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The Contribution of ATP and Endothelium-Dependent Vasodilation to Exercise Hyperemia in Canine Resistance Blood Vessels

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Sharon S. Kelley

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THE CONTRIBUTION OF ATP AND ENDOTHELIUM-DEPENDENT VASODILATION TO EXERCISE HYPEREMIA IN CANINE RESISTANCE BLOOD VESSELS

By

Sharon S. Kelley

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ABSTRACT

THE CONTRIBUTION OF ATP AND ENDOTHELIUM-DEPENDENT VASODILATION TO EXERCISE HYPEREMIA IN CANINE RESISTANCE BLOOD VESSELS

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The contribution of ATP and endothelium-dependent vasodilation to exercise hyperemia was investigated in the blood-perfused canine hindlimb resistance vessels. The P2-purinoceptor antagonist $ANAPP_3$ (arylazido aminopropionyl ATP), antazoline and ATP desensitization were used to inhibit ATP vasodilation. Methylene blue (MB) and 5,8,11,14-eicosatetraynoic acid (ETYA) were used to inhibit endothelium-dependent vasodilation. The blood supply to the hindlimb was vascularly isolated and the muscle pump perfused at a constant pressure. Exercise was performed at 1.5, 3, and 6 twitches/sec.

In the presence of $ANAPP_3$, the vasodilator response to ATP was increased at low doses of ATP and decreased at higher doses. The hyperemic response during exercise was increased at an oxygen consumption of 6 ml/min/100g. Peak reactive hyperemia was increased in the presence of $ANAPP_3$. These results suggest that ATP elicits a vasoconstrictor influence during exercise and reactive hyperemia. Two other putative ATP antagonists, antazoline and ATP desensitization, reduced exercise hyperemia, but these agents were not specific antagonists to ATP.

The vasodilator responses to ATP and ACH were antagonized by treatment with MB. The vasodilator response to ACH was antagonized by treatment with ETYA. The vasodilator response to one dose of ATP was reduced by ETYA. Oxygen consumption and tension development during 1.5, 3, and 6 Hz exercise were reduced by MB and the peak reactive hyperemic response attenuated. ETYA reduced blood flow and oxygen consumption at 1.5 Hz. The decrease in oxygen consumption during 1.5 Hz exercise by MB and ETYA was due to a depression of blood flow. The decrease in oxygen consumption during 3 and 6 Hz exercise by MB was due to both a decrease in blood flow and a direct effect on muscle metabolism.

These results indicate that the vasodilator responses to ATP and ACH are mediated through endothelial cells in this preparation. Endothelium-dependent vasodilation contributes to exercise hyperemia during 1.5 Hz exercise in the canine resistance vessels. Two lines of evidence rule out a vasodilator influence of ATP during exercise. First, the P2-purinoceptor antagonist $ANAPP_3$ does not reduce exercise hyperemia. Second, ETYA does not reduce ATP vasodilation yet it reduces 1.5 Hz exercise hyperemia. This suggests only a vasoconstrictor role for ATP during exercise.

This dissertation is dedicated with love, to my family, Gayle, Josie and Emma; and to my mother, Sandie, Connie and Brooke.

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INTRODUCTION

Skeletal muscle blood flow can increase 10-15 times above resting levels in response to muscular contraction (Shepherd, 1983). Since skeletal muscle comprises up to 50% of total body mass (Laughlin and Armstrong, 1985), blood flow can reach levels of 20-25 liters/minute during exercise (Shepherd, 1983). It is the caliber of skeletal muscle resistance vessels that determines the blood flow response. A decrease in vascular resistance of skeletal muscle is responsible for distributing a large proportion of cardiac output to this region. Mean arterial pressure is maintained by a reflex increase in cardiac output, which in turn, maintains muscle perfusion pressure.

Natural exercise consists of a mixture of twitch and tetanic type contractions (Laughlin and Armstrong, 1985). Tetanic contractions cause transient flow interruptions due to compression of the blood vessels by the force of contraction (Mohrman and Sparks, 1974). Twitch contractions produce a flow response that is more easily measured, for this reason, are often studied experimentally.

The changes in blood flow that accompany exercise can be divided into three temporal phases: initiation, steady state, and post contraction (Laughlin and Armstrong, 1985). Initiation occurs within the first minute of muscular twitch contraction (Laughlin and Armstrong, 1985). It is sometimes characterized by a vasodilation that peaks at a level greater than the steady state blood flow while at

other times there is an increase in flow to steady state levels without a peak response (personal observation and Sparks, 1978). Within 1 minute of twitch contraction steady state hyperemia is reached. The duration of hyperemia following contraction depends upon the oxygen debt incurred during exercise (Sparks, 1978).

In addition, exercise hyperemia is characterized by the species, muscle fiber type (glycolytic, oxidative or a mixture, fast or slow ATPase activity) and muscle perfusion pressure (restricted vs. free flow) (Shepherd, 1983; Laughlin and Armstrong, 1985). Various phases and types of exercise exhibit unique regulation. There is no one mediator of blood flow changes; multiple mechanisms insure adequate flow delivery.

