscle (*Smith* et al. 1993). BRL 49653 (10 μmol/kg body weight for 4 days) has been reported also to increase significantly muscle glucose disposal under euglycaemic clamp conditions in high-fat-fed insulin resistant rats but it has no effect in control rats (*Kraegan, Oakes, Kennedy, Sader, Laybutt* and *Chisholm* 1993).

In the present study, we have investigated further the effect of BRL 49653 on insulin sensitivity of the obese *fa/fa* insulin-resistant rat, by measuring the insulin sensitivity of the perfused hindlimb.

Materials and Methods

Mature (20 and 27 week old) male genetically obese (*fa/fa*) and lean (Fa/? Zucker rats were obtained from Harlan Olac Ltd., Bicester, Oxfordshire, U.K. The effect of BRL 49653 on glucose uptake was determined in the 20 week old male *fa/fa* rats. The differences in glucose uptake between obese and lean animals were determined in the 27 week old male rats. Animals were housed in groups under climate-controlled conditions (20 ± 2°C, 12 h light/dark cycle) and provided with R & M 1 (rat and mouse diet) made by SDS, Manea, Cambridgeshire, U.K., and water *ad libitum*. Rats were dosed once daily by oral gavage for 7 days with either BRL 49653 as the maleic acid salt (3 μmol/kg body weight) or water (vehicle).

For isolated hindlimb perfusion, animals were given an injection (i.p.) of heparin sodium (2500 U/kg) and then anaesthetised with an injection (i.p.) of pentobarbitone sodium (60

Age (wk)	Phenotype and treatment	n	Body wt. (g)	Heart wt. (g)	Perfused Hindlimb		Table 1 Body weight, heart weight and perfused hindlimb analysis of non-obese and obese Zucker rats treated with BRL 49653.
					Muscle (g)	Fat (g)	
27	Lean	5	438.0 ± 11.4	1.61 ± 0.05	31.47 ± 1.15	3.83 ± 0.59	
27	Obese	4	587.5 ± 5.5ᵃ	1.70 ± 0.09	21.62 ± 0.57ᵃ	10.69 ± 2.53ᵃ	
20	Obese + BRL 49653	5	579.0 ± 24.4	1.90 ± 0.09	20.28 ± 1.36	10.37 ± 0.41	
20	Obese + vehicle	5	584.0 ± 12.20	2.05 ± 0.08	24.43 ± 0.82ᵇ	14.49 ± 1.38ᵇ	

Values are means ± S.E. for 27 week old male lean and obese rats, and for two groups of 20 week old male obese rats that had received BRL 49653 or vehicle for 7 days.
ᵃ $p < 0.05$, significantly different from lean
ᵇ $p < 0.05$, significantly different from BRL 49653-treated

mg/kg). Rats were then pithed and maintained on a respirator via a tracheal tube. Hindlimb perfusion was conducted as described previously (*Colquhoun, Hettiarachchi, Ye, Richter, Hniat, Rattigan* and *Clark* 1988). Flow was diverted to one hindlimb by ligating the contralateral common iliac artery. Perfusion was conducted at constant-flow and 32 °C using an erythrocyte-free modified Krebs-Ringer bicarbonate buffer containing 2.5 mM $CaCl_2$, 8.3 mM glucose and 4 % undialysed bovine serum albumin (Sigma, fraction V). The flow rate was adjusted at the start of each perfusion to give comparable venous PO_2 values [nominally set at a minimum of 350 mmHg to ensure normoxia, as established in previous studies (*Ye, Colquhoun, Hettiarachchi* and *Clark* 1990)]. Perfusion of the hindlimb was conducted at 32 °C to both ensure normoxia in the absence of red blood cells and to allow a sufficient rate of glucose uptake. Insulin (Calbiochem, bovine soluble insulin, 25 U/mg) was infused into a small stirred bubble trap proximal to the arterial cannula. Glucose uptake across the hindlimbs was determined by glucose analyser (YSI 2300 STAT, Yellow Springs Instruments, U.S.A.) using samples of the inflow and outflow perfusates. Upon completion of the perfusion, a 1 % solution of Evans blue was infused into the arterial cannula at a similar flow rate to that used throughout the experiment. The resultant stained tissue was subsequently dissected out, blotted dry and weighed. Perfusion was largely confined to the muscle and fat; other tissues (bone and skin) contained very little dye.

Results are given as means ± S.E. The significance of differences between means was analysed using Student's t-test.

Results

Table 1 shows body weight, heart weight and perfused hindlimb muscle and fat content of lean, obese and obese Zucker rats treated with BRL 49653 for 7 days. At 27 weeks of age, the obesity was pronounced and the homozygote (*fa/fa*) rats showed significant differences from the age-matched lean (Fa/?) rats in terms of body weight, hindlimb muscle and hindlimb fat content (Table 1). Treatment of the 20-week old obese animals with BRL 49653 for one week lowered significantly the amounts of hindlimb muscle and fat when compared with age-matched obese animals given vehicle alone. The proportion of muscle to fat in the hindlimb was not affected by treatment with BRL 49653.

Treatment of the obese rats with BRL 49653 resulted in a significant increase in weight gain of approximately 10 g over 7 days (Fig. 1). However comparison with the data of Table 1
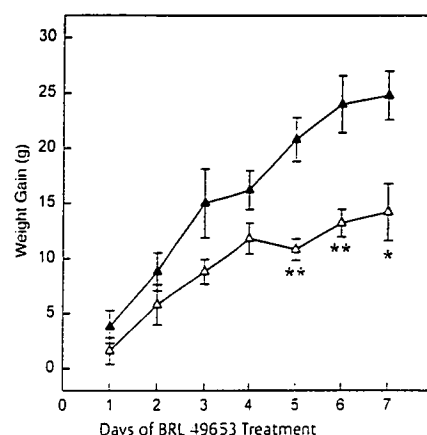
Fig. 1 Effect of BRL 49653 on body weight gain of 20 week old male obese Zucker rats. BRL 49653 (3 μmol/kg) (▲) or vehicle (△) was administered daily as an oral gavage. Individual weight gains were used to calculate the mean ± S.E. *$p < 0.05$; **$p < 0.01$, significantly different from BRL 49653-treated.

show that this effect was not apparent when body weights were averaged and that the weight gain was not reflected by changes in hindlimb fat or muscle mass.

Fig. 2 shows the insulin dose-response curves for glucose uptake by perfused hindlimbs of 27-week old obese and lean Zucker rats. Basal (pre-insulin) values for glucose uptake tended to be greater in the lean hindlimbs relative to the obese hindlimbs. Hindlimbs from lean rats were more responsive than those from obese rats and the trend became significant at ≥ 10 nM insulin. The half maximal effect of insulin for both lean and obese hindlimbs was 40 nM insulin but the obese hindlimb showed only approx. 50 % of the glucose uptake capacity of the lean hindlimb (Fig. 2) at all insulin concentrations.

Fig. 3 shows the effect of 7 days of treatment of 20-week old obese animals with BRL 49653 on insulin-mediated glucose uptake by the perfused hindlimbs. Hindlimbs from vehicle-treated 20-week old *fa/fa* rats had a lower basal (pre-insulin) glucose uptake rate as well as a smaller insulin response
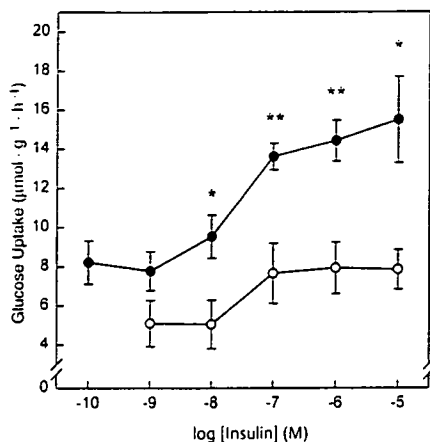
Fig. 2 Insulin dose-response curves for glucose uptake by perfused hindlimbs from obese and lean Zucker rats. Details for perfusion and determination of glucose uptake are given in the text. Values are mean ± S.E. for five lean(●) and four obese (○) animals all of 27 weeks of age, and are expressed in terms of perfused mass of muscle + fat.
*p < 0.05, **p < 0.01, significantly different from obese.
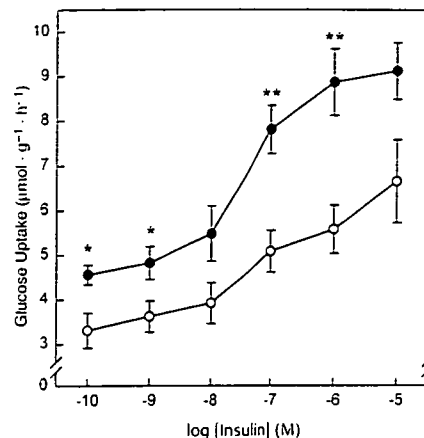
Fig. 3 Insulin dose-response curves for glucose uptake by perfused hindlimbs from 20-week old obese Zuckers that were treated with BRL 49653 (3 μmol/kg. p.o.) (●) or vehicle (○) for 7 days. Valaues are means ± S.E. for five animals in each group, and are expressed in terms of perfused mass of muscle + fat. *p < 0.05; **p < 0.01, significantly different from vehicle treated.

(Fig. 3) than hindlimbs from the older obese animals (Fig. 2). However treatment with BRL 49653 increased significantly the basal rate of glucose uptake and, at maximal doses of insulin (≥ 1 μM), there was a 50 % increase in glucose uptake. The sensitivity to insulin remained unaltered by BRL 49653 treatment with the half maximal concentration remaining at 40 nM.

Discussion

BRL 49653 is a new potent insulin sensitizer. In two models of genetic obesity, the *ob/ob* mouse and the *fa/fa* rat (*Smith* et al. 1993; *Cawthorne, Lister, Holder, Kirkham, Young, Cantello, Hindley* and *Smith* 1993), one of diet-induced obesity (*Kraegen* et al. 1993), and one diabetic strain (*db/db: Cawthorne* et al. 1993) all of which are insulin resistant, BRL 49653 has been found to normalize oral glucose tolerance (*Smith* et al. 1993; *Cawthorne* et al. 1993) and reduce the serum levels of insulin (*Smith* et al. 1993; *Cawthorne* et al. 1993). An improved whole body insulin sensitivity under euglycaemic clamp conditions has also been reported (*Smith* et al. 1993; *Kraegen* et al. 1993; *Cawthorne* et al. 1993), and there is indirect evidence to suggest that glucose uptake by muscle has been increased. In the present study oral treatment with BRL 49653 at 3 μmol/kg/day for 7 days was found to increase significantly insulin mediated glucose uptake of the hindlimb of the obese Zucker rat.

The effect of BRL 49653 on the hindlimb of the obese rat was manifest as an increased responsiveness to insulin without a change in EC_{10}. It is also important to note that the basal (preinsulin) rate of glucose uptake was also enhanced following BRL 49653 treatment making it unlikely that the thiazolidinedione was simply enhancing the effect of insulin. It is possible that as in adipose tissue of *ob/ob* mice, treatment with BRL

49653 increases the tissue content of glucose transporters (*Young, Cawthorne, Coyle, Holder, Holman, Kozka, Kirkham* and *Smith* 1993). Further studies will be needed to assess not only this but also which tissue(s) within the hindlimb is affected by the agent.

Acknowledgements

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Vascular and endocrine control of muscle metabolism

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Clark, Michael G., Eric Q. Colquhoun, Stephen Rattigan, Kim A. Dora, Tristram P. D. Eldershaw, Jenny L. Hall, and Jiming Ye. Vascular and endocrine control of muscle metabolism. *Am. J. Physiol.* 268 *(Endocrinol. Metab.* 31): E797–E812, 1995.—Important differences exist between perfused and incubated (or perifused) skeletal muscle preparations with regard to their metabolism and control. A growing body of evidence suggests that the differences may be due to the role played by the vascular system. In the constant-flow perfused rat hindlimb preparation, a group of vasoconstrictors has been identified that enhance muscle metabolism and aerobic contractility. Another group of vasoconstrictors decrease muscle metabolism and aerobic contractility even though perfusate flow remains constant. All effects of both groups of vasoconstrictors are opposed by vasodilators. Because none of the vasoconstrictor effects is evident when isolated muscles are incubated or perifused, involvement of an active vascular system is indicated. Although some hormones may act directly on muscle by purely endocrine effects, a vascular component of their actions is now emerging. Mechanisms to account for vascular control of perfused skeletal muscle metabolism may involve *1*) functional vascular shunts where the proportion of flow processed by these is regulated by site-specific vasomodulators, *2*) a direct response to a change in the rate of supply of nutrients and removal of products, and *3*) a signal substance released by vascular tissue in association with vasoconstriction that interacts with surrounding skeletal muscle cells. Impaired control at the level of the vascular system may have implications for long-term access of nutrients and hormones and therefore the control of skeletal muscle metabolism and contractile performance.

hemodynamic effects of hormones; functional vascular shunts; vascular system control of muscle metabolism; nutritive flow; nonnutritive flow; paracrine relationship between vasculature and skeletal muscle

WHEN CONSIDERING FACTORS controlling the metabolism of skeletal muscle, use of preparations isolated from central, neural, and hormonal influences allows the investigator to obtain a clearer understanding of individual treatments. This review will focus on the differential effects of vasoconstrictor agents that are apparent in perfused, but not incubated, muscle systems. An emerging theme will be the role of the vasculature, which in conjunction with endocrine effects acts to control the metabolism and performance of muscle.

HISTORICAL ASPECTS

Two systems have largely been used to explore factors controlling skeletal muscle metabolism. These are the perfused hindlimb preparation (77) and the isolated incubated (or perifused) muscle preparation. Many metabolic controlling influences have been identified with the perfused hindlimb system, but because most of these could be more readily studied with the simpler isolated incubated muscle preparation workers have favored this latter approach [see recent review evaluating the two systems (5)]. However, an important omission in comparing perfused with incubated muscle preparations is the possible role played by the vascular system, which would show little, if any, involvement in studies with isolated incubated muscles.

Initially, vascular effects of various agents were studied independently of metabolic effects. For example, Folkow and his associates (74) developed the perfused hindlimb system especially to study vascular effects, including capillary filtration capacity. In this respect, the isolated perfused rat hindlimb was seen as a logical extension of the autoperfused (essentially in vivo) preparations of hindlimbs of dogs extensively studied by Pappenheimer and Soto-Rivera (63) and Stainsby and Renkin (84). It was Folkow's laboratory (74) that noted that the isolated perfused rat hindlimb was largely fully dilated, and this was based on the observation that very

high flow rates of perfusate could be obtained at remarkably low perfusion pressures even when viscous perfusates containing red blood cells were used.

By the mid-1980s use of the perfused hindlimb preparation was widespread, and there was interest in carbohydrate metabolism, thermogenesis, exercise physiology (see Ref. 5 and references therein), vascular pharmacology (49), and vascular properties (74) using this experimental model. Although there had been three reports of stimulatory effects of norepinephrine (NE) and epinephrine on oxygen uptake associated with increased perfusion pressure (18, 35, 72), not one of the groups had identified a relationship between the vascular and metabolic changes.

VASOCONSTRICTORS OF PERFUSED HINDLIMB THAT STIMULATE BASAL METABOLISM

Findings from this laboratory in 1988 (17) linking the vasoconstrictor effects of angiotensin and vasopressin to increased oxygen uptake by the constant-flow perfused rat hindlimb were unexpected. Up to that point in time, we and others might have predicted that vasoconstrictors, such as angiotensin and vasopressin, would have strong vasoconstrictor activity with little or no effect on hindlimb metabolism. This led to a detailed study of effects of vasoconstrictors on hindlimb metabolism. Table 1 summarizes the findings and shows that the constant-flow perfused rat hindlimb responds to many different vasoconstrictors that stimulate basal oxygen uptake. Although not all of these vasoconstrictors have been examined in detail, all that have showed changes consistent with increased metabolism. The changes observed with the nonrecirculating perfusion system included increased lactate efflux by angiotensin (44), vasopressin (44), NE (up to 10^{-5} M; see Ref. 44), methoxamine (44), [6]-gingerol (28), and [6]-shogaol (28), increased glycerol efflux by NE (12) and by NE plus propranolol, and increased urate and uracil efflux by NE (13), vasopressin (13), and angiotensin (13). An α-adrenergic effect of catecholamines to increase glucose uptake by the perfused rat hindlimb was first observed by Richter and colleagues (72) and was also accompanied by increases in perfusion pressure and oxygen uptake.