The focus of the present study was on steady state hyperemia, under conditions of free flow, twitch exercise in the canine hindlimb. During steady state free flow twitch exercise, blood flow reaches a steady state by one minute after the onset of contraction. The skeletal muscles of the canine hindlimb consist predominantly of fast oxidative-glycolytic and slow-twitch oxidative fibers (Maxwell et al., 1977; Shepherd, 1983; Sparks, 1978). During steady state exercise it is the oxidative fibers rather than the glycolytic fibers that maintain the contractile performance (Sparks, 1978; Laughlin and Armstrong, 1985). This is because glycolytic fibers fatigue more readily that oxidative fibers (Laughlin and Armstrong, 1985).

Regulation of the peripheral circulation involves local, neural and myogenic mechanisms (Sparks, 1978; Shepherd, 1983). Each of these mechanisms contribute to altering blood flow in response to exercise. It is generally believed that local mechanisms dominate over myogenic

or neural mechansims during steady state hyperemia. A review of the literature and data that led to the formulation of the hypothesis that local factors dominate in regulating exercise hyperemia will be presented. The major emphasis of the review will be on the mechanisms responsible for steady state free flow hyperemia and the evidence for ATP as a possible mediator will be extensively reviewed.

Myogenic Mechanisms

In response to stretch, vascular smooth muscle contracts, which is the basis for the myogenic (Bayliss, 1902) control of vascular smooth muscle (Shepherd,1983). In terms of peripheral blood flow regulation, a change in vessel wall transmural pressure results in changes in vascular smooth muscle diameter, which alters resistance to blood flow (Sparks and Phair, 1978).

During tetanic muscular exercise the external force of muscle contraction should decrease transmural pressure in the vessel wall, eliciting relaxation of vascular smooth muscle and a fall in resistance. An inflatable cuff placed around the dog calf muscle was used to simulate the increase in extravascular and therefore, intramuscular pressure of tetanic muscle contractions (Mohrman and Sparks, 1974). Intramuscular pressure was increased to levels observed during one second tetnus (16 and 64 impulses/sec). It was concluded from this study that 30%-50% of the hyperemic response to a 1 second tetanus can be attributed to a myogenic mechanism (Mohrman and Sparks, 1974).

If external compression of skeletal muscles is used to mimic the

compression of twitch exercise, the vascular response is small (Sparks and Phair, 1978). However, these investigators cautioned against ruling out a role for the myogenic mechanism in twitch exercise because exercise-induced shearing forces may create compressions in resistance vessels. The compressions could cause transient changes in transmural pressure distally and elicit a myogenic response (Sparks and Phair, 1978). By use of a specially designed muscle chamber which can create changes in extravascular pressure that are similar to those of twitch exercise (2-5 Hz) no evidence for the participation of a myogenic mechanism was found under free or constant flow (Bacchus, 1981). The results of these experiments demonstrate that it is unlikely that the myogenic mechanism is important for sustained hyperemia of twitch exercise. The myogenic response contributes to the hyperemia of tetanic contractions and probably the initiation of exercise hyperemia, where changes in vascular resistance are too rapid to be controlled by oxidative metabolism (Phair and Sparks, 1978; Mohrman and Sparks, 1973).

Neural Mechanisms

The involvement of the central and peripheral nervous systems in controlling blood flow during exercise is important for redistribution of cardiac output to working muscles. Skeletal muscle blood vessels are innervated by sympathetic adrenergic fibers and in some species, sympathetic cholinergic fibers (Shepherd, 1983 and Sparks, 1978). Resistance vessels have both alpha and beta adrenergic receptors that mediate vasoconstriction and vasodilation, respectively (Sparks, 1978).

When sympathetic nerve fibers are stimulated electrically or by exogenously administered norepinephrine, vasocontriction is the dominant response (Sparks, 1978; Shepherd, 1983). When an antagonist of alpha adrenergic receptors is given, the response to sympathetic nerve stimulation and to norepinephrine is vasodilation (Shepherd, 1983; Sparks, 1978).

Adrenergic nerves function to increase vascular resistance, decrease capacitance and decrease capillary surface area under resting conditions (Sparks, 1978). The sympathetic outflow to skeletal muscle blood vessels contributes to a relatively high resting vascular resistance. Inhibition of sympathetic tone can approximately double resting skeletal muscle blood flow and maximum sympathetic vasocontrictor activity can decrease resting blood flow by about 75% (Shepherd and Vanhoutte, 1975).

A vasodilator tract originating from the motor cortex has been traced via the brainstem to the spinal lateral horns (Uvnas, 1967). The vasodilator tract has a relay station in the hypothalamus (Uvnas, 1967). This vasodilator tract is probably responsible for changes in sympathetic outflow to various vascular beds that occurs during the mental preparation before exercise (Bolme and Novotny, 1969). This increase in blood flow prior to exercise may provide an immediate source of extra blood for working muscles (Shepherd and Vanhoutte, 1975). In the human and dog, sympathetic neurons release acetylcholine as the neurotransmitter for the vasodilation that accompanies the anticipation of exercise (Burnstock, 1980 ; Shepherd, 1983). Treatment with atropine, a specific muscarinic antagonist, does not reduce the blood flow response to muscle contraction (Anrep and Saalfeld, 1935;

Burnstock, 1980). Sympathetic cholinergic fibers do not therefore, mediate steady state hyperemia.

In some species histamine may play a role as a neural mediator of the resistance vessels. Stimulation of the sympathetic nerves to the hindlimb of the dog after adrenergic neuron block and atropine causes a vasodilation that is reduced by antihistamines (Heitz and Brody, 1975). These authors suggested that histamine may mediate some of the reflex vasodilation produced by baroreceptor stimulation. Histamine may contribute to the sympathetic cholinergic vasodilator pathway. The evidence for this is that antihistamines can decrease the vasodilation induced by acetylcholine in some but not all species, and acetylcholine stimulates histamine release from muscle (Brody, 1978 and Shepherd, 1983). Neural involvement of sympathetic histaminergic vasodilation during free flow steady state exercise has not been tested.

From these studies it can be concluded that the sympathetic nervous system is capable of releasing vasodilator substances (e.g., acetycholine, ATP and histamine) which mediate changes in blood flow independent of muscular contraction. However, a role for sympathetic-induced release of these vasodilators during steady state hyperemia is unlikely, since exercise hyperemia can occur in the abscence of extrinsic neural innervation. Donald et al. (1970) compared blood flow during graded treadmill exercise in the normal and sympathectomized dog hindlimb. The animals were chronically instrumented with flow probes and unilaterally sympathectomized by removal of lumbar chain intact from L-2 through L-7. Studies were initiated 12-14 days after surgery. Oxygen saturation and limb blood flow were compared in the denervated and innervated limbs and were

similar in both cases in response to treadmill running. Thus, vasodilator(s) released from sympathetic nerves are not required for steady state hyperemia. However, if the intact lumbar chain is electrically stimulated (6 volts, 6 Hz, 5 msec) for 30 seconds during exercise, there is a decrease in exercise hyperemia up to 50% (Donald et al., 1970). The per cent reduction in blood flow decreased as the blood flow response to exercise increased.

Strandell and Shepherd (1967) showed that increases in sympathetic nerve activity produced by lower body suction could reduce the hyperemic response to mild forearm exercise but had less effect as exercise intensity increased. The decrease in flow caused by the increase in sympathetic action was compensated for by an increase in oxygen extraction. They postulated that sympathetic nerves may modulate local vasodilator mechanisms to maintain a constant ratio of blood flow:oxygen consumption. Thompson and Mohrman (1983) found that sympathetic nerve stimulation to canine muscle (0.2 msec, 30-60 V, 0.25, 0.5 and 1 Hz) decreased the blood flow response at a given oxygen consumption by the same absolute amount at rest, 1 and 4 Hz exercise. Thompson and Mohrman observed that at a higher exercise rate the vasoconstrictor response to sympathetic nerve stimulation was attenuated. Sympathetic stimulation reduced muscle oxygen consumption during 4 Hz, but not rest or 1 Hz exercise. The decrease in oxygen consumption elicited by sympathetic stimulation was attributed to a decrease in blood flow that could not be compensated for by increased oxygen extraction. The sympathetic nervous system can thus modulate exercise hyperemia by decreasing the blood flow response, especially at rest and low exercise levels. Vasoconstriction elicited by the

sympathetic nervous system competes with local vasodilators to control blood flow. At higher metabolic rates the sympathetic nervous system can reduce oxygen consumption, although increased levels of vasodilator metabolites accumulate and dominate the vascular response.

Honig and co-investigators have described neurons that are intrinsic to the vasculature. Following two weeks of extrinsic denervation, nonadrenergic and a small population of adrenergic cells were histologically identified within the walls of dog gracilis muscle arterioles (Myers, et al., 1975). The nonadrenergic axons stained for acetylcholinesterase, and reportedly originated from ganglia in the vessel wall. The investigators cautioned that the staining technique utilized was not specific evidence for acetylcholine-containing cells. These investigators also suggested that post ganglionic, sympathetic vasodilator fibers appeared to terminate on the cell bodies of the intrinsic neurons, rather than the vascular smooth muscle. They propose that acetylcholine is an intermediate neurotransmitter that is released from sympathetic nerves to stimulate intrinsic neurons, which release a yet unidentified mediator.

The evidence for participation of intrinsic neurons during exercise is that local anesthetics can block the vasodilation to brief trains of twitches (Honig, 1979). Local anesthetics are thought to block local, intrinsic neuron conduction. Another piece of evidence that supports the local intrinsic nerve hypothesis is that the onset of exercise hyperemia occurs within 500msec, which is consistant with a neurally-mediated time course (Honig, 1979; Shepherd, 1983). A possible involvement of intrinsic neurons in steady state hyperemia has not been tested. More data are needed to confirm or deny their

existance and contribution to blood flow regulation. Whether intrinsic neurons exist and contribute to exercise hyperemia remains undetermined, but they would be acting locally.