In general, we have perfused the rat hindlimb at 25°C under conditions of constant flow with 95% O_2-5% CO_2 as the gas phase (Table 1). However, qualitatively similar changes occur when the hindlimb is perfused at 37°C under constant flow with medium containing bovine red blood cells and with 95% air-5% CO_2 as the gas phase (Fig. 1).

The close association between the increase in perfusion pressure and change in oxygen uptake by vasoconstrictors acting on the constant-flow perfused rat hindlimb is readily evident if flow rate, temperature, and rat size remain constant (Fig. 2). Vasopressin, angiotensin II, and NE each increased oxygen uptake and lactate efflux in a dose-dependent manner following closely the rise in perfusion pressure (44). In addition, the vasoconstrictor effects of these agents are inseparable from the metabolic changes as evidenced by the findings that vasodilators, regardless of mode of action, block both the

Table 1. *Vasoconstrictor stimuli that increase (type A) or decrease (type B) oxygen uptake in perfused rat hindlimb*

| Agonist | Change From Control at Maximum Dose of Agonist, % | | Ref. No. |
	Perfusion pressure	Oxygen uptake	
Type A			
Norepinephrine	52, 130, NA	46, 52, 77	17, 18, 35
Epinephrine	67, NA	25, 67	72, 35
Phenylephrine	160, NA	50,* 57	81, 35
Methoxamine	167	25	44
Amidephrine	24	28	12, 42a
Ephedrine	70	10	12, 42a
Norephedrine	57	18	43
Angiotensin II	133	64	17
Vasopressin	121	66	17
Capsaicin	54.5	22	8
Dihydrocapsaicin	49	18	8
[6]-Gingerol	30	21	28
[6]-Shogaol†	30	21	28
Low-frequency sympathetic nerve stimulation (0.5–4 Hz)	6	6	12‡
Type B			
Norepinephrine (≥ 1 μM)	700, 200, 380	−22.7, −15, −10	24, 18, 34
Serotonin	76	−30.3	24
Capsaicin (> 1 μM)	110	−35	§
Dihydrocapsaicin (> 1 μM)	+	−	12
[6]-Gingerol (≥ 20 μM)	96	−21.2	28
High-frequency sympathetic nerve stimulation > 4 Hz	37	−16	12‡

Hindlimbs were perfused at 25°C with constant flow. * Cold-acclimated rats. † 1-(4'-hydroxy-3'-methoxyphenyl)dec-4-en-3-one. ‡ See also Fig. 3. § Unpublished. NA, not available. + and −, indicative of an increase or a decrease, respectively, when compared with control (vehicle only) perfusions.

metabolic and vasoconstrictor effects of the vasoconstrictors (44). The list of vasodilators includes nitrovasodilators (16), nifedipine (Ca²⁺ channel blocker; see Ref. 16), isoproterenol (β_1,β_2-adrenergic agonist; see Ref. 17), adenosine, AMP, ADP, ATP, and UTP (70).

A further link between the vascular and metabolic effects of vasoconstrictors was noted when metabolic poisons were used (24, 69). As shown by Richards et al. (69), 1 mM cyanide totally blocked the pressor effect of 5 nM angiotensin II as well as the increases in oxygen consumption and lactate efflux. Contrary to expectation, cyanide was slower than angiotensin II at inducing an increase in lactate efflux under the perfusion conditions used of constant flow at 25°C (69). Cyanide or hypoxia (95% N_2-5% CO_2) also blocked the pressor effect of 50 nM NE (24).

The association between increased lactate efflux and increased oxygen uptake by the perfused rat hindlimb during vasoconstriction might at first appear puzzling. The first report of lactate release by the perfused rat hindlimb in association with vasoconstriction was by Richter et al. (72) using the α-adrenergic combination of epinephrine plus propranolol in a recirculating perfu-

sion system. Lactate release is more commonly the result of exercise, hypoxia, high K^-, and β-adrenergic action of catecholamines (see Ref. 44 and references therein) where in each case high rates of glycolysis in hindlimb skeletal muscle cells would be expected. The magnitude of the release can also be affected by the perfusate concentration of lactate. In our studies, oxygen uptake and lactate efflux have been jointly monitored in a nonrecirculating perfusion system with no added lactate, and reciprocity between the two does not occur unless poisons such as cyanide are present (69).

In addition to the vasoconstrictor-induced increases in oxygen uptake, lactate, and glycerol output (Table 1), many of the more potent members of this group have been found to increase the release of breakdown products of both purine and pyrimidine nucleotides (13, 69). Uric acid and uracil are the most prominent of the products released, and although the origins and precursors are unknown their time course of release is similar to that of lactate (69).

VASOCONSTRICTORS OF PERFUSED HINDLIMB THAT INHIBIT BASAL METABOLISM

Vasoconstriction of the constant-flow perfused rat hindlimb is not always a stimulus for increased oxygen uptake. Indeed several vasoconstrictors (which we now categorize as type B) lead to decreased oxygen consumption with increased vascular resistance. This group includes serotonin (5-HT; 22, 24, 67), NE at high doses
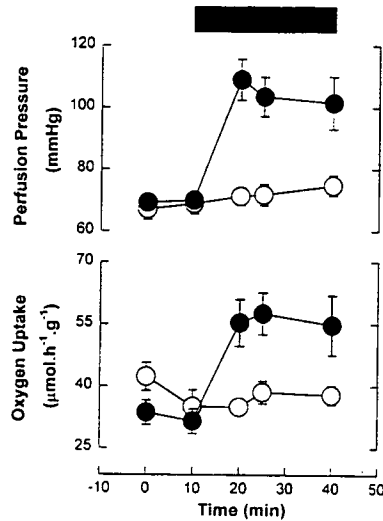
Fig. 1. Time course for effect of angiotensin II on perfusion pressure and oxygen uptake by constant-flow (0.97 ml·min⁻¹·g⁻¹) perfused rat hindlimb. Perfusion media contained 4% bovine serum albumin, bovine red blood cells (32.5% hematocrit), 2.5 mM CaCl₂, and 5 mM glucose in Krebs-Ringer bicarbonate buffer. pH 7.4 at 37°C, and was gassed with 95% air-5% CO₂. Bar indicates period of infusion of angiotensin II (●) or vehicle (○). Values shown are means ± SE for 4 perfusions. Data are similar to those previously published (17) using different condition of perfusion.

Δ Perfusion Pressure (mmHg)
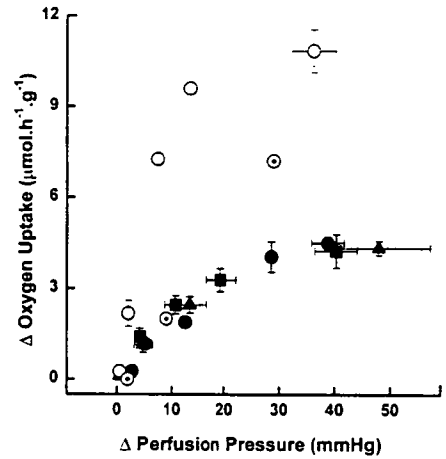
Fig. 2. Relationship between change in oxygen uptake and perfusion pressure for constant-flow perfused rat hindlimb. Values are from the following perfusions: angiotensin II (■), vasopressin (▲), and norepinephrine (●) dose curves. 180–200 g rats, 25°C, 0.27 ml·min⁻¹·g⁻¹, with no red blood cells (n = 4–5 for each) from Ref. 17; circle with plus inside, norepinephrine dose curve, 180–200 g rat, 37°C, 0.27 ml·min⁻¹·g⁻¹, with red blood cells (n = 1) and ⊙, norepinephrine dose curve, 70–80 g rat, 37°C, 0.34 ml·min⁻¹·g⁻¹, with no red blood cells (n = 3–4) from Ref. 68.

(18, 24, 34, 68), high frequency sympathetic nerve stimulation (Fig. 3), and high-dose vanilloids (Table 1). Overall, the metabolic effects of the type B vasoconstrictors are the opposite to those of type A and are therefore potentially negatively thermogenic.

The absence of a single simple direct relationship between the increase in perfusion pressure and change in oxygen uptake by vasoconstrictors acting on the constant-flow perfused rat hindlimb is readily evident. Figure 2 shows that, for relatively little pressure change [e.g., NE in young (70–80 g) rats], a large increase in oxygen uptake occurs. This contrasts with the effects of higher concentrations of NE in slightly older (180–200 g) rats where very high pressures occur and there is a relatively small increase in oxygen uptake. Furthermore, at high doses, NE further increases pressure in rats of either age, but oxygen uptake shows a net inhibition (24, 68).

Figure 4 shows the time courses for low-dose NE (type A) and 5-HT (type B) vasoconstrictors on perfusion pressure, oxygen uptake, lactate, and glycerol release. Although each agent produces a similar increase in perfusion pressure, there are opposite changes in oxygen uptake and release of metabolites.

The effects of NE at high concentrations [e.g., ≥ 1 μM is the concentration that is thought to occur at vascular smooth muscle synapses (29)] are similar to those of 5-HT. In fact the dose-response curve for NE-mediated changes in oxygen uptake is bell-shaped even though the corresponding dose-dependent rise in pressure continues over the full range of concentrations (24). At least two other groups (18, 34) have noted the bell-shaped nature of the NE dose curve for oxygen uptake. In
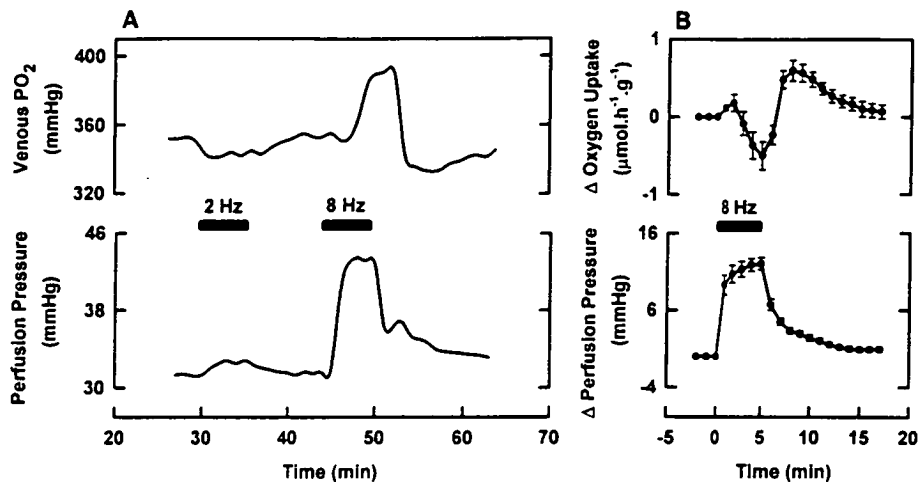
Fig. 3. Time course for effect of sympathetic nerve stimulation on oxygen uptake and perfusion pressure of constant-flow (0.27 ml·min⁻¹·g⁻¹) rat hindlimb. See Colquhoun et al. (17) for perfusion details. Paravertebral sympathetic chain of rat was cut at level of 4th lumbar vertebra and was electrically stimulated with pair of electrodes at 5 V at either 2 or 8 Hz. Stimulation was given for 1-s duration every 2 s to preserve nerve integrity. Tubocurarine was included in perfusate to prevent any skeletal muscle contraction during hindlimb perfusion. *A*: typical trace; *B*: *n* = 5 (Hall, Clark, and Colquhoun, unpublished data).

addition, there is analogous evidence from sympathetic nerve stimulation studies in the autoperfused dog hindlimb that frequency-dependent changes in oxygen extraction occur. Thus, at low frequency, sympathetic nerve stimulation leads to increased oxygen uptake in association with mild vasoconstriction, but at higher frequencies the increase changes to inhibition even though the vascular resistance continues to rise (26, 62). Similar observations have been made using the perfused rat hindlimb (Fig. 3).

As with type A vasoconstrictors, known vasodilator substances act to oppose type B vasoconstrictor-mediated increases in pressure and metabolism although, in some cases, with considerably less efficacy (12, 67). Thus, again, an association between vasoconstriction and metabolism is evident even though the effects of type B vasoconstrictors are the opposite to those of type A. It follows therefore that vasodilators that oppose type B vasoconstrictors revert the hindlimb to a more thermogenic state than that existing when type B constrictors are acting alone.

Several lines of evidence suggest that both 5-HT and NE [≥ 1 μM at 25°C (24) or ≥ 10 μM at 37°C (68)] inhibit metabolism in perfused hindlimb by similar vascular mechanisms but involving distinctly different receptors. First, 5-HT and high-dose NE each inhibit oxygen uptake (22, 24), lactate output (12), and uracil and uric acid output (12). Second, 5-HT and high-dose NE each inhibit aerobic muscle contraction and the associated increase in contraction-induced oxygen uptake (23 and see CORRELATION BETWEEN VASCULAR EFFECTS TO ALTER METABOLISM AND VASCULAR EFFECTS TO ALTER CONTRACTILITY OF PERFUSED HINDLIMB MUSCLE).

Third, high-dose NE, like 5-HT, exerts a vasoconstrictor effect at sites on the vasculature of the hindlimb system that are independent of extracellular Ca^{2-} (12) and that are largely unaffected by anoxia or respiratory poisons (24). Finally, NE effects are blocked by prazosin, but 5-HT effects are not (24).

POSSIBLE MECHANISMS TO ACCOUNT FOR THE OBSERVED CHANGES IN PERFUSED HINDLIMB METABOLISM

The fact that several groups (17, 18, 35, 72) have noted marked effects of NE acting via α-adrenergic mechanisms (17, 35, 72) to increase oxygen uptake in the perfused rat hindlimb (30–100% over basal) under a variety of perfusion conditions (i.e., with and without red blood cells, carbogen, or air-CO_2 as gas phase and perfusion temperature at 25, 32, or 37°C) is indicative of a thermogenic mechanism that is activated. However, the mechanism by which site-specific vasoconstriction leads to changes in metabolism and thermogenesis (either increases or decreases) remains unknown. At present, there appear to be four possibilities that could account for the alterations in metabolism.

Direct Effects on Skeletal Muscle

One possibility that we have argued (17) is that the pressure increase is unrelated to the change in oxygen uptake (and other metabolism) and that receptors for all type A and B vasoconstrictors (see VASOCONSTRICTORS OF PERFUSED HINDLIMB THAT STIMULATE BASAL METABOLISM and VASOCONSTRICTORS OF PERFUSED HINDLIMB THAT INHIBIT BASAL METABOLISM). in addition to those on the

A

Norepinephrine

Praz

B
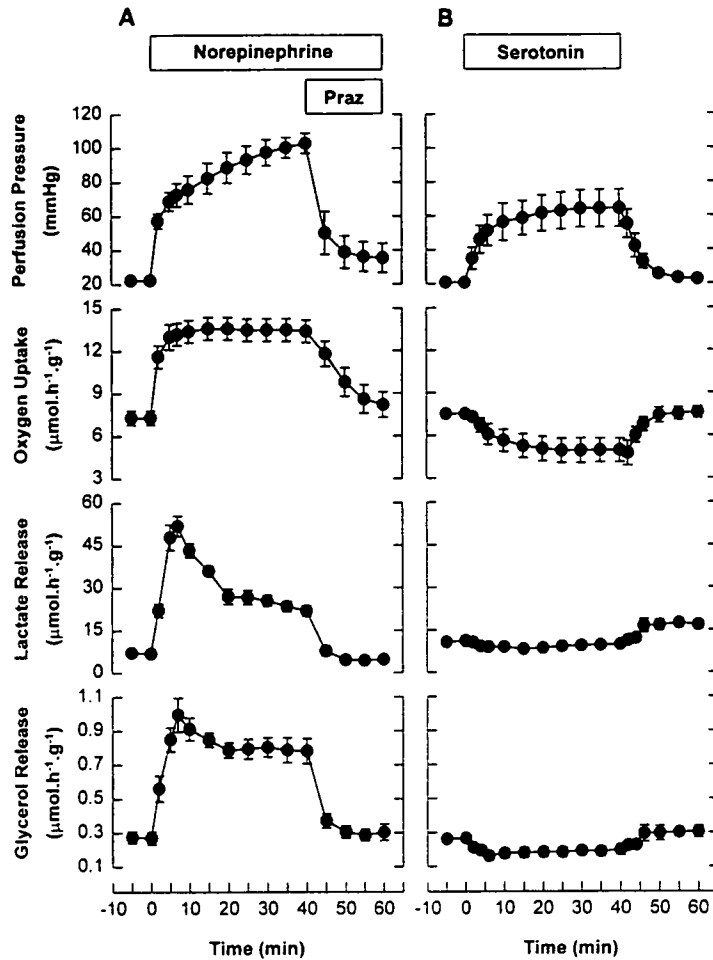
Serotonin

Fig. 4. Time course of effect of norepinephrine (A) and of serotonin (B) in constant-flow perfused rat hindlimb. See Colquhoun et al. (17) for perfusion details. Values are shown as means ± SE for 4-5 perfusions. Increase in lactate efflux after serotonin removal is transient and returns to preinfusion values. Data are similar to those previously published (24, 44) and include some unpublished data for lactate and glycerol release. Praz, prazosin.

vasculature, are present on skeletal muscle and act directly to alter skeletal muscle fiber oxygen uptake and metabolism. However, if this were the case, it would be difficult to explain why direct effects of NE or other type A vasoconstrictors have no effect on oxygen uptake or metabolism by isolated incubated muscles (Table 2 and Ref. 44).

In rats, whole body oxygen uptake (and therefore thermogenesis) increases by up to 100% when NE is injected (12 and references therein). It follows therefore that an effective procedure to address the question of which tissue(s) is involved in this response has been to assume that the effects of NE in vivo can be effectively mimicked in vitro by exposing individual tissues to NE. For brown adipose tissue, this is certainly the case, and all preparations (tissue fragments, isolated cells, and slices) respond markedly to the addition of catecholamine with values for oxygen uptake and heat produc-

tion consistent with estimates for this tissue in vivo (31). However, unlike brown adipose tissue, isolated skeletal muscles incubated or perifused in vitro with NE plus propranolol (or angiotensin II; Table 2) do not respond by showing an increase in oxygen uptake, heat flux, or lactate output. These findings contrast markedly with our own observations (12, 16, 17, 94) and those of other groups (see Ref. 15 and references therein) who reported that infused sympathomimetic substances increased oxygen uptake in noncontracting skeletal muscle receiving its nutrient supply by the vascular route.

Vascular Thermogenesis

A second possibility that we have considered (see Ref. 15 and references therein) is that the increased metabolism (including oxygen uptake) is due to the work performed by the vascular smooth muscle itself as it

Table 2. *Effects of norepinephrine and other vasoconstrictors on metabolism by isolated incubated muscles*

Muscle	Addition	Oxygen Uptake	Lactate Output	Ref. No.
Rat soleus	None	13 ± 2	10 ± 2	44
	Norepinephrine (100 nM)	12 ± 2	7 ± 3	44
	Angiotensin II (100 nM)	18 ± 4	8 ± 2	44
Rat epitrochlearis	None		27 ± 2	44
	Angiotensin II (5 nM)		28 ± 1	44
	Vasopressin (0.5 nM)		29 ± 2	44
Mouse soleus	None	2.6 ± 0.2		25
	Norepinephrine (1 μM)	2.7 ± 0.2		25

Values are means ± SE. Units for rat soleus and epitrochlearis muscles are $\mu mol \cdot g^{-1} \cdot h^{-1}$. Units for mouse soleus muscle are $\mu W/mg$ wet wt. See also conclusions by Eaton (27).

contracts to increase and hold perfusion pressure (i.e., hot pipes). Indeed, the close association between type A vasoconstriction and increased hindlimb oxygen uptake for animals of similar weight and age (Fig. 2) and the release of lactate have led us to propose that both aerobically and anaerobically generated ATP may be consumed by the working vascular tissue as it constricts (15) or as it resists an increase in pressure due to flow increase (15). The dependence of type A vasoconstrictors on coupled oxidative phosphorylation to mediate rises in perfusion pressure (24, 69) supports the notion of vascular thermogenesis.

Attempts in this laboratory to demonstrate high rates of oxygen consumption by the isolated perfused rat tail artery have shown that mitochondrial content and cytochrome oxidase activity may be high enough to allow for the predicted high rates of oxygen consumption (i.e., of the order of 800 $\mu mol \cdot h^{-1} \cdot g$ wet wt^{-1}). If rates of oxygen consumption of this order were attainable by the vascular elements, 3.4% by weight of the skeletal muscle would need to be vascular smooth muscle, and both arterial and venous structures would need to be involved in responding to the vasoconstrictors that increase oxygen consumption. However, the potential for oxidative metabolism appears to rise with a decrease in vessel size, and thus the values for perfused rat tail artery may underestimate rates for arterioles. Hence, metabolic studies using small-resistance vessels will be beneficial.

It is of interest that selected vasoconstrictors increase oxygen uptake by perfused intestine, kidney, and mesenteric artery in association with increases in perfusion pressure (93). Thus the thermogenic mechanism associated with vasoconstriction is not restricted to skeletal muscle, whether it derives from working vascular tissue (i.e., "hot pipes") or another unidentified process.

The obvious lack of a positive relationship between perfusion pressure and metabolism observed upon infusion of type B vasoconstrictors into the perfused hindlimb (see VASOCONSTRICTORS OF PERFUSED HINDLIMB THAT INHIBIT BASAL METABOLISM) is difficult to explain simply according to vascular thermogenesis. Despite marked rises in perfusion pressure upon infusion of 0.1 μM NE or 0.25 μM 5-HT (Fig. 4), the effects on metabolism were

opposite with NE stimulating O_2 but 5-HT inhibiting O_2. It has been proposed that, in these situations, site-specific vasoconstriction, perhaps of larger vessels, reduces the pressure load on the metabolically active working resistance vasculature and/or reduces the delivery of nutrients to working vascular smooth muscle (22).

Heterogeneity of Perfusion

An alternative possibility to the notion of hot pipes stems from the observation that all type A vasoconstrictors in the constant-flow perfused rat hindlimb induce an efflux of red blood cells on initial infusion(s) after equilibration with perfusate not containing red blood cells (unpublished observations). This coincides with the early efflux of lactate, purines, and pyrimidines and may reflect pressure-induced clearance of regions and vessels not accessed during equilibration. The vasoconstricting agents may act at specific sites on terminal arterioles to increase perfusion pressure resulting in perfusion of regions of the hindlimb that were previously underperfused, eliminating a prexisting state of microheterogeneity. Such heterogenous perfusion has been noted in the autoperfused tenuissimus muscle of the rabbit (55). When perfusion pressure was decreased <50 mmHg using an occluder, decreases in both blood flow and the number of perfused capillaries resulted (55). The constant-flow hindlimb perfusion system used in the author's laboratory is characterized by a minimal or nonfunctional neural component, and equilibration leads to the washout of humoral vasoconstrictive agents. Thus basal perfusion pressures are low. However, the overall flow through the hindlimb skeletal muscle is kept at a constant high rate (0.27 ml·min^{-1}·g^{-1}). Furthermore, the effects of NE to cause vasoconstriction-associated increases in oxygen uptake are evident across a full range of flow rates from 0.13 to 1.2 ml·min^{-1}·g^{-1} (94). Thus, if heterogeneity is an issue, then the degree of heterogeneity is not decreased by simply increasing flow. Indeed, studies by Grubb and Snarr (36) led them to conclude that high perfusate flow rates preferentially increased heterogeneity by increasing nonnutritive flow in the rat hindlimb.

A consequence of the isolated perfused rat hindlimb being fully dilated is that flow rates, which are supraphysiological, fail to achieve basal perfusion pressures approaching those in vivo. Thus it could be argued that vasoconstrictors that act to increase metabolism do so by decreasing heterogeneity. Although this cannot be entirely ruled out, constant-flow perfusions at pressures approaching in vivo values (i.e., 90 mmHg) respond similarly to those at lower pressures and flow rates with marked responses to NE or vasopressin in terms of increased oxygen uptake (94). Furthermore, perfusions with red blood cells at 37°C, constant flow, and 60 mmHg, under basal conditions, show marked increases in oxygen uptake with angiotensin II (Fig. 1). Overall, the results are qualitatively similar regardless of the conditions of perfusion.

Our explanation to account for the type B effects of 5-HT (67), and the recent findings for high-dose NE as a

type B agent (68), focuses on the notion of functional vascular shunting in the hindlimb in which agonists are proposed to mediate a marked constriction in large arterioles (92) downstream from branch points for functional shunts leading to the venous circulation (22). As a result, 5-HT- or NE-mediated partial closure at these points returns perfusate to the venous circulation, allowing constant flow while decreasing access of the perfusate to significant areas of the microvasculature. Consistent with this notion, Saltzman et al. (79) have suggested that constriction at the transverse arteriole root restricts perfusion of muscle transverse arterioles and capillaries while still allowing flow through the arcade arterioles to connective tissue. These effects would not be evident when unperfused muscle is incubated where access is governed by diffusion alone but would markedly affect nutrient delivery in perfused hindlimb. We would propose therefore that sites in the muscle vasculature where low doses of NE (≤ 1 μM) mediate an increase in oxygen uptake are compromised by high-dose effects to divert flow to functional shunts thus giving rise to a marked inhibition of oxygen uptake at higher doses of NE. Hence a bell-shaped curve for oxygen uptake, as a function of NE concentration, results even though perfusion pressure continues to rise in proportion to the concentration of catecholamine (24). In addition, the parallels between 5-HT and high-dose NE-mediated effects suggest that the proposed sites for 5-HT on arterioles larger than 90 μm (50) are probably near the sites for high-dose NE-mediated vasoconstriction but are anatomically and pharmacologically distinct from those constricted by low-dose NE, which increase oxygen uptake.

The anatomy of the so-called functional vascular shunts is an important issue for which there is very little hard information. Early studies reporting on changes in oxygen consumption of resting muscle during stimulation by vasoconstrictor (62) or by vasodilator (75) innervations ascribed the findings to a shunting of blood into areas of low metabolic rate or through arteriovenous anastomoses that were considered to be nonfunctional in nutritional exchange with the muscle tissue itself. The anatomy of these structures was not defined even though clearance studies for certain effects of vasomotor innervation and epinephrine injection as well as microparticle injection studies and anatomical procedures suggested their presence in muscle (see Ref. 46 and references therein). Such findings provided the basis for discussions of "nonnutritional" flow during the 1960s. However by 1970 it was clear that anatomical arteriovenous shunts of the kind found in other tissues were not present in skeletal muscle (37). Work in our laboratory has confirmed that passage of 12-μm microspheres through rat hindlimbs is minimal (0.7 ± 0.3%; see Ref. 21). Thus recent argument by ourselves and others focuses on the notion of functional vascular shunts which, although still undefined, involve vessels of < 15 μm in diameter, possibly capillaries. Chinet (11) has argued for a microscale heterogeneity of blood flow to muscle that is controlled predominantly by flow and favors the view that part of the physiological control of

muscle respiration would be by intraorgan control of O_2 availability to cells. This general line of reasoning is also adopted by Harrison et al. (38) in their attempt to explain intravenous hydrogen clearance measurements before and during femoral nerve stimulation. These workers propose two sets of capillaries with a small population of capillaries of relatively large diameter that carry a disproportionately large amount of flow. Arguments along these lines might also be applicable to the findings of Vetterlein and Schmidt (89) who noted that the increase in total blood flow in autoperfused cat and rat hindlimbs induced by the intra-arterial infusion of vasodilating agents appeared to be associated with a reduced perfusion of certain parts of the striated muscles. Potter and Groom (66) using corrosion casts in rat gastrocnemius muscle revealed a bimodal distribution of capillary diameter with modes at 5.5 and 7.5 μm. Despite the population of large-diameter capillaries representing only 13%, Harrison et al. (38) argue, according to the Hagen-Poiseuille law, that they would carry 71% of the flow. The remaining 29% would flow through 87% of the capillaries. During motor nerve stimulation, it was proposed that oxygenated blood was probably diverted from the high flow (albeit functional shunts) to the normal capillaries to meet the increased local oxygen demand (38). Monitoring of the intravenous hydrogen clearance kinetics indicated that, during motor nerve stimulation, the slope of the fast component of the curve was reduced and that of the slower component increased until, in almost all cases, they became indistinguishable from each other (38). Kinetics of sodium fluorescein under similar experimental conditions also supported the shift in flow from shunts to nutritive capillaries (39). The hypothesis put forward by Harrison et al. (38) also encompasses the notion of differing capillary length. Thus the longer narrower capillaries contribute more toward gas (nutrient) exchange than the shorter wider ones.

Paracrine Control of Skeletal Muscle Metabolic Rate

Increasing the supply of oxygen and nutrients to muscle is not, in itself, a stimulus for increased metabolism [e.g., red blood cell vs. nonred blood cell perfusions (5)], and there is now reason to suspect that site-specific vasoconstriction within the hindlimb, and possibly other tissues, leads to the release of a signal molecule. Thus the fourth possibility involves an endocrine relationship between the vasculature and the skeletal muscle fibers. This is based on a growing body of evidence that vasoconstriction and increased flow are two principal mechanisms of shear stress-dependent endothelial autacoid release. For example, Hecker et al. (40) have shown that either addition of acetylcholine to or increasing flow through isolated endothelium-intact rabbit femoral arteries increased nitric oxide and prostaglandin I_2 release. Moreover Suárez and Rubio (86) have shown that increases in coronary flow in guinea pig hearts resulted in a linear increase in glycolytic flux of the underlining parenchymal heart cells. Although the endothelial-derived signals were not identified, disruption of the endothelial cell membrane intravascular glycocalyx by

Fig. 5. Proposed relationship between vasculature and skeletal muscle. *A*, sites for type A vasoconstrictors; *B*, sites for type B vasoconstrictors.
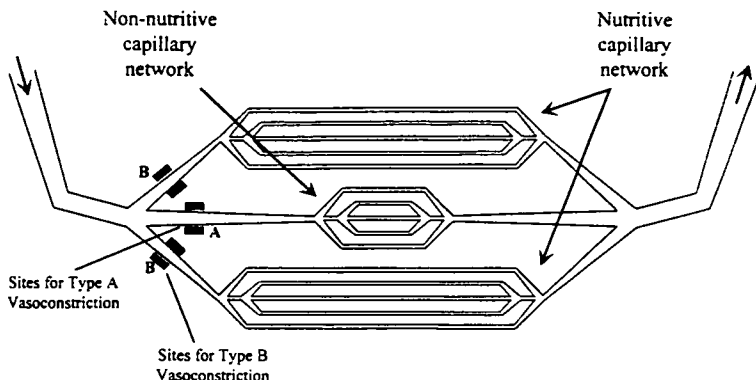
heparinase inhibited the coronary flow-induced increase in cardiac glycolysis (86).