The experimental evidence suggests that the extrinsic nervous system is a modulator during steady state hyperemia. In response to muscular contraction, the major decrease in vascular resistance is mediated locally.

Local Vasodilator Mechanisms

Gaskell was the first to suggest that vasodilator metabolites are responsible for exercise hyperemia. Over 100 years ago, he suggested that "dilator fibers" co-existed with motor fibers in the motor nerve trunk (Gaskell, 1878). Later, in perfusion experiments, he demonstrated that lactic acid caused vasodilation and proposed that blood vessels were dilated by vasodilator metabolites coming from skeletal muscle fibers (Gaskell, 1880). Since Gaskell's original studies, it has become generally accepted that exercise hyperemia is the result of locally released vasodilator metabolites.

Local regulation of blood flow is based upon the hypothesis that changes in metabolism are followed by alterations in oxygen or vasodilator metabolites, which mediate changes in blood flow. Therefore, blood flow is adjusted to meet metabolic demand of the tissue (Haddy and Scott, 1968). Another way to describe this hypothesis would be that it is the flow-to-metabolism ratio that determines vascular resistance (Haddy and Scott, 1968). During muscular contraction, metabolism is increased, therefore resistance

must decrease to maintain the flow-to-metabolism ratio. The decrease in resistance is mediated by a signal that links metabolism to blood flow: vasodilator metabolites. Metabolites act locally, at the site of muscular contraction, to elicit relaxation of vascular smooth muscle and hyperemia.

A significant mediator (i.e. vasodilator metabolite) of exercise hyperemia should satisfy certain criteria: (1) a mediator should produce a potent vasodilation when administered exogenously, (2) the mediator should elicit a sustained vasodilation when infused, similar to steady state exercise hyperemia, (3) agents which inhibit or augment the mediator should have a similar effect on exercise hyperemia, (4) an elevated concentration of the mediator should be measurable in venous effluent of exercising muscle, (5) endogenous concentration of the mediator must be sufficient and present at the site of vascular smooth muscle relaxation (Shepherd, 1983).

Although many vasodilators have been tested as possible mediators, none have fulfilled all the criteria for mediating steady state hyperemia. Testing of metabolites can be difficult due to experimental and physiological limitations, such as rapid breakdown in plasma, inaccessability to site of action, and lack of specific pharmacological antagonists. A review of the literature pertaining to the mediators that have been considered and tested will be presented. A major emphasis will be placed on the experimental work that has led to the hypothesis of this dissertation, that ATP is a significant mediator of exercise hyperemia.

Vasodilator Metabolites

Potassium ion and plasma osmolarity

The rise in potassium ion concentration very closely parallels the rise in vascular conductance at the initiation of twitch exercise (Sparks, 1980). An increase in arterial blood osmolarity causes transient vasodilation in skeletal muscle and during exercise, both tissue and venous osmolarity increase (Sparks, 1980 and Sparks and Belloni, 1978). However, potassium ion and plasma osmolarity do not play a large role in steady state hyperemia of oxidative muscle because increases in release of these substances is not maintained (Radawski et al., 1972; Sparks, 1980; Stowe, 1975). Furthermore, they have minimal vasodilator potency in oxidative muscle during steady state exercise (Mohrman, 1982). In glycolytic muscle fibers, potassium ion and osmolarity probably contribute to sustained exercise hyperemia (Bockman, 1983; Kapin and Bockman, 1984; Mellander and Lundvall, 1971; Mellander and Lundvall, 1971). It was suggested by Mellander (1981) that glycolytic fibers produce more osmotic particles for a given exercise intensity because of a greater increase in lactic acid. This differential sensitivity between glycolytic and oxidative fiber types raises the possibility that in a muscle with mixed fiber types, potassium ion and osmolarity contribute to functional hyperemia, especially as more glycolytic fibers are recruited during exercise. However, steady state exercise hyperemia is mediated mostly by oxidative fibers. Therefore, the relevance of potassium ion and plasma osmolarity relates to exercise conditions in which glycolytic fibers are involved, such as short bursts of activity or the initiation of

exercise hyperemia.

Oxygen

One of the most striking characteristics of exercise hyperemia is the excellent correlation between blood flow and oxygen consumption under steady state conditions. One explanation of this relationship is that a fall in vessel wall PO_2 during exercise results in vascular smooth muscle relaxation (Guyton et al., 1964). If this hypothesis is correct two conditions must be met. First, decreased vessel wall PO_2 must cause relaxation of vascular smooth muscle. This has been shown to be true (Detar and Bohr, 1968). Secondly, the vessel wall PO_2 must fall during exercise. During exercise of the rat cremaster preparation arteriolar PO_2 increased (Duling and Pittman, 1975). The increase can be explained by the increased flow delivery of oxygen to the wall which accompanies the hyperemia (Duling and Pittman, 1975; Sparks, 1980). Duling and Pittman (1975) have demonstrated that vessel wall PO_2 is influenced more by blood PO_2 than tissue PO_2. Thus, although tissue PO_2 drops during exercise, the increased supply of oxygen in the blood maintains or actually increases vessel wall PO_2 (Gorczynski and Duling, 1976; Sparks, 1980). These results suggest that relative tissue or arterial vessel wall hypoxia is not a direct mediator of exercise hyperemia.