For the skeletal muscle vasculature, a similar relationship may exist (Fig. 5). Thus vasoconstriction by type A constrictors at site A decreases flow through nonnutritive capillaries and redirects flow to nutritive regions. Increased flow at sites along the nutritive capillary network leads to autacoid(s) release. These could have the following two functions: *1*) an autacoid role to relax vessels distal to the impending flow and *2*) a paracrine role to increase metabolism in underlining muscle fibers. Likely metabolic changes in muscle include uncoupling or decoupling of mitochondria and substrate cycling (including ion pumping). Because concentrations of high-energy phosphates and the redox ratio do not change significantly before and after addition of type A vasoconstrictor (16), it appears unlikely that metabolism increases without an accompanying energy-dissipating process. Further studies are needed to identify the proposed paracrine substance(s) and to show whether they directly alter skeletal muscle metabolism (e.g., by uncoupling of mitochondria) or act via receptor-mediated signal-transducing systems. In any event, all observed metabolic changes would need to be accounted for, including the sustained steady-state increases in oxygen uptake, glucose uptake, and lactate. glycerol, purine, and pyrimidine catabolite release.

Type B vasoconstrictors may act at the level of larger vessels to either redirect flow away or reduce the load from the vessels responsible for the autacoid release.

ENDOCRINE EFFECTS OF VASOACTIVE AGENTS ON MUSCLE METABOLISM

Isolated Incubated Skeletal Muscle

A consideration of direct endocrine influences on skeletal muscle assumes that the effects seen are exclusively the result of the hormone, neurotransmitter. or related substance reacting with receptors on the skeletal muscle sarcolemma and that an intermediate involvement by the vasculature has not occurred. Thus discussion in this section is directed at studies where effects on skeletal muscle have been observed with muscle(s) that have been removed from the animal and incubated or perfused with buffer containing the proposed agent. Under these conditions, the vasculature receives no flow and cannot distribute hormone or substrate. Similarly, the release and distribution of a signal substance arising from the vasculature due to changes in flow or vasoconstriction are minimized. It is also assumed that the hormone, neurotransmitter, or related substance as well as substrate has reached the muscle sarcolemma by diffusion from the bathing incubation buffer. Thus data for observed metabolic effects are considered in conjunction with evidence for the corresponding receptors on skeletal muscle sarcolemma.

Table 3 lists the metabolic effects of several vasoactive agents observed with isolated skeletal muscle preparations. Relatively few agents produce unequivocal effects. These include insulin, which has been shown with a variety of incubated muscles to increase glucose transport and glycogen synthesis (see Ref. 5 and references therein). Other insulin-sensitive processes include glycolysis (53), amino acid uptake (42), and lactate formation (51). There seems little doubt that insulin's effect to alter these parameters is a direct result of insulin interacting with insulin receptors located on the skeletal muscle sarcolemma. Support for this notion comes from binding studies for insulin to intact isolated mouse and rat soleus muscles (Table 4). Closely related insulin-like growth factor I receptor mechanisms may also be present as effects of this growth factor have been reported on amino acid uptake by isolated epitrochlearis (42) and on glucose transport. glycolysis, glycogen synthesis. and glucose oxidation by isolated soleus muscles independent of the effects of insulin (19).

Catecholamine effects can be broken down into those arising from the β-adrenergic receptor mechanisms and into those arising from α-adrenergic receptor mechanisms. β-Adrenergic effects demonstrable with isolated muscles include the production of adenosine 3',5'-cyclic monophosphate (cAMP). activation of phosphorylase (30), stimulation of glycogen breakdown (95), stimulation of lactate formation. and an inhibition of alanine and glutamine release as a consequence of an inhibition of proteolysis (Table 3). Glucose transport appears to be inhibited by isoproterenol (95), although there is a recent report (1) that the β_3-adrenergic agonist BRL

Table 3. *Endocrine effects of vasoactive hormones, neurotransmitters, and related substances on isolated skeletal muscle metabolism*

Addition	Muscle Preparation	Effects	Ref. No.
Insulin	Soleus, soleus strips	Glucose transport	Table 6 of Ref. 5
	Epitrochlearis	Glycogen synthesis	Table 7 of Ref. 5
	EDL, EDL strips	Lactate formation	51
		Glycolysis	53
		Amino acid uptake	42
IGF-I	Epitrochlearis	Amino acid uptake	42
	Soleus	Glucose transport & metabolism	19
β-Adrenergic agonists	Soleus	Glycogen breakdown	48, 60, 95
		Lactate formation	44, 48
		Inhibition of 2-DG uptake phosphorylation	48
		Increased Na/K transport and membrane potential	14
	Epitrochlearis	Alanine + glutamine release, cAMP production, phosphorylase α formation	30
β₃-Adrenergic agonist	Soleus	2-DG uptake increased	1
α-Adrenergic agonists	Rat diaphragm	Glucose uptake	78
	BC3H1 muscle cell lines	Mobilization of intracellular Ca^{2+}	6
Adenosine (or adenosine receptor agonist)	Soleus	Decreases sensitivity to insulin for glucose uptake	51 and Refs. therein
Serotonin	Epitrochlearis	Alanine + glutamine release, cAMP production, phosphorylase α production, depletion of glycogen	30

IGF-I. insulin-like growth factor I; EDL. extensor digitorum longus; 2-DG. 2-deoxy-D-glucose.

37344 increased 2-deoxyglucose uptake by 30% at 100 pM in isolated soleus muscles from young rats.

It is generally assumed that so-called "skeletal muscle membrane preparations" predominantly contain membranes from skeletal muscle; however, contamination by membranes from the vascular elements cannot be avoided. Thus, if the density of a receptor for a particular hormone or neurotransmitter is rich on the vasculature, even a small contamination of "skeletal muscle membranes" by vasculature elements can confuse the picture. For example, the relative density of β-adrenergic receptors on skeletal muscle vasculature may be higher than the surrounding muscle fibers (cf. Refs. 52 and 57), and it is therefore possible that the presence of β-adrenergic receptors of vascular origin could significantly affect data for β-receptor binding or adenylyl cyclase activity measurements with isolated membrane

Table 4. *Hormone and neurotransmitter receptors identified by binding to isolated skeletal muscle, skeletal muscle plasma membrane preparations, or skeletal muscle nuclear fractions*

Receptor	Muscle Preparation	Ref. No.
Insulin	Mouse soleus	53
	Rat soleus	96
Catecholamines		
β general	Rat muscle membranes	52, 57, 59
β₂	Rat muscle membranes	91
	Human muscle membranes	54
	Cultures of neonatal rat skeletal muscle	20
Putative β₃	Rat soleus muscle membranes	82
α₁	Rat skeletal muscle	58
α₁	Rabbit intrafusal muscle spindle fibers	33
α₂	Rabbit intrafusal muscle spindle fibers	33

For brevity. only sample references are given.

preparations. The purported status of α-adrenergic receptors on skeletal muscle is even more precarious because the relative densities strongly favor the vasculature. Autoradiographic analysis after long exposure times to [³H]prazosin (±phentolamine) showed a low but significant level of specific binding in muscle fibers (58). No difference in α₁-receptor density was observed among types I, IIa. and IIb fibers. However, small blood vessels have a high α₁-receptor density, with resistance arterioles (20–100 μm diameter) and small arteries (100–500 μm diameter) containing 6- and 32-fold more binding sites per unit section area, respectively, than surrounding muscle fibers.

To date, there have been relatively few reports of α-adrenergic metabolic effects on isolated muscle preparations. Saitoh et al. (78) reported an α-adrenergic receptor-mediated stimulation of glucose utilization by isolated rat diaphragm. However, Young et al. (95) were unable to find an α-adrenergic effect of phenylephrine to stimulate 3-O-methylglucose uptake by isolated epitrochlearis despite a report by Richter et al. (72) of an α-adrenergic effect to increase arteriovenous glucose uptake by the perfused hindlimb (see POSSIBLE MECHANISMS TO ACCOUNT FOR THE OBSERVED CHANGES IN PERFUSED HINDLIMB METABOLISM for our proposed explanation). There has been no follow-up of a report of α₁- and α₂-adrenergic receptors on rabbit intrafusal muscle spindle fibers (33). It is also important to note that the glycogenolytic effects of either epinephrine or NE with isolated rat soleus or epitrochlearis muscles are completely blocked by propranolol and are not affected by phentolamine (60. 95). Thus a role of α-adrenoceptors in muscle metabolism is not evident. A second important point is that the BC₃H1 muscle cell line. although possessing α₁-adrenergic receptors and mechanisms. is considered to be more representative of smooth rather than striated muscle (6). Finally. the functional role of

the atypical sites labeled by [^{125}I]iodocyanopindolol (putative β_3-sites; see Ref. 82) in membranes from rat soleus muscle or evenly distributed over gastrocnemius, plantaris, and soleus (richest in soleus) remains unknown (59).

Another candidate substance that may directly affect skeletal muscle metabolism is adenosine. In a series of experiments, Langfort et al. (51 and references therein) have shown that the local hormone adenosine modulates the sensitivity to insulin of the rate of glucose transport in isolated incubated skeletal muscle. Addition of adenosine deaminase or an adenosine receptor antagonist to incubated skeletal muscle preparations caused an increase in the sensitivity, whereas addition of an adenosine receptor agonist decreased the sensitivity to insulin. Even though adenosine is a strong vasodilator in perfused skeletal muscle vasculature (70), for the various reasons given above, it is unlikely that the insulin-antagonistic effects of adenosine indirectly result from receptor-mediated changes initiated at the vasculature. Indeed Challiss et al. (10) concluded that the effects of adenosine to oppose insulin-mediated glucose uptake were mediated at a "postreceptor" level. Although binding studies have not been conducted, various selective agonists and antagonists suggest that the receptor mechanism involved is of the A_1 adenosine type (10). A recent study (88) involving perfused rat hindlimb concluded that stimulation of sarcolemmic adenosine receptors during contractions was involved in the synergistic stimulation of muscle glucose transport by insulin and by contractions. Again, contrasting findings between incubated isolated muscle and perfused hindlimb could imply a vascular involvement. Clearly, further studies are required to resolve these questions.

Serotonergic receptor mechanisms appear to be present on rat epitrochlearis muscles (30). 5-HT inhibited alanine and glutamine release, increased cAMP and phosphorylase levels, and promoted the depletion of glycogen; all of these effects were blocked by methysergide or cyproheptadine (30). Although unconfirmed by binding studies, it would appear likely that 5-HT acts via 5-HT$_1$ receptors coupled to adenylyl cyclase.

COORDINATED VASCULAR AND ENDOCRINE EFFECTS

Catecholamines

This subsection attempts to draw together observations from in vivo as well as in vitro studies to present an overview of the likely coordinated effects of catecholamines on skeletal muscle metabolism. To account for the full range of physiological states, two concentration levels of NE and/or the equivalent intensity of sympathetic nerve stimulation need to be addressed. Thus low to moderate levels of circulating NE are considered to be between 0.1 and 100 nM and at the lower end are in the physiological range (41). Low-frequency sympathetic nerve stimulation is from 1 to 6 Hz and, for this range of frequencies, gives rise to data similar to the low levels of NE (12). Higher levels of NE are those exceeding 100 nM and up to 10 μM and those are believed to be approximately the levels likely to occur at vasoconstric-

tor synapses (29). Frequencies of the sympathetic nervous system (SNS) > 6 Hz are considered intense and may occur in some states of hypertension (56).

At low to moderate levels of NE and/or low levels of SNS stimulation, the final effects on skeletal muscle metabolism can be broken down into four separate aspects. First, increased cardiac output mediated by α- and β-adrenergic (61) mechanisms may lead to net increased blood flow to muscle if unconstrained by increased total vascular resistance within the muscle beds. Any net increase in flow could predictably increase the net recruitment of nutritive capillaries without a change in heterogeneity and, through mechanisms unknown, could lead in turn to increased metabolism. Flow-dependent increases in oxygen uptake in perfused hindlimb preparations have been noted by several laboratories (see Ref. 5 and references therein), although there has been one exception (36). Increased flow also leads to increased lactate (94). Second, and as discussed in VASOCONSTRICTORS OF PERFUSED HINDLIMB THAT STIMULATE BASAL METABOLISM, low concentrations of NE lead to marked increases in metabolism, including oxygen uptake, lactate, glycerol, purine, pyrimidine efflux, glucose uptake, and insulin-mediated glucose uptake. Under some conditions (e.g., hindlimbs from young rats), the increase in perfusion pressure resulting from the vasoconstriction is remarkably small (5–10 mmHg) even though the increase in metabolism is large. We and others (75) have proposed that the effects of vasoconstrictors to increase metabolism result from site-specific vasoconstriction to change flow patterns within muscle. The nature of high-capacity nonnutritive flow in anatomical terms is discussed in POSSIBLE MECHANISM TO ACCOUNT FOR THE OBSERVED CHANGES IN PERFUSED HINDLIMB METABOLISM. For low-dose NE, the sites of vasoconstriction are Ca^{2+} dependent, have a 100-fold higher sensitivity to prazosin than yohimbine (21), and may fall in the category of putative α_{1L}-receptors (7). Third, β-adrenergic receptor mechanisms of a vasodilatory nature may also operate on the skeletal muscle vasculature. Indeed isoproterenol, when added with low-dose NE, completely opposes the NE-mediated vasoconstriction and associated metabolic effects (16). Thus vasodilators, in general, appear to oppose the changes in nutritive and nonnutritive flow brought about by putative α_{1L}-adrenergic receptor activation involving low-dose NE and return metabolism to pre-NE values. Despite this possible scenario, the β-adrenergic vascular activity of NE in the rat hindlimb appears remarkably weak. Fourth, NE exerts a strong β_2-adrenergic effect directly on skeletal muscle leading to the activation of adenylyl cyclase and the various cAMP-dependent processes. Although the role may be to support the energetic requirements of working skeletal muscle, the action on resting muscle is less clear. Overall, the coordinated response resulting from low-level NE and/or low-frequency SNS stimulation is increased basal metabolism, resulting partly from increased blood flow to muscle and site-specific vasoconstriction within the muscle vasculature to increase the proportion of nutritive nonnutritive flow and hence metabolism. This may

have benefits by increasing nutrient delivery (putative α_{1L}) and mobilization of endogenous fuel reserves (β_2) in anticipation for future work by the muscle fibers or for a thermogenic intent, presumably to warm muscle or the body generally.

At higher levels of NE and/or intense SNS stimulation, the positive metabolic effects of increased blood flow to muscle and site-specific α_{1L} vasoconstriction are overridden by a strong putative α_{1H} vasoconstriction (7) to open functional vascular shunts (nonnutritive). The consequence of this is to dramatically decrease nutritive flow, and the coordinated response of high-level NE and/or intense SNS stimulation is decreased metabolism (see VASOCONSTRICTORS OF PERFUSED HINDLIMB THAT INHIBIT BASAL METABOLISM and POSSIBLE MECHANISMS TO ACCOUNT FOR THE OBSERVED CHANGES IN PERFUSED HINDLIMB METABOLISM) with nonnutritive flow predominating. This scenario would have decidedly negative effects on nutrient and hormone delivery (68), metabolic end product efflux, and contractility of skeletal muscle (see CORRELATION BETWEEN VASCULAR EFFECTS TO ALTER METABOLISM AND VASCULAR EFFECTS TO ALTER CONTRACTILITY OF PERFUSED HINDLIMB MUSCLE).

A major difficulty in this consideration is the set point of the ratio of nutritive/nonnutritive flow in muscle in vivo. The reasoning put forward above is based on the assumption that, under basal conditions, the extent of nonnutritive flow is relatively low. If this is the case, then the positive metabolic effects of putative α_{1L}-adrenergic vasoconstriction would be relatively small, and the negative metabolic effects of putative α_{1H}-adrenergic vasoconstriction would be profound. Alternatively, if the extent of nonnutritive flow is relatively high in skeletal muscle in vivo, the opposite conclusion would prevail with α_{1L}-adrenergic vasoconstriction having a marked effect to increase metabolism. There are reasonable grounds to predict (71) that lack of physical activity and/or aging may cause a shift from predominantly nutritive to predominantly nonnutritive flow.

Insulin

Recent interest in the cardiovascular actions of insulin has highlighted the possible effect of this hormone to increase its own access to skeletal muscle by hemodynamic effects (see Ref. 4 and references therein). Although there is some controversy as to whether insulin increases blood flow to skeletal muscle (4), it is quite possible that it does so. A correlation between the increase in cardiac output and leg blood flow suggests that the increase in cardiac output is a major contributor to the increased flow. Insulin-mediated vasodilation and lowering of leg vascular resistance by a nitric oxide-dependent mechanism is believed to partially account for the increase in leg blood flow and to act as an amplifier of insulin action (4). However, questions arise concerning the mechanisms by which insulin increases cardiac output. Whereas previous explanations have focused on a putative activation of the SNS by insulin with the assertion that mean arterial blood pressure would rise (76), recent findings suggest that this may not be so simple. Thus Anderson et al. (2) reported that

systemic hyperinsulinemia produced a marked increase in muscle sympathetic neural outflow with a simultaneous reduction of forearm vascular resistance and a small fall in blood pressure. Data from Baron (4) would suggest that the combined effect of increased cardiac output and vasodilation within skeletal muscle vasculature improves insulin (and glucose?) access. It is too soon to speculate whether insulin directly, or indirectly, improves access by modulating the proportion of nutritive/nonnutritive flow. Clearly the potential is there for a coordinated response involving the combination of a vascular effect of insulin with actions through the insulin receptors located on skeletal muscle fibers.

In perfused hindlimb studies, insulin has been reported to cause a small vasodilatory effect without SNS involvement (68), and there have been some reports that insulin appears to lower intracellular Ca^{2-} concentration of vascular smooth muscle cells in culture (85).

CORRELATION BETWEEN VASCULAR EFFECTS TO ALTER METABOLISM AND VASCULAR EFFECTS TO ALTER CONTRACTILITY OF PERFUSED HINDLIMB MUSCLE

Although the inotropic effect of epinephrine on contracting skeletal muscle has been known for some time (see Ref. 90 and references therein), the effects seen were invariably the result of systemic injection of extracts of the adrenal gland or epinephrine itself. Thus the cardiovascular effects of epinephrine, including changes in cardiac output, peripheral vascular resistance, and within-muscle redistribution of flow (see below), could not be separated from direct effects on muscle. Furthermore, it was clear that not all skeletal muscle types were alike in their response to the inotropic action of epinephrine. In fast-contracting muscles, twitch tension and the degree of subtetanic fusion were increased, the rate of relaxation was slowed, and tetanic tension was unaltered by epinephrine. Conversely, in slow-contracting muscles, epinephrine reduced twitch tension and increased the rate of relaxation while it decreased unfused tetanic contraction tension and the degree of fusion (see Ref. 90 and references therein). The question of concentration of catecholamine used and hence its action as type A or type B vasoconstrictor also seems to be important, and in previous studies this issue may not have been considered. Thus recent findings show that low- and high-dose NE have opposite effects on the contractility performance of working skeletal muscle (Table 5).

Rather surprisingly, there are only a few reports where the specific effects of vasomodulators (vasoconstrictors or vasodilators) on skeletal muscle contractility have been explored. Most information in this general area has been concerned with effects of sympathetic vasoconstriction. At the whole body level, the redistribution of cardiac output from inactive areas to exercising muscle is controlled by sympathetic vasoconstriction of the vasculature supplying the gut and kidneys (see Ref. 80 and references therein). Curtailment of blood flow to muscles generally is mediated by the SNS acting on the resistance vasculature with preferential flow to acti-

Table 5. *Effects of vasomodulators on contractile performance by striated muscle*

Preparation	Addition	Aerobic Phase Tension Development, % of control	Ref. No.
Autoperfused cat gastrocnemius-soleus preparation at constant flow	Acetylcholine (2 μg/min)	50	45
	Histamine (5 μg/min)	75	45
	Isoxsuprine (500 μg/min)	45	45
	Bradykinin (3.5 μg/min)	72	45
Autoperfused canine diaphragm strip at constant flow	Norepinephrine (20 μg/min)	100	87
Autoperfused canine diaphragm strip without flow constraint	Norepinephrine (20 μg/min)	177	87
Isolated perfused rat hindlimb at constant flow	Norepinephrine (1 μM)	155	†
	Norepinephrine (10 μM)	36	†
	Angiotensin II (1 nM)	187	†
	Serotonin (0.25 μM)*	58	23
	Epinephrine (24 nM)	167	73
Isolated perifused rat epitrochlearis muscle	Epinephrine (10 μM)	125	60
	Norepinephrine (10 μM)	115	60

*Anaerobic phase contraction was not inhibited by 0.25 μM serotonin (23). † Unpublished observations.

vated muscle facilitated by the release of unknown vasodilator substances produced by the working muscle.

There is histochemical evidence for the presence of adrenergic nerves throughout the skeletal muscle arteriolar network, excluding capillaries and venules. However, adrenergic receptors are present on venules, and thus control of blood flow can be manifest at various sites by blood-borne catecholamines as well as those diffusing upon release from nerve endings on nearby arterioles (for review see Ref. 85 and references therein). Anderson and Faber (3) have noted a differential location of α_1- and α_2-adrenoceptors on large and small arterioles, respectively, with differential sensitivity to metabolic inhibition during muscle contraction. A further control of nutrient supply may occur at the level of the feed arteries although these may be less susceptible to the influence of the vasoactive products of muscle contraction, as they are external to the muscle parenchyma (80).

Studies using the constant-flow perfused rat hindlimb (23, 73) and earlier observations from the autoperfused dog and cat muscle preparations lend support to the notion that vasomodulators exert a marked regulatory influence on contractility performance of skeletal muscle. Table 5 shows the effects of several vasomodulators (neural and blood borne) on contractile performance of skeletal muscle. Data for the diaphragm are included because differences between types of striated muscles may occur. For example, Supinski et al. (87) noted that NE had little or no effect on tension development at constant flow but increased contractility when NE was able to increase blood flow. Thus a simple relationship between oxygen supply and tension development may

apply to the diaphragm. For hindlimb skeletal muscle, a more complex relationship appears to occur even though, for an individual, muscle changes in total blood flow (delivery) result in a proportional change in contractile performance (32).

Infused agents that act to reduce aerobic tension development thus far would appear to fall into two categories. In the first category are the vasodilators (Table 5), which when used in the constant-flow autoperfused cat gastrocnemius-soleus preparation led to decreased tension (45). The second category includes the type B vasoconstrictors described above (see Table 1). Of these, 5-HT and high-dose NE (≥10 μM) were most prominent. Thus, under the already fully vasodilated conditions of constant flow, infusion of each of these markedly reduced aerobic tension development and tension-dependent oxygen consumption while raising perfusion pressure (12, 23). Of particular importance were the additional findings that the inhibitory effects on contractility were blocked by vasodilators and that the inhibitory effect could not be observed when representative muscles were isolated and tested in vitro (23). Such findings suggest that the inhibitory effects of 5-HT (23) or high doses of NE (12) are due to a vascular effect.

More recent observations, again using the isolated constant-flow perfused rat hindlimb, have confirmed that epinephrine (73) and other vasoconstrictors belonging to the type A group improve contractility of working muscle (Table 5). Again, these vasoconstrictors have little or no effect on tension development when muscles are isolated and stimulated to contract as incubated preparations in vitro. Indeed, the metabolic control of isolated incubated muscle exerted by endocrine effects may be reduced in the perfused muscle preparations because of vascular effects. Thus β-agonists enhance contractility of isolated perifused rat epitrochlearis muscles by 25% (Table 5); these effects are also visible in constant-flow perfused rat hindlimb when α-antagonists are present (72) but additivity of the α- and β-adrenergic effects in perfusion may not be quantitative, as β-agonists oppose the constrictor effects of type A vasoconstrictors (16), including NE.

Attempts to explain the basis by which vasomodulators affect muscle contractility have in the past considered the possibility that there is a distribution of flow within skeletal muscle into two components of "nutritional" and "nonnutritional" (46) that are altered by various procedures as outlined in POSSIBLE MECHANISM TO ACCOUNT FOR THE OBSERVED CHANGES IN PERFUSED HINDLIMB METABOLISM.

Data supporting a functional (nonnutritional) shunt of 30–40% in the dog gastrocnemius muscle, both at rest and during stimulation, have been observed using the local xenon clearance method (9) and by the inert gas washout (65), and Piiper and Haab (64) have proposed an unequal blood flow model for muscle without diffusion limitation that allows adaptation when blood flow, arterial O_2 content, or O_2 requirement are changed. Thus, according to these authors (64), unequal distribution of blood flow and shunt are important factors limiting O_2 uptake in the tissues when the ratio O_2

delivery/O_2 requirement is reduced. Such a proposal may account for the findings of Hogan et al. (47) that maximal O_2 uptake decreased in proportion to venous Po_2 of the muscle under conditions of reduced O_2 delivery. In addition, the proposal is consistent with the data of Stainsby et al. (83) where blood flow through muscle was well correlated with the development of fatigue and was decreased as fatigue developed in a manner that kept the blood arteriovenous oxygen difference nearly constant.

A VASCULAR BASIS FOR ACUTE INSULIN RESISTANCE AND OBESITY ARISING FROM SITE-SELECTIVE VASOCONSTRICTION

As indicated earlier in this review, the effects of vasoconstrictors can be divided into two types. In all respects, the metabolic effects of the type A vasoconstrictors were the opposite to those of type B (compare VASOCONSTRICTORS OF PERFUSED HINDLIMB THAT STIMULATE BASAL METABOLISM with VASOCONSTRICTORS OF PERFUSED HINDLIMB THAT INHIBIT BASAL METABOLISM).

The possibility that type B vasoconstrictors inhibit nutrient delivery and end-product efflux by the constant-flow perfused rat hindlimb was taken a stage further by a series of experiments that focused on glucose uptake (Table 6). In these experiments, rat hindlimbs were perfused with medium containing glucose and a tracer amount of 2-deoxy-D-[1-^{3}H]glucose with and without 5-HT, insulin, and a combination of the two (67). 5-HT was found to inhibit insulin-mediated stimulation of glucose uptake by ~30%. In addition, 5-HT inhibited insulin-mediated 2-deoxy-D-[1-^{3}H]glucose uptake by perfused muscles with an inhibition ranging from 32% (soleus) to 80% [extensor digitorum longus (EDL)]. Furthermore, the effects of 5-HT on insulin-mediated glucose uptake were partially opposed when the vasodilator carbachol was coinfused with 5-HT (67).

To examine the possibility that 5-HT might directly affect glucose uptake/metabolism in skeletal muscle, isolated soleus and EDL muscles were incubated with 5-HT. In contrast to the results for the hindlimb, 5-HT had no significant effect on either basal glucose uptake

Table 6. *Effects of serotonin and high-dose (10 μM) norepinephrine on glucose uptake and 2-deoxyglucose uptake by isolated perfused rat hindlimb*

Additions	Hindlimb A-V Glucose Uptake, $\mu mol \cdot h^{-1} \cdot g^{-1}$	R_g for Thigh Muscle Group, $\mu mol \cdot h^{-1} \cdot g^{-1}$
None	8.5 ± 1.0	15.0 ± 1.0
5-HT (10 μM)	10.0 ± 1.5	11.0 ± 0.5
Insulin (15 nM)	22.4 ± 1.3	67.0 ± 7.0
5-HT (10 μM) + insulin (15 nM)	16.2 ± 0.8	30.0 ± 3.0
None*	16.0 ± 4.2	20.0 ± 5.0
NE (10 μM)	16.0 ± 3.0	3.8 ± 1.5
Insulin (15 nM)	42.0 ± 5.0	86.0 ± 7.0
NE (10 μM) + insulin (15 nM)	24.0 ± 4.0	22.0 ± 3.0

Values are from constant-flow perfusions at 32°C using 240- to 270-g animals or at 37°C using 72-g animals (*) and are means ± SE for $n = 4-5$ animals in each group (67, 68). A-V, arteriovenous; 5-HT, serotonin; NE, norepinephrine; R_g, 2-deoxyglucose uptake.

or the stimulation of glucose uptake mediated by insulin with either muscle (67). These findings, overall, suggest that 5-HT acting via the vasculature is able to control glucose and insulin access, thus imposing a vascular effect on the endocrine effect of insulin to stimulate skeletal muscle glucose uptake.

Parallels between the effect of 5-HT and of a high concentration of NE to inhibit oxygen uptake during vasoconstriction in the perfused rat hindlimb (24) led to an exploration of the effects of NE on insulin-mediated glucose uptake (68). In that study (68), the effects of low- and high-dose NE and adrenergic blockers on insulin-mediated 2-deoxyglucose uptake by muscles and muscle groups of the perfused rat hindlimb were investigated. In general, the inhibitory effects of the low- and high-dose NE on insulin-mediated glucose uptake were present in all three muscle preparations but were most marked in EDL and the thigh muscles where complete inhibition occurred with 10 μM NE. At low-dose NE the less marked inhibitory effect was blocked by either α (soleus)- or β (soleus, EDL, and thigh)-blocker. At the higher dose of 10 μM NE the more marked inhibitory effect on insulin-mediated uptake of 2-deoxyglucose was again partly blocked by either propranolol (EDL and thigh) or prazosin (thigh) and was totally blocked by a combination of the two (soleus, EDL, and thigh: see Ref. 68). Thus this recent study (68) identifies an α-adrenergic effect of NE to inhibit insulin-mediated glucose uptake in perfused rat hindlimb. The inhibitory effect is evident at doses thought to occur at sympathetic vasoconstrictor synapses (≥ 10 μM; see Ref. 29) and is similar in character to that seen for 5-HT (67; see Table 6). In addition, the α-adrenergic-mediated effect was undetectable when muscle was incubated rather than perfused.

On the basis of these findings, we propose that there is a hemodynamic basis for insulin resistance in skeletal muscle. Our hypothesis is that a high proportion of functional vascular shunting occurs early in the development of insulin resistance as a result of hypertension-associated site-specific vasoconstriction in skeletal muscle vasculature to reduce access for glucose, insulin, other nutrients, and hormones. As the hypertension-associated site-specific vasoconstriction persists with time, the impaired access for nutrients and hormones leads to permanent changes to both the vasculature (rarefaction; see Ref. 97) and skeletal muscle (alterations to the expression of proteins for insulin signal transduction, glucose metabolism, and contraction), characteristic of long-term insulin resistance. A key issue in this hypothesis is the presence within skeletal muscle of functional vascular shunts of small diameter along the lines discussed in POSSIBLE MECHANISMS TO ACCOUNT FOR THE OBSERVED CHANGES IN PERFUSED HINDLIMB METABOLISM and different from the large anatomical arteriovenous shunts of other tissues.

CONCLUSION

Recent data have led to a revision of some of the older concepts of nutritive and nonnutritive flow (functional vascular shunts) in muscle, reinforcing the view that metabolism and hemodynamics are interdependent.

A major advance is the discovery that site-specific vasomodulators control muscle metabolism and performance by apparently regulating the proportion of nutritive to nonnutritive flow within muscle. Imperfections in the control of flow distribution within muscle may arise in hypertension and may have implications for insulin, glucose, and O_2 access.

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FUNCTIONAL AND METABOLIC EVIDENCE FOR TWO DIFFERENT VANILLOID (VN₁ and VN₂) RECEPTORS IN PERFUSED RAT HINDLIMB

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Summary

Vanilloid spice principles, including capsaicin, stimulate vasoconstriction in the rat hindlimb perfused at constant flow and, depending on dose, either stimulate or inhibit oxygen consumption by this vascular bed. We now present metabolic and functional evidence for two different vanilloid (VN_1 and VN_2) receptor types. These receptors can be distinguished on the basis of their differing agonist affinity for capsaicin, their different calcium and oxygen dependencies for inducing vasoconstriction, and whether they stimulate, or inhibit, oxygen consumption. The higher affinity vanilloid receptor, VN_1 can be distinguished on the basis of initiating vasoconstriction at low doses of capsaicin and simultaneously stimulating oxygen consumption. Its apparent biological function is dependent on the presence of oxygen and external calcium. In contrast, the lower affinity receptor, VN_2 induces vasoconstriction associated with inhibition of oxygen consumption. Its vasoconstriction action can occur independently of either external calcium ions, or the presence of oxygen in the perfusate.