Adenosine

Adenosine has received much attention as a candidate for mediating steady state exercise hyperemia. In 1963 the adenosine hypothesis for local blood flow regulation was first formally stated by

Berne. Adenosine is a metabolite of adenine nucleotides (Berne et al., 1971). It is vasoactive (Thompson et al., 1986; Bockman et al., 1975; Bockman et al., 1976) and can diffuse or be transported across cellular membranes (Gorman et al., 1986; Sparks and Bardenheuer, 1986). There is evidence from experiments on heart and leucocytes that adenosine release is regulated by a signal related to the adenine nucleotide phosphorylation potential (Rubio et al., 1974; Worku and Newby, 1976). A reasonable hypothesis is that exercise would reduce the phosporylation potential and this would result in release of vasoactive adenosine from adenine nucleotides.

The involvement of adenosine in skeletal muscle exercise hyperemia has been studied by several investigators. In the canine blood-perfused hindlimb, Thompson et al. (1986) tested the hypothesis that adenosine mediates steady state free flow exercise of 3 twitches per second. The hypothesis was tested by predicting interstitial fluid adenosine concentration during exercise and by antagonizing adenosine P1-purinoceptors with aminophylline during exercise. By use of a mathematical model it was determined that venous concentration of adenosine was a better index of interstitial adenosine than release of adenosine. Venous plasma concentration of adenosine was not elevated during 3 Hz twitch exercise. This result suggests that interstitial fluid adenosine levels are not significantly increased with 3 Hz exercise. A selective adenosine receptor antagonist, aminophylline, did not reduce exercise hyperemia. It was concluded that adenosine does not contribute to sustained 3 Hz exercise hyperemia. The venous concentration of adenosine was elevated after ten minutes of free flow 6 Hz twitch exercise in the canine hindlimb (Fuchs et al., 1981; Sparks

and Fuchs, 1983). The results of these two studies, which were obtained from the canine hindlimb, suggest adenosine may be involved in mediating the more strenuous 6 Hz exercise hyperemia, but not 3 Hz. Klabunde (1986) found that adenosine contributed to active hyperemia only under conditions of partial ischemia. In early studies, muscle adenosine content was used as an indicator of interstitial fluid adenosine. Phair and Sparks (1979) did not find a rise in canine calf muscle adenosine during free flow exercise; however, Steffen and co-workers observed an increase in canine gracilis muscle adenosine content with exercise (1983). The gracilis muscle may contain a higher proportion of glycolytic fibers compared to the highly oxidative calf muscle. The higher adenosine content of gracilis muscle may reflect an increased metabolic demand on this muscle compared to the calf muscle during steady state hyperemia. Arteriolar diameter changes in the cremaster muscle were decreased by lowering the adenosine availability during muscle contraction (Proctor and Duling, 1982). Levels of adenosine were decreased by suffusing the cremaster muscle preparation with the enzyme, adenosine deaminase, which catalyzes the conversion of adenosine to inosine. In the presence of adenosine deaminase, the arteriolar dilator response during 1 Hz stimulation was decreased by 20-25%. Mohrman and Heller (1984) tested the response of cremaster muscle arterioles to aminophylline. For a range of stimulation frequencies, from 0.2 to 2.0 Hz, aminophylline had no effect on arteriolar diameter. The overall implication of these studies is that adenosine contributes to steady state exercise hyperemia under conditions of ischemia or strenuous, fatiguing exercise but is not a significant mediator of free flow steady state exercise hyperemia in

oxidative muscle.

Prostaglandins

Prostaglandins are vasoactive autacoids produced from arachidonic acid via cyclooxygenase in platlets, endothelial cells and vascular smooth muscle (Smith, 1986). Prostaglandins of the E series are potent vasodilators in oxidative skeletal muscle and therefore could theoretically contribute to exercise hyperemia (Young and Sparks, 1980). Despite the fact that prostaglandin release into the venous effluent is increased with exercise (Young and Sparks, 1980) a number of investigators have failed to reduce free flow exercise hyperemia with cyclooxygenase inhibitors (Beaty and Donald, 1979; Weiner et al., 1977; Young and Sparks, 1979). Under conditions of restricted blood flow, cyclooxygenase inhibition reduces exercise vasodilation in human forearm (Kilbom and Wennmalm, 1976). Thus, prostaglandins may be involved with ischemic exercise conditions, but probably are not significant contributors to free flow, steady state hyperemia.