The active pungent ingredients of hot peppers and chillies from the genus *Capsicum* (family Solanaceae) are the capsaicinoids, a family of closely related acid amides of vanillylamide (1). The major naturally occurring capsaicinoids, capsaicin (8-methyl-N-vanillyl-6-nonanamide) and its reduced form, dihydrocapsaicin, both appear to increase in concentration in proportion to the increase in 'hotness' or pungency of fruits (2). Other spices such as ginger and black pepper contain chemically related compounds, each characterised by a homovanillyl moiety so that the general class of molecules may be called vanilloids (3).

Most research interest into capsaicin and other vanilloids has centered on their well known actions

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on unmyelinated sensory nerves in the periphery as well as as in the spinal cord and the brain. Recently, the role of vanilloid or capsaicin-like molecules in the depolarisation of these nerves and the mediation of pain and other effects has been reviewed extensively (4).

Capsaicin-sensitive primary afferent neurones release a number of neuropeptides when stimulated by capsaicin or other active vanilloids. These include substance P, neurokinin A, calcitonin gene-related peptide, galanin, dynorphin, cholecystokinin, vasoactive intestinal peptide and somatostatin (1). These peptides all appear to play a role in the communication of primary sensory neurones with other neuronal and non-neuronal cells.

The sensitivity of the capsaicin-sensitive neurones to vanilloids is most likely due to the presence of a cation channel which when stimulated by capsaicin, allows the influx of calcium and sodium ions and the efflux of potassium ions (5). Capsaicin and other vanilloids are relatively lipophilic molecules and it is suggested that capsaicin may have a binding site on the surface of the cation channel proteins within the lipid bilayer (6). The binding of capsaicin then results in the opening of the channel and the initiation of an impulse, the release of neuropeptides and ultimately the acute, painful, burning sensation associated with capsaicin (7).

The acute actions of capsaicin are not restricted to neurones and there are a number of reports of capsaicin influencing non-neuronal systems. These effects of capsaicin and functional analogues include inhibition of cardiac muscle excitability, inhibition of visceral smooth muscle activity and contraction of vascular smooth muscle (1).

Capsaicin and dihydrocapsaicin (8), gingerols and shogaols (9) and resiniferatoxin and piperine (10) have been shown in our laboratory to directly stimulate oxygen uptake at low concentrations when infused into the perfused iliac bed of the rat hindlimb. The vanilloids stimulated a maximum increase of approximately 20-25% in oxygen consumption, and a concomitant increase of approximately 50% in perfusion pressure. At higher doses of vanilloid, the perfusion pressure continued to rise, but the oxygen consumption fell with increasing dose to reach frank inhibition relative to the starting oxygen consumption.

In contrast to the findings of Kawada et al. (11) and Watanabe et al. (12) in whole rats, neither α-nor β-adrenergic antagonists significantly altered the vanilloid-induced effects in the perfused rat hindlimb (8,9,10). However, the vanilloid actions were largely, but not completely, blocked by the vascular smooth muscle relaxants nitroprusside and glyceryl trinitrate (GTN). Thus it appears that the vanilloids may act via a non-adrenergic mechanism, perhaps similar to their actions on the cation channel in sensory nerves. Such an action might be directly on smooth muscle or alternatively by the release of tachykinins from perivascular nerves which then cause vasoconstriction and produce either a stimulation of oxygen consumption at lower doses, or an inhibition of $\dot{V}O_2$ at higher doses.

The bidirectional effect on oxygen consumption found with each vanilloid (8,9,10) suggests that two different vanilloid receptors might be present on or near vascular smooth muscle, or that the same receptor could be located on different responsive cell types having different post-receptor events. This communication presents further functional and metabolic evidence showing two distinctly different sets of actions of capsaicin in the perfused rat hindlimb.

Methods

Chemicals:
Bovine serum albumin (Fraction V), NAD$^+$ (free acid) and lactate dehydrogenase (5 mg/ml) were

purchased from Boehringer Mannheim (Australia); capsaicin, ethylene-glycol-bis(β-aminoethyl-ether) N,N-tetraacetic acid (EGTA), xylazine, polyoxyethylene sorbitan monooleate (Tween 80) from Sigma (USA); sodium azide from Merck (Germany); potassium cyanide from B.D.H. Laboratory Chemicals Division (Poole, England); pentobarbitone sodium (Nembutal, 60 mg/ml) from Bomac Laboratories Pty. Ltd. (Australia); heparin sodium from David Bull Laboratories (Australia); paracetamol drops from Mead Johnson (Australia) and ketamine from Aldrich Chemical Co. (USA). All other chemicals were of analytical grade and were purchased from Ajax Chemicals Ltd (Australia).

Rat Hindlimb Perfusion:
All anaesthetic, surgical and subsequent experimental procedures were approved by the University of Tasmania Animal Ethics Committee under the Australian code of practice for the care and use of animals for scientific purposes (13).

Experiments were performed using male, 180 to 200 g hooded Wistar rats raised on a commercial rat chow diet containing 21.4% protein, 4.6% lipid, 68% carbohydrate and 6% crude fibre with added vitamins and minerals (Gibson's, Hobart) together with water *ad libitum*. Animals were housed in groups at $21\pm1°C$ under a 12 h:12 h light/dark cycle. Anaesthesia, surgery and perfusion procedures were performed as described previously (14). One hindlimb was perfused at a constant flow rate of 4 ml/min, at $25°C$. The perfusion medium was a modified Krebs-Ringer bicarbonate buffer containing 8.3 mM glucose, 1.27 mM $CaCl_2$ and 2% (w/v) dialyzed bovine serum albumin (Fraction V).

The perfusion buffer reservoir was gassed with 95% O_2-5% CO_2 at 4°C and the perfusate was further oxygenated by pumping it through a silastic lung gassed with 95% O_2-5% CO_2. This ensured constant arterial PO_2 levels. During hypoxic perfusions, 95% N_2-5% CO_2 replaced the 95% O_2-5% CO_2 mixture. Calcium-free ("zero calcium") perfusions were performed by omitting calcium from the perfusate and adding 0.1 mM Na_2EGTA.

The oxygen content of venous effluent was measured continuously *via* an in-line 0.5 ml capacity Clark-type oxygen electrode. The method of calculation of oxygen uptake has been described previously (14). When required, oxygen consumption and perfusion pressures were calculated from both peak and steady state values on the chart recorder.

The infusion of the various agents into the rat commenced only after the hindlimb had reached steady state oxygen uptake and pressure values (approximately 30 min). Agents infused during the perfusion were freshly prepared prior to use. Due to the lipophilic nature of vanilloids and their apparent affinity for silastic tubing, capsaicin was dissolved in 50% ethanol and infused using a syringe pump (Model 355, Sage Instruments, Orion Research Inc., USA) driving a 1.0 ml glass syringe equipped with teflon tubing. All other agents were dissolved in isotonic saline and infused using a LKB 2132 Microperpex peristaltic pump (Bromma, Sweden) at rates between 5 and 40 ml/min. Controls were conducted with vehicle alone.

Lactate assay was based on that of Gutman and Wahlefeld (15). Perfusate samples were kept on ice and centrifuged at 3000 g, 5°C for 5 min to sediment any remaining red blood cells and the supernatant transferred to a new tube and stored at -20°C until assay.

Statistical Analysis:
The statistical significance of differences between groups of data was assessed by the unpaired Student's *t* test. Significant differences were recognised at $P<0.05$. All values given are the mean $\pm$ standard error (SE).

Results

After perfusions had reached steady state, the mean arterial PO_2 was 672.5±8.0 mm Hg (n=31) and the unstimulated mean venous PO_2 was 372.7±7.9 mm Hg (n=31) with a basal $\dot{V}O_2$ of 7.0±0.2 $\mu mol.g^{-1}.h^{-1}$ (n=31) and a mean perfusion pressure of 24.5±0.6 mm Hg (n=31). Infusion of capsaicin over its effective range gave similar but slightly more potent data to that of Cameron-Smith et al. (8). At the lower end of the dose range (0.125 µM), capsaicin showed a monophasic stimulation of oxygen consumption (Fig. 1A.) and the expected vasoconstriction-induced rise in perfusion pressure (Fig. 1B). Infusion of higher doses of capsaicin (>0.5 µM) led to further vasoconstriction (Fig. 1B). However, the effects on oxygen consumption became triphasic with an initial stimulation followed by a steady state inhibition and a third phase of transient stimulation of oxygen consumption upon cessation of the infusion of capsacin (Fig. 2A) as previously observed by Cameron-Smith et al. (8). Steady state values for the high dose inhibition are shown in Fig. 1A. Other vanilloids show similar triphasic effects at high dosage (9,10).
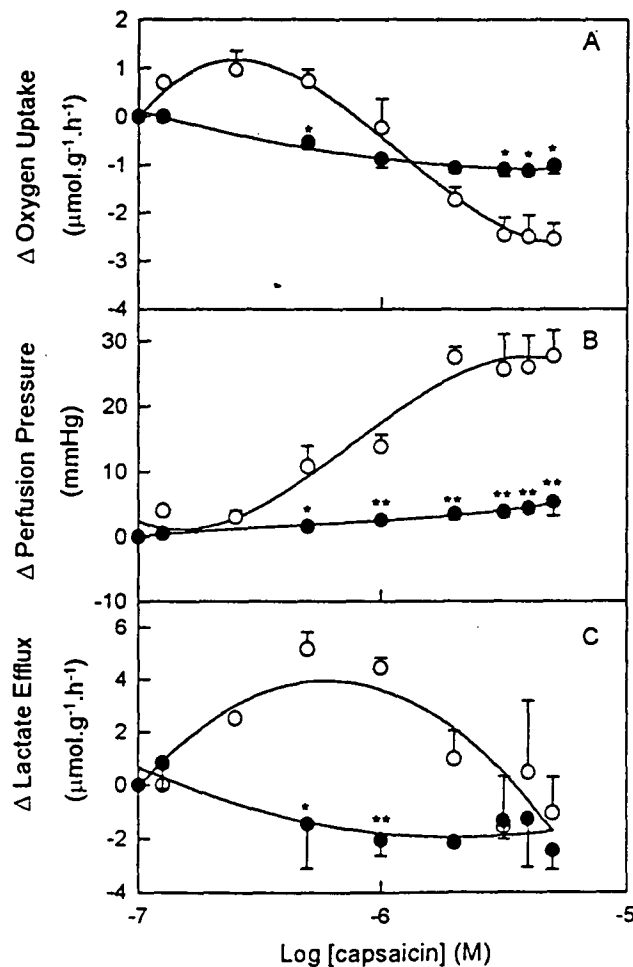
Fig. 1

Dose response curve for changes in steady state oxygen consumption (panel A), plateau perfusion pressure (panel B) and lactate efflux (panel C) in response to capsaicin in perfused rat hindlimbs perfused with medium containing 1.27 mM calcium (O) or with medium containing 0.1 mM EGTA and no added calcium (●). Points are the mean±S.E. of 3-5 observations. Where error bars are not visible they are within the symbol. *P<0.05 **P<0.01

Infusion of large concentrations (2 μM) of capsaicin increased the perfusion pressure (Fig. 2B) by a maximum of 39.1±2.4 mm Hg (173.4±12.3% above basal, n=5) followed by a steady change of 29.4±1.1 mm Hg (130.1±6.2% above basal, n=5). In association with the rise in perfusion pressure, 2 μM capsaicin exhibited a triphasic oxygen consumption response. Initially $\dot{V}O_2$ increased (P_vO_2 decreased) transiently above basal oxygen consumption during Phase 1, and was then inhibited to below basal (P_vO_2 increased) by 1.9±0.5 μmol.g^{-1}.h^{-1} (27.3±5.3%, n=5) during steady state (Phase 2). High dose capsaicin-induced effects approached steady state $\dot{V}O_2$ inhibition within 5 min of infusion and remained constant provided capsaicin was not withdrawn. The removal of capsaicin resulted in a period of increased oxygen uptake (Phase 3). The $\dot{V}O_2$ transiently increased above basal by 1.5±0.1 μmol.g^{-1}.h^{-1} (22.2±2.3%, n=5), while perfusion pressure rapidly

Fig. 2

Time courses of the oxygen and pressure responses exhibited by 2 μM capsaicin in the hindlimb perfused with: medium containing 1.27 mM calcium (n=5) (A,B) or medium containing 0.1 mM EGTA and no added calcium (n=3) (C,D). Values are the mean ± S.E.. Resting values of venous PO$_2$ were (A) 395.9±24.7 mm Hg and (C) 394.4±21.9 mm Hg. Basal perfusion pressures were (B) 21.6±0.9 mm Hg and (D) 23.7±0.9 mm Hg.

fell to resting values. The steady state dose response curves for $\dot{V}O_2$, perfusion pressure and lactate efflux in response to capsaicin are shown in Fig. 1. In general, the pattern of lactate efflux (Fig. 1C)

followed the oxygen consumption; it being increased during stimulatory phases and inhibited in Phase 2 in which steady state $\dot{V}O_2$ was inhibited.

The infusion of capsaicin in the presence of an effectively zero external concentration of Ca^{2+} (0.1 mM EGTA) led to marked diminution of the perfusion pressure (Figs. 1B and 2D) and changes in the $\dot{V}O_2$ (Figs. 1A and 2A,C) and lactate efflux responses (Fig. 1C). Vasoconstriction was less at all effective concentrations of capsaicin, and the steady state $\dot{V}O_2$ (normally either Phase 1 for low dose capsaicin, or Phase 2 for higher inhibitory doses in the presence of Ca^{2+} ions, Fig. 2A) was either zero at low doses, or inhibited (P_vO_2 increased) at higher doses (see Fig. 1), However the inhibition was less in magnitude than the inhibition of $\dot{V}O_2$ observed in the presence of Ca^{2+} ions. Zero Ca^{2+} reduced the steady-state efflux of lactate during infusion of 2 μM capsaicin (Fig. 1C) followed by a transient increase after capsaicin removal (data not shown).

The effects of hypoxia, cyanide and azide on resting hindlimb $\dot{V}O_2$ and perfusion pressure are summarised in Fig. 3 and Table 1. Changes in $\dot{V}O_2$ were calculated from the basal values of those perfusions before additions. No significant effect on basal perfusion pressure was observed for any of these treatments. Infusion of 1 μM potassium cyanide inhibited $\dot{V}O_2$ by 4.6±0.4 μmol.g^{-1}.h^{-1} (77.9±5.2%, n=5) below basal value. Infusion of sodium azide increased $\dot{V}O_2$ by 4.4±0.1 μmol.g^{-1}.h^{-1} (73.2±7.1%, n=5) above basal value. Gassing the perfusion medium with 95% N_2-5% CO_2 decreased the arterial partial pressure of oxygen (PO_2) from 652 mm Hg to 18.5±2.6 mm Hg. Representative traces for the action of high dose capsaicin in the rat hindlimb when mitochondrial respiration was impaired are shown in Fig. 3.

When steady state inhibition of oxygen consumption was reached with 1 mM potassium cyanide, shown in Fig. 3C as an increase in venous PO_2, 2 μM capsaicin was infused. This resulted in a transient and rapid increase in perfusion pressure of approx. 19.2±1.1 mm Hg before falling by approx. 14 mm Hg to be maintained at 4.8±0.3 mm Hg above the basal value (see Table 1). In addition, there was a small increase in oxygen consumption of 0.3±0.1 μmol.g^{-1}.h^{-1} (n=5) that was not sustained but rapidly returned to the level existing prior to the infusion of capsaicin. Upon removal of capsaicin, the perfusion pressure returned to the basal value.

At the peak stimulation of oxygen consumption (deepest trough in venous PO_2) achieved by 1 mM sodium azide (Fig. 3E), subsequent infusion of 2 μM capsaicin similarly resulted in a rapid and transient increase in perfusion pressure of 24.9±1.9 mm Hg. As with the cyanide experiments, the pressure was not sustained and fell by approx. 12 mm Hg to a steady state of 12.5±1.5 mm Hg above basal (n=5). Capsaicin clearly induced a biphasic inhibition of azide-stimulated oxygen consumption. In the first rapid phase, $\dot{V}O_2$ was inhibited by 0.9±0.2 μmol.g^{-1}.h^{-1} before the second phase of inhibition of $\dot{V}O_2$ which was decreased by 1.0±0.4 μmol.g^{-1}.h^{-1}. Removal of capsaicin resulted in the pressure returning to the basal value and the $\dot{V}O_2$ returning to the azide-alone stimulated value observed prior to capsaicin infusion.

Under hypoxic conditions achieved by gassing with 95% N_2-5% CO_2, 2 μM capsaicin induced a maximal perfusion pressure increase of 19.2±3.2 mm Hg above basal, as indicated in Table 1 and Fig. 3H. The pressure fell back to be maintained for the remainder of the infusion period at a value of 40.9±2.7 mm Hg or 15±2.7 mm Hg above basal. Once the capsaicin was removed, pressure again returned to resting values. In all three methods of disturbing mitochondrial action in the perfused hindlimb, the infusion of a low dose of capsaicin (0.25 μM) had no discernible effect on perfusion pressure nor oxygen uptake (data not shown).

TABLE I

Effects of 2μM Capsaicin on Oxygen Uptake and Perfusion Pressure during Cyanide, Azide and Hypoxia

Treatment	phase (n)	Arterial PO₂ (mm Hg)	Venous PO₂ (mm Hg)	V̇O₂ (μmol·g⁻¹·h⁻¹)	Perfusion pressure (mm Hg)
Vehicle only control	(8)	662.1±10.9	359.8±15.6	7.2±0.4	23.1±1.2
+2μM Capsaicin phase 1	(8)			7.8±0.5	58.6±2.1
phase 2	(8)			5.3±0.4	50.8±1.7
phase 3	(8)			8.6±0.5	24.6±1.8
control	(5)	710.5±17.6	411.4±16.6	5.9±0.3	26.9±0.6
Cyanide (1mM) alone	(5)	670.7±1.7	613.3±12.2	1.3±0.3	25.3±0.8
+2μM Capsaicin phase 1	(5)			1.6±0.3	48.5±2.5
phase 2	(5)			1.0±0.3	34.1±2.9
control	(5)	681.3±11.1	396.3±16.4	6.9±0.6	24.9±1.8
Azide (1mM) alone	(5)	680.7±14.5	239.3±13.9	10.1±0.5	26.1±0.5
+2μM Capsaicin phase 1	(5)			9.1±0.6	54.0±1.9
phase 2	(5)			9.1±0.8	42.1±3.9
phase 3	(5)			9.9±0.5	35.8±4.3
control	(5)	666.9±26.1	359.2±12.5	6.8±0.8	25.5±0.8
N₂:CO₂(95%:5%)alone	(5)	18.5±2.6	5.8±1.5	0.3±0.1	25.9±1.1
+2μM Capsaicin phase 1	(5)			0.3±0.1	45.1±3.5
phase 2	(5)			0.3±0.1	40.9±2.7

Values are means±S.E.; n = number of hindlimb perfusions. The effects of cyanide, azide and hypoxia on basal venous and arterial oxygen tension, $\dot{V}O_2$, and perfusion pressure are shown. A gas mixture of 95% N₂-5% CO₂ was used to induce hypoxia. Control values are basal levels before treatment with cyanide, azide or nitrogen. Phase 1 is the initial stimulatory phase of oxygen uptake associated with peak pressure development. Phase 2 is steady state. Phase 3 is following the removal of capsaicin. (See also Fig 3.)
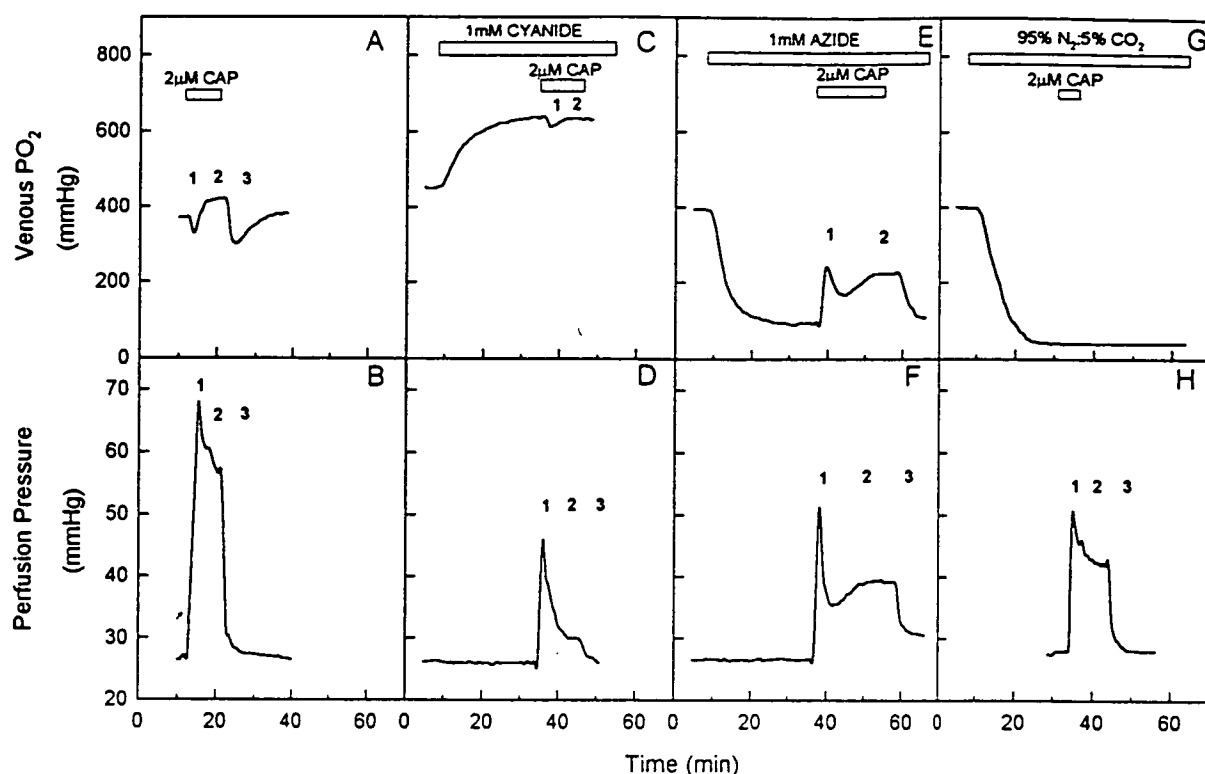
Fig. 3

Representative tracings of changes in venous PO_2 and perfusion pressure in response to infusion of 2 μM capsaicin (CAP) in the presence of potassium cyanide (1 mM), sodium azide (1 mM) or hypoxia. Perfusion medium, initially equilibrated against 95% O_2-5% CO_2, was either maintained (A-F) or switched to one equilibrated against 95% N_2-5% CO_2 (G,H) as shown. Capsaicin was infused for varying times until apparent steady state conditions of oxygen consumption were obtained. Phases of oxygen consumption and pressure change are labelled as follows: initial respose, phase 1; steady-state response, phase 2; recovery phase, phase 3. Mean values from all experiments are given in Table 1.

Discussion

This study has extended the findings of previous work with vanilloids in the perfused rat hindlimb model (8-10) by examining the responses to both high and low dose vanilloid stimulation under conditions of metabolic challenge. These included low external calcium concentration, hypoxia, and disruption to mitochondrial function using both cyanide and azide. The main finding to emerge from these present studies is that the stimulation of $\dot{V}O_2$ at low concentrations of capsaicin and the inhibition of $\dot{V}O_2$ at high concentrations of capsaicin appear to result from activation of two different mechanisms. Both responses are independent of secondary release of catecholamines (8,9,10). We propose that these different actions of capsaicin are activated through two receptor types (presumptive VN_1 and VN_2).[1] The key reasons for the proposed classification into VN_1 and VN_2 receptors are summarised in Table 2 and discussed below.

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[1]We have followed the suggestion of Szállasi and Blumberg (3) that the receptors for capsaicin and its structural analogues be called vanilloid receptors. Since V is used to denote vasopressin receptors we have used VN with subscripts 1 and 2 as the appropriate abbreviation.

Both mechanisms of vanilloid action are vasoconstrictive and appear to be additive because the perfusion pressure continues to rise with increasing capsaicin concentrations despite $\dot{V}O_2$ becoming inhibitory (Fig. 1). Similar patterns are seen with other active vanilloids such as gingerols and shogaols (9) and piperine and resiniferatoxin (10). The two oxygen consumption responses of stimulation and inhibition occur at low and high doses respectively, suggesting differing affinities of the two presumptive receptors for capsaicin. Thus the receptors stimulated by lower concentrations of capsaicin (high affinity) and which stimulate $\dot{V}O_2$ are nominated as VN_1 and the receptors stimulated by higher concentrations of capsaicin (low affinity) and which inhibit $\dot{V}O_2$ are nominated as VN_2. The generation of bell-shaped response curves or related curves by the overlapping actions of both stimulatory and inhibitory receptors has been reviewed recently by Rovati and Nicosia (16) and previously by Szabadi (17). Such ideas are consistent with the premise of two vanilloid receptors acting in concert in the hindlimb to firstly stimulate, and then inhibit oxygen consumption with increasing dose of vanilloid.

TABLE II

Proposed Classification Criteria for VN_1 and VN_2 Vanilloid Receptors
in Perfused Muscle

Receptor type	VN_1	VN_2
Oxygen consumption	increased	decreased
Vasoconstrictor	strong	moderate
Affinity for vanilloid	high	low
Dependent on external Ca^{2+}	yes	no[1]
Dependent on O_2	yes	no
Lactate production (steady state)	increased	decreased[2]

[1] Independent of $[Ca^{2+}]$ but may require some Ca^{2+} for full agonist effect as inhibition of $\dot{V}O_2$ is less than in the presence of Ca^{2+}.

[2] After removal of capsaicin there is a "wash-out" peak of lactate.

The absence of external calcium inhibited the observed maximal capsaicin-induced vasoconstriction and shifted the dose curve markedly to the right (Figs. 1 and 2). Remarkably, the absence of external Ca^{2+} ions led only to an inhibition (Phase 2) of both $\dot{V}O_2$ and lactate efflux in response to capsaicin. Neither Phase 1 nor Phase 3 stimulation of $\dot{V}O_2$ were observed. However, inhibition of $\dot{V}O_2$ at high doses of capsaicin was less pronounced than observed with equivalent doses of capsaicin in the hindlimb perfused with buffer containing 1.27 mM Ca^{2+}. Thus, this

vasoconstriction effect was substantially independent of Ca^{2+} but required external calcium for full agonist effect.

The proposed VN_1 site appears to be calcium dependent and to stimulate increases in $\dot{V}O_2$ and lactate production, whilst the proposed VN_2 receptor inhibits $\dot{V}O_2$ and lactate efflux and is largely independent of the need for external calcium ions. The simultaneous increase in lactate and oxygen consumption without hypoxia has been seen previously in the perfused rat hindlimb in response to a number of different vasoconstrictors (18)

A recent, preliminary communication has suggested the presence of two capsaicin receptors in cells from the rat dorsal root ganglion based on the different ability of capsazapine (a new competitive vanilloid inhibitor) to block the actions of capsaicin and the highly potent vanilloid agonist, resiniferatoxin (19). Lou et al. (20) have described two mechanisms of action of capsaicin in the perfused lung which depend on the concentration of capsaicin present. Low dose effects (10^{-8} M capsaicin) were blocked by tetrodotoxin (TTX) whereas high dose effects (10^{-6} M capsaicin) were not. These authors modified an earlier suggestion that there were two mechanisms of action of capsaicin in which low concentrations of capsaicin stimulated the influx of limited amounts of Na^+ or Ca^{2+} ions which then triggered voltage sensitive Na^+ channels to conduct depolarisation to other varicosities or collaterals (21) whereas high dose capsaicin stimulated sufficient influx of ions to cause depolarisation without the need for Na^+-induced depolarisation. Recently, using patch-clamp methods, rat trigeminal cells have been shown to exhibit two different capsaicin-induced currents, one being fast and the other, slow (22).

Implicit in these observations is the premise that there may be two different receptor sites, one of which has a higher affinity for capsaicin and the other with lower affinity for capsaicin. Alternatively, there may be only one receptor type which is coupled to two different post receptor mechanisms. However, to explain the different affinities that we have observed for the stimulation and inhibition of oxygen consumption would require that the microenvironments of the receptors and hence their protein conformations to be different, to produce the different affinities for capsaicin.

The use of alterations in external Ca^{2+} ions to discriminate between α-adrenergic receptor subtypes has been used by Minneman (23) and by Han et al. (24,25). They have suggested that the α_{1a} subtype is coupled to external Ca^{2+} ions and that the α_{1b} subtype is coupled to internal Ca^{2+} stores. Similarly, studies in this laboratory have shown that the presence or absence of external Ca^{2+} can distinguish between two presumptive α_1-adrenoceptor subtypes in the control of oxygen uptake by noradrenaline in the perfused rat hindlimb (26).

On the other hand Ruffolo and coworkers have argued strongly (27) that the same α-receptor may be present, but coupled to different effector mechanisms. Were a similar arrangement to underlie the present study, with one post receptor mechanism stimulating $\dot{V}O_2$ and another inhibiting $\dot{V}O_2$, it is hard to reconcile how such receptors could exist simultaneously on the same cell. It would thus seem more appropriate to postulate the presence of the same receptor on two different smooth muscle cell types. As both sites are associated with vasoconstriction, the receptors may thus be on different calibre arteries or arterioles. Supporting this idea is the general correlation beween artery size and dependency on external calcium ions for contraction, with smaller vessels showing the greatest dependency (28). This is also true in the rat hindlimb (29).

Such an idea of the presumptive differently VN_1 and VN_2 being distributed on vessels of different calibre, is consistent with the different anatomical distribution of $5HT_1$ and $5HT_2$ (30, 31) receptors and of α_1 and α_2 (32, 33) receptors on the arterial vasculature that has been suggested by others.

The present data shows that each of: hypoxia (N_2 gas); cyanide (cytochrome oxidase inhibitor) and azide block some, but not all, of the perfusion pressure. Azide at 1 mM acts as though it is an uncoupler of mitochondria in the perfused hindlimb (33). These data taken together, suggest that the inhibitory receptor or site for $\dot{V}O_2$ (VN_2) is not functionally dependent on O_2, even in the presece of O_2.

The presence of an inhibitory receptor for the vanilloids may explain why the consumption of such spice principles has not uniformly shown a thermogenic or weight-loss effect. The data suggest that it might be possible to synthesise drugs that selectively stimulate VN_1 or inhibit VN_2 receptors which would have important thermogenic or weight loss potential. We are currently examining the effects of known vanilloid antagonists and newly synthesised agonists to further test these possibilities.

Acknowledgements

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Vasoconstrictor-induced thermogenic switching in endotherm and ectotherm muscle

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Introduction

The perfused rat hindlimb preparation has proven to be a reliable model for the investigation of muscle metabolism (Bonen *et al.* 1994). Studies in this laboratory, reviewed by Clark *et al.* (1995), have refined this technique for use at 25°C without red blood cells. A major thrust of this work has been to demonstrate that rat skeletal muscle has the potential to regulate whole body thermogenesis, as measured by oxygen consumption (MO_2) changes, via a non-shivering thermogenic mechanism controlled by site-specific vascular switching. However, assessing the influence of such a mechanism on overall non-shivering thermogenesis is clouded by the presence in the rat of brown adipose tissue (BAT), a highly active non-shivering thermogenic tissue.