Histamine

Histamine is a potent systemic and local vasodilator that acts via H_1 and/or H_2 receptors (Marshall, 1984). Histamine is found in high concentrations in the walls of both arteries and veins (Brody, 1977). Several investigators have examined the role of histamine during skeletal muscle contraction and found no evidence for its participation during ischemic exercise (Daniel and Honig, 1980; Morganroth et al., 1977). The vasodilation observed in response to 30 seconds of constant flow exercise was not reduced by separate or combined treatment with H_1 or H_2 receptor antagonists (Powell and

Brody, 1976). Antazoline, a H_1 receptor antagonist, reduced neurogenic vasodilation in the rabbit skeletal muscle, but exercise hyperemia was not tested (Shimada and Stitt, 1984). Histamine mediates a portion of postcontraction hyperemia under restricted flow conditions (Sparks, 1980). No one has reported the effects of selective histamine antagonists on steady state exercise hyperemia and histamine may have been prematurely rejected because of its lack of contribution to ischemic exercise. Its potency and availability make it a decent candidate for mediating free flow exercise hyperemia and studies are needed to ascertain its role.

Adenosine 5'-Triphosphate (ATP)

Development of ATP Hypothesis

Fifty years after Gaskell first advanced the theory that vasodilator metabolites mediate exercise hyperemia, Drury and Szent-Gyorgyi (1929) discovered the vasoactivity of purines. When purines from heart muscle were applied externally to blood vessels, they caused vasodilation. In the late 1940's several investigators reported the systemic effect of exogenously administered ATP. ATP and other purines produced a potent vasodilation in the peripheral circulation.

Rigler, Folkow and Holton were among the first to suggest that ATP could mediate changes in blood flow. Folkow (1949) demonstrated the potency of intra-arterial injections of ATP in skeletal muscle. The dilations produced by ATP were not blocked by atropine or an

antihistamine, which suggested ATP's ability to elicit vasodilation was not via stimulating acetylcholine or histamine release. Rigler (1932) noted that purines may be important regulators of vasodilation in contracting frog muscle. Infusion of ATP into the human forearm by Duff et al., (1954) elicited a sustained vasodilation. The vasodilation lasted during 10 minutes of steady state infusion, although in some of their subjects, blood flow at the beginning of infusion was greater than at the end of infusion. Boyd and Forrester (1968) and Forrester and Lind (1969) demonstrated that ATP levels were elevated in the venous effluent of contracting skeletal muscle.

Data from these early studies provided evidence that ATP could be a mediator of exercise hyperemia based upon 3 criteria: (1) vasoactivity of exogenously administered ATP, (2) increased levels of ATP in effluent draining contracting muscle and (3) sustained vasodilation over a sufficient period of time to account for steady state hyperemia. Since these early studies, researchers have pursued the hypothesis that ATP mediates exercise hyperemia. The methods used to test this hypothesis have involved quantifying ATP release during exercise hyperemia. Since these early studies, we now have a better understanding of ATP's metabolism, sources of ATP, conditions which stimulate ATP release and vascular mechanisms that mediate ATP's effect. Thus, we have a substantial amount of evidence suggesting that ATP is a potential candidate for mediating exercise-induced blood flow changes. A critical review of the literature pertaining to ATP's potential for mediating local blood flow will be presented. From this information the basis for this dissertation was derived: despite a wealth of information concerning ATP's ability to produce changes in

blood flow and data from earlier experiments linking ATP release with vasodilation, a functional role for ATP as a mediator of local blood flow has not been established.

Cellular Sources of ATP

ATP is a relatively large, negatively charged molecule. Because of its size and charge the ability of ATP to traverse bilipid membranes as an intact molecule has been doubted (Forrester, 1981). Forrester suggested that muscle membrane depolarization may be the mechanism or stimulus for ATP release from hypoxic myocytes (Forrester, 1981). Abood et al. (1962) measured the outflux of ATP and other phosphates from frog nerve and muscle and determined that release was related to changes in membrane potential and physical changes in the membrane. Therefore, ATP release may be a non-selective process, possibly the result of changes in membrane permeability.

Platelets contain dense granules that store nucleotides and amines (Gordon, 1986). Degranulation and release of nucleotides occurs during platelet aggregation, where ATP may mediate further platelet aggregation and vasoactivity. Platelets can contribute significantly to blood ATP levels. In a study by Forrester and Lind (1969) platelets contributed up to half of the ATP in plasma from the human forearm (Forrester, 1981). ATP was measured in the blood of rats, rabbits and man during hemostasis (Born and Kratzer, 1984). The initial concentration of free ATP emerging 2-4 seconds after a puncture of rat or rabbit artery was 2×10^{-7}M and after an incision to human skin was 2×10^{-6}M. The source of ATP was identified as damaged cells. The

concentration of ATP increased 3-5 minutes after injury to 2 X 10^{-5}M and this second peak of ATP release was attributed to platelets, since it was inhibited by heparin (Born and Kratzer, 1984). The concentration of ATP in the blood without hemostasis was about 1000X lower. Therefore, in response to tissue injury, there is release of ATP by platelets.