Although endothermy has been a major evolutionary progression, the non-shivering component of facultative thermogenesis (NST) is relatively poorly understood. Shivering is clearly a function of skeletal muscle, but the site(s) and mechanism(s) of NST are less certain. BAT makes a large contribution to facultative thermogenesis in some species, yet a growing body of evidence suggests that BAT is not essential for endothermy. Marsupials (Hayward and Lisson, 1992) and birds (Saarela *et al.* 1989) represent large groups of endotherms in which BAT is most likely absent. Furthermore, in many species such as adult humans (Astrup, 1986), BAT presence is too limited to account for the magnitude of the observed NST.

The evidence for NST in marsupials is limited. However, Nicol (1978) and Ye *et al.* (unpublished) have demonstrated *in vivo* NST upon noradrenaline (NOR) infusion in the potoroo and Tasmanian bettong respectively. In birds, a growing body of evidence supports claims that skeletal muscle is a major non-shivering thermogenic site (Duchamp *et al.* 1993). Representatives from these groups are therefore potentially good models for investigating the presence and magnitude of alternative mechanisms of NST.

The present investigation aims to extend the findings made using perfused rat muscle by examining and comparing the effects of vasoconstrictors on perfused muscle from birds (chickens, *Gallus domesticus*) and marsupials (Tasmanian bettongs, *Bettongia gaimardi*), and from an ectotherm group, the cane toad (*Bufo marinus*).

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Material and Methods

Animals

All procedures adopted and experiments undertaken were approved by the University of Tasmania Ethics Committee under the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (1990). Male hooded Wistar rats (180-200 g at experiment) and bettongs (male and female, 1.2 ± 0.11 kg at experiment) were housed as described by Colquhoun et al. (1988) and Ye et al. (1995) respectively. Chickens (male and female, 400-800 g at experiment) of local Hyline and Leghorn strains were obtained from a commercial hatchery and kept for 2-14 days in large cages at $21 \pm 1°C$ with ad libitum access to commercial pellets and water. Cane toads (male and female, 129.4 ± 16.6 g at experiment) from Queensland, Australia were housed for 1-2 months at $21 \pm 1°C$ in a 12h light/12h dark cycle prior to experiment. Toads were fed beetles during this period.

Hindlimb Perfusions

The endotherm (rat, chicken and bettong) hindlimb vascular beds were perfused at 25°C in similar fashion to that previously described for the rat (Colquhoun et al. 1988). Perfusion flow rates were constant and set to maintain satisfactory muscle phosphagen concentrations relative to in vivo values. Thus the flow rates used were 0.27 $ml.min^{-1}.g^{-1}$ in the rat (Colquhoun et al. 1990); 0.33 $ml.min^{-1}.g^{-1}$ in the chicken; and 0.28 $ml.min^{-1}.g^{-1}$ in the bettong (Ye et al. 1995).

The ectotherm (cane toad) hindlimb vascular bed was also perfused at 25°C and constant flow. The perfusion medium was a modified amphibian Hepes-buffer solution, gassed with 100% O_2 at pH 7.4 (Pelster et al. 1993). The constant perfusate flow was initially set to give a basal perfusion pressure of 15 mmHg, reported to be the approximate physiological value in vivo (Pelster et al. 1993).

Surgical Procedures

The surgical protocols used in rats and bettongs are described by Colquhoun et al. (1988) and Ye et al. (1995) respectively.

Chickens were anaesthetised using 60 $mg.kg^{-1}$ i.p. sodium pentobarbital. The major skin vessels of the lower leg were ligated, and the popliteal fossa incised to expose the popliteal artery and vein. The popliteal nerve was divided and the hamstring muscles were ligated and resected proximal to the fossa to give good access for cannulation of the popliteal artery and vein. Heparin (2 $IU.g^{-1}$) was administered i.v. (brachial vein). Tight ligatures were positioned around the ankle and the lower thigh above the cannulation site in order to restrict flow to other tissues. Following commencement of perfusate flow, the bird was killed with a lethal cardiac injection of sodium pentobarbital. Infusion of 1% (w/v) Evans blue dye confirmed that perfusate flow was confined to the lower limb in both hormone-stimulated and non-stimulated preparations. The perfused muscle mass and hence appropriate flow rate was estimated by similarly ligating and subsequently excising the contralateral limb, enabling removal and weighing of the muscles (generally 14-17 g).

Toads were anaesthetised (sodium pentobarbital, 100 $mg.kg^{-1}$ i.p.) and two incisions were made parallel to the abdominal mid line leaving a strip of the anterior abdominal wall with the inferior abdominal vein intact. The upper part of the strip was ligated and cut to expose the abdominal cavity. After removal of the abdominal contents, vessels crossing cut edges were ligated. A suture ligation was performed around the middle of the ilium and pubis bones. Following heparin administration (i.v. 200 IU, renal portal vein), both renal

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portal veins were ligated. The dorsal artery and the anterior abdominal vein were cannulated, both hindlimbs receiving perfusate. Perfused muscle mass was 18.4 ± 3.2 g (Evans blue dye).

Results and Discussion

All species studied were able to demonstrate increased MO_2 (type A) in response to infused NOR. Basal perfusion parameters and the magnitude of the type A NOR responses are shown in Table 1.

In perfused rat muscle, vasoconstrictors were capable of both increasing MO_2 (type A response, e.g. low NOR concentrations, angiotensin II, and vasopressin; Clark et al. 1995) and decreasing MO_2 (type B response, e.g. serotonin and high NOR concentrations; Clark et al. 1995) in association with increased perfusion pressure. Fig. 1 shows dose-response curves for NOR and serotonin (5-HT) in the rat, illustrating both type A (< 1 μM NOR) and type B (> 1 μM NOR and all 5-HT concentrations) MO_2 responses.
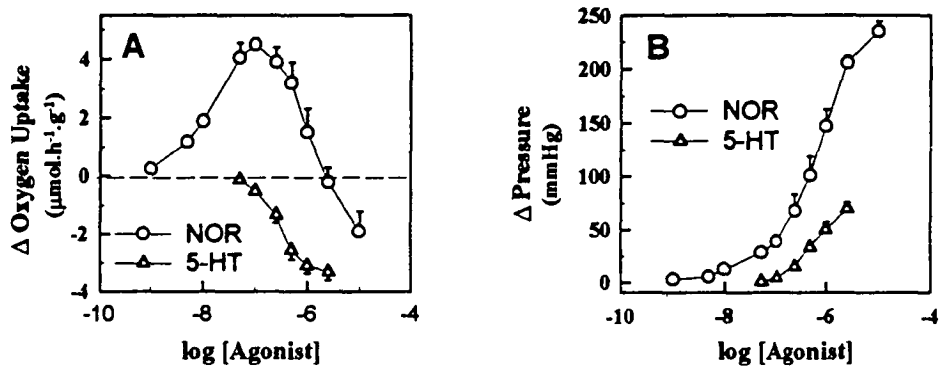

Fig. 1. Δ MO_2 (A) and Δ perfusion pressure (B) concentration-response curves for NOR and 5-HT in perfused rat hindlimb. Basal values for MO_2 and perfusion pressure are given in Table 1. Data points are means ± SE and are all n = 5.

Similarly, perfused chicken muscle gave a biphasic MO_2 curve in response to NOR and adrenaline (Fig. 2), both of which are present in significant concentrations in chicken plasma (9.9 ± 4.6 nM and 1.8 ± 1.2 nM respectively, Fujita et al. 1992). The actions of 5-HT were qualitatively similar but quantitatively less than those of NOR in perfused chicken muscle. The biphasic MO_2 response to 5-HT is different to the actions of this agonist in perfused rat and bettong (data not shown, Ye et al. 1995) hindlimbs where MO_2 is inhibited at all effective doses. We have suggested that this type B behaviour was due to the operation of functional flow shunts in the microvasculature, effecting at least a partial bypass of perfusate flow from actively respiring tissue (Dora et al. 1991, 1992). Although anatomically defined large diameter arterio-venous shunts are rarely seen by histological means in mammalian muscle beds (Hammersen, 1970), capillary based non-nutritive vasculature may exist within intermuscular septa (Lindbom & Arfors, 1984). We have extended this reasoning to propose that the inhibitory effect of high dose NOR may also be due to functional vascular shunting in the rat (Clark et al. 1995) and in the bettong (data not shown, Ye et al. 1995). The biphasic nature of the MO_2 curves in the chicken (Fig. 2)

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show that similar type B effects can occur, although the differing response pattern to 5-HT suggests that the control of such flow patterns may be quite different to that in mammalian models, possibly reflecting differing distributions and/or relative abundancies of 5-HT receptor subtypes.

Table 1. Basal and NOR-stimulated perfusion data for the four species studied.

Species	Flow rate $(mL.min^{-1}.g^{-1})$	Basal MO_2 $(\mu mol.g^{-1}.h^{-1})$	Basal perfusion pressure (mm Hg)	Max. NOR-stimulated ΔMO_2 $(\mu mol.g^{-1}.h^{-1})$
rat[1] (*Rattus rattus*)	0.27	6.6 ± 0.1 (n = 23)	29.1 ± 0.6 (n = 23)	4.4 ± 0.2 (67%) (n = 5)
chicken (*Gallus domesticus*)	0.33	7.4 ± 0.3 (n = 31)	44.8 ± 2.2 (n = 44)	2.6 ± 0.3 (35%) (n = 5)
bettong[2] (*Bettongia gaimardi*)	0.28	4.18 ± 0.35 (n = 8)	32.0 ± 2.3 (n = 8)	4.7 ± 0.4 (112%) (n = 5)
cane toad (*Bufo marinus*)	0.20	1.36 ± 0.10 (n = 3)	15.8 ± 3.2 (n = 3)	0.35 ± 0.06 (26%) (n = 3)

[1]Data taken from Dora *et al.* (1992); [2]data taken from Ye *et al.* (1995). Data are means ± SE.
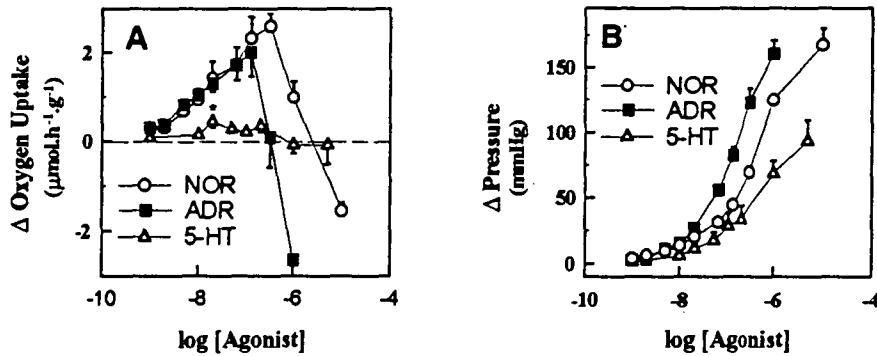

Fig. 2. ΔMO_2 (A) and Δ perfusion pressure (B) concentration-response curves for NOR, 5-HT, and adrenaline (ADR) in perfused chicken lower limb. All data are means ± SE and represent 4-7 experiments. Basal values are as shown in Table 1. * Significant MO_2 increase ($P < 0.05$). Perfusion pressure and MO_2 increases were significant ($P < 0.05$) at 10 nM concentrations of both NOR and ADR.

The actual tissue(s) responsible for, and the mechanism(s) underlying type A vasoconstrictor-induced MO_2 are still not certain. We have previously proposed that vascular smooth muscle (VSM) may be at least partially responsible for the increased MO_2 (Colquhoun & Clark, 1991). However, the possibility of a substantial VSM contribution hinges on sufficient VSM being present in muscle to account for the size of the responses recorded, particularly in the mammalian models. Further, this proposal cannot easily explain type B (inhibitory) MO_2 effects without invoking flow redistribution away from a

large number of small vessels (Dora *et al.* 1992) or other respiring tissue. An alternative hypothesis is that type A vasoconstrictors act in a site-specific fashion to switch flow to actively respiring skeletal muscle whilst maintaining overall normoxia. A variation of this scenario may be site-specific release of a local second messenger which induces a temporary state of mitochondrial uncoupling in skeletal muscle (Clark *et al.* 1995).
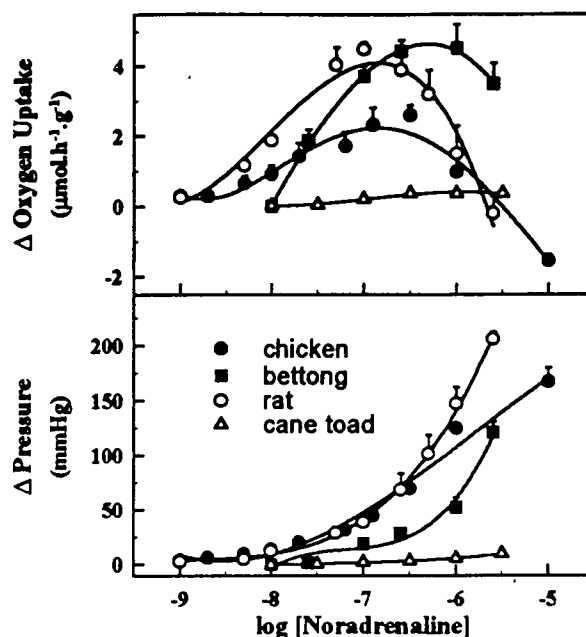

Fig. 3. Δ MO$_2$ (A) and Δ perfusion pressure (B) concentration-response curves for NOR in perfused chicken (n = 4-7, Eldershaw *et al.* unpublished), bettong (n = 5), rat (n = 5), and toad (n = 3, J-M. Ye, PhD thesis, University of Tasmania, 1995) muscle preparations. All data points are means ± SE. Toad data points are significantly different (P < 0.05, paired *t* test) to basal values at all concentrations greater than 10^{-7} M NOR.

Given that all four species show a similar response to catecholamines, it seems likely that vascular control of resting muscle thermogenesis is an underlying non-shivering thermogenic mechanism, common to all vertebrate species. The relatively minor MO$_2$ effect in the toad at physiological temperature and perfusion pressure is consistent with the relative inability of ectotherms to respond to thermal challenge. Nevertheless, the response to NOR in this species, although indicative of a less complex system of vascular control, suggests that this mode of thermogenesis may have evolved prior to the occurrence of BAT in eutherians. The evolutionary appearance of BAT may have been due to the requirement of a supplementary thermogenic mechanism in juvenile and smaller mammals.
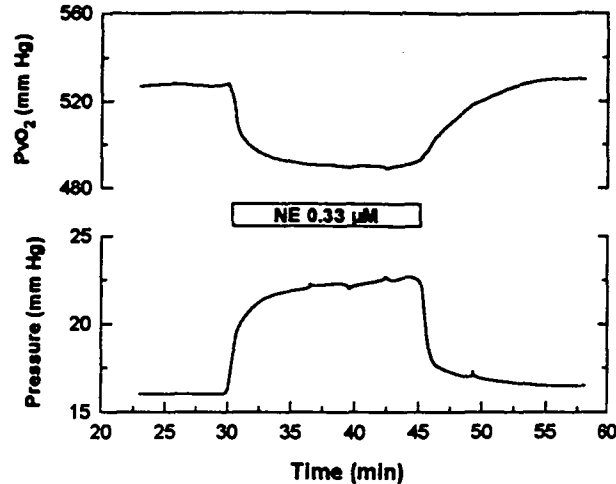
315

Fig. 4. Representative perfusion tracing of the effect of infused NOR on venous oxygen partial pressure (Pvo$_2$) and perfusion pressure in the constant-flow perfused toad hindlimbs (Ye, 1995).

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