Cellular sources of ATP include endothelial cells, red blood cells, smooth muscle, and skeletal muscle (Dixon and Forrester, 1985; Forrester, 1981; Pearson and Gordon, 1979; Pearson and Gordon, 1985). Pearson and Gordon (1979) demonstrated that thrombin and trypsin stimulate ATP release from cultured endothelial and smooth muscle cells. They concluded this release is a selective process because lactate dehydrogenase, an enzyme released during cellular damage, was not detected extracellularly (Gordon, 1986). Dixon and Forrester (1985) noted that ATP accumulated in suspensions of frog red blood cells diluted with Ringer's solution in proportion to the concentration of red blood cells. Release of ATP from contracting muscle will be discussed in detail in the following section.

The intracellular concentration of ATP is $\geq$ 5 mM in most cells and only a small fraction of this is required in the vasculature to affect tone and platelet function (Gordon, 1986; Pearson and Gordon, 1985). Whether or not intracellular stores of ATP contribute to in vivo ATP release has not been determined. Gordon (1986) suggested that the high level of intracellular ATP could result in perivascular ATP in the micromolar range. As pointed out by Burnstock and Kennedy (1986), the concentration of ATP in cultured cardiac endothelial cells is greater than the combined concentrations of adenosine 5'-diphosphate

(ADP), adenosine 5'-monophosphate (AMP) and adenosine and is also 3 times the concentration of ATP in cardiac myocytes (Nees and Gerlach, 1983). Gordon (1986) pointed out that the local concentration of ATP will depend on the amount released, volume of distribution in the extracellular space, capacity and accessability of catabolic enzymes (Gordon, 1986).

Circulating ATP and nucleotides are rapidly catabolized by ectoenzymes located on the luminal endothelial plasma membrane (Pearson and Gordon, 1985). Paddle and Burnstock (1974) demonstrated that when ATP is infused into guinea pig hearts in situ, only 1% is recovered in the venous effluent. A bolus of ATP is almost entirely removed by a single passage through the lung (Gordon, 1986). The contribution of endothelial ectonucleotidases to ATP metabolism is significant. The half life of exogenous ATP (100 uM) is about 30 minutes in cell-free plasma, about 5 minutes in whole blood, and <.1 minute in the microcirculation (Pearson and Gordon, 1985). ATP is broken down into ADP, AMP and adenosine, all of which are vasoactive. There are apparently three separate enzymes that sequentially break down ATP to adenosine (Gordon, 1986). The relevance of rapid and efficient extracellular ATP catabolism is that the vasoactive metabolites might prolong the local vascular response (Pearson and Gordon, 1985).

ATP and its degradation products are taken up by endothelial cells in the vasculature, which results in preservation of the purine moeity (Pearson and Gordon, 1985). Uptake of ATP by cells has been extensively studied by Chaudry and co-workers. Infusions of ATP, but not ADP, AMP, or adenosine restored adenine nucleotide levels in liver and kidney following shock (Chaudry, 1982). In the rat soleus muscle,

experiments with radiolabelled ATP at carbon and phosphate positions showed that the intact nucleotide was taken up by these muscle cells (Chaudry, 1982). However, another study showed that more of the adenine moiety of ATP than the phosphorylated compound was taken up in frog skeletal muscle, suggesting the nucleoside is carried across cell membranes, not the nucleotide (Woo et al., 1977). Rovetto (1985) concluded that in striated muscle, the majority of adenine nucleotide uptake was from nucleotide-derived adenosine. At least 78% of adenosine is removed from the circulation in a single passage through the canine capillary bed (Gorman et al., 1986). Substantial adenosine uptake by endothelium has also been demonstrated for the heart and lung (Pearson and Gordon, 1985). This process is saturable and, therefore, carrier-mediated in the capillary endothelium of the heart, lung and skeletal muscle (Pearson and Gordon, 1985; Gorman et al., 1986). Following adenosine uptake by endothelial cells, the major product produced intracellularly is ATP (Pearson and Gordon, 1985).

ATP Release From Contracting Muscle

Investigators have measured the release of ATP from the working rat heart during coronary vasodilation in response to hypoxia. The concentration of ATP increased from 0.63 ± 0.18 nM during rest to 4.7 ± 0.39 nM during hypoxia (Clemens and Forrester, 1980). Paddle and Burnstock (1974) similarly found that in guinea pig hearts, hypoxia caused an increase in ATP release with an accompanying vasodilation. Since both ATP levels and coronary flow increased during hypoxia it was postulated that ATP may be an important regulator of blood flow changes

in response to metabolic demand.

Boyd and Forrester found that ATP was released from active frog skeletal muscle (1968) and Forrester and Lind (1969) demonstrated ATP release during contraction of the human forearm. The bathing solution obtained from contracting frog sartorious muscle caused a positive inotropic response when perfused over a frog heart. The inotropic response mimicked the action of exogenous ATP. The bathing solution contained purines as determined by ultraviolet spectroscopy. In addition, the action of the bathing solution was inhibited by apyrase, an enzyme that breaks down ATP to AMP. This was one of the first experiments to demonstrate ATP release during muscle contraction.

In further experiments, Forrester and Lind (1969) measured venous ATP concentrations in human forearms during exercise. In these experiments, ATP was measured by the firefly luciferin-luciferace assay. This assay was shown to be specific and sensitive for ATP (Forrester, 1972; Clemens and Forrester, 1980). Isometric contractions with a hand-grip dynamometer were done at 10% and 20% maximum voluntary contraction (mvc). Muscle blood flow and venous ATP levels were measured during and following muscle contraction. During 10% mvc, blood flow and ATP concentration were elevated and maintained at a steady state level for four minutes of contraction. Following contraction forearm blood flow fell immediately below exercise steady state values and returned to resting levels in about 1.5 minutes (Lind and McNicol, 1967). ATP levels peaked following contraction at 10% mvc at a time when blood flow was decreasing. During 20% mvc, muscle fatigue ocurred, ATP concentration increased initially, then fell throughout the four minutes of contraction, despite a continued rise in

blood flow. After muscle contraction blood flow rose to a peak value higher that that obtained during exercise and fell to resting levels in 3 minutes. ATP levels steadily increased 15 minutes following fatiguing exercise, during a time when blood flow was decreasing back to resting level. With both exercise levels, blood flow and ATP levels were inversly related, except during 10% mvc, when both increased. The highest ATP values were measured during post contraction hyperemia, at a time when blood flow was decreasing. Forrester and Lind (1969) concluded that the venous concentration of ATP directly correlated with exercise blood flow. However, close examination of these results suggests that following 10% and 20% mvc and during 20% mvc, there is an inverse relationship between blood flow and venous ATP concentration.

In 1973, Parkinson assayed human plasma for adenine nucleotides after exercise on a rowing ergometer. ATP levels were increased after 5 minutes of mild, moderate, and severe exercise, but not 1 minute after exercise (Parkinson, 1973). In this study, as with the Forrester and Lind experiments, ATP levels increased in the post contraction period, at a time when blood flow was decreasing to resting values.

Forrester (1972) found the amount of ATP released from exercising human forearm was 5-7 ug/ml plasma. In making this determination Forrester was careful to minimize possible sources of error, which included platelet destruction, the diluting effects of exercise hyperemia, and the loss of ATP during sample handling. Duff et al. (1954) infused 16 ug/min of ATP into the human forearm which produced a change in blood flow equivalent to the exercise hyperemia observed in Forrester's study. Forrester suggested that the results his and Duff's studies together indicate that sufficient ATP is released to mediate

exercise hyperemia (Forrester, 1972). Therefore, ATP given exogenously in amounts equivalent to that measured in the venous effluent during exercise produced a vasodilation sufficient to satisfy exercise hyperemia. However, we do not know if intraarterially administered ATP acts at the same site(s) and in the same manner as endogenous ATP.

Conflicting measurements of ATP have been obtained in the dog hindlimb during exercise. In blood-perfused muscle, Chen et al. (1972) measured an increase in the concentration of ATP in venous effluent during 6 Hz, 25 Hz, and prolonged (45-70 minutes) exercise. Bockman, Berne and Rubio (1975) were unable to detect increased ATP levels consistantly with 25 Hz tetanic contractions. In both of these studies, blood samples were collected in EDTA in order to inhibit degradation of ATP by ATPases of blood. However, EDTA also causes release of ATP from blood cells and this interfered with accurate ATP measurements. Bockman, Berne and Rubio concluded that it was not possible to make valid measurements of plasma ATP by this method. Despite efforts to measure ATP carefully in blood samples, it is difficult to quantify due to rapid breakdown in the vasculature. Furthermore, we do not know what the actual concentration of ATP is locally, at the site(s) where ATP elicits changes in vascular resistance.

Improved techniques to measure ATP have confirmed what we knew for 30 years: ATP release increases during contraction and so does blood flow. However, we are not any closer to knowing whether ATP mediates the increase in blood flow. In fact, in some instances, there is apparently an inverse relationship between blood flow and ATP levels. More specific analysis of ATP's role in exercise is lacking and needed.

Data supporting adenine nucleotide involvement in vascular regulation however, is substantial. ATP contributes to blood flow adjustments via two major mechanisms: (1) as a neurotransmitter in the autonomic nervous system and (2) as an autacoid, stimulating endothelial cell and/or vascular smooth muscle. Burnstock was the first to suggest that ATP provides vascular regulation by these two mechanisms (Burnstock and Kennedy, 1985; Burnstock, 1987).

ATP as a Neurotransmitter

In 1953, Holton and Holton first described ATP release from rabbit ear artery via antidromic nerve stimulation. ATP caused local vasodilation and could be measured in the venous effluent following stimulation. In 1959, Holton identified the substance liberated by antidromic stimulation as ATP and a neural source was confirmed. When the calculation of ATP output was corrected for blood flow, there was no difference in ATP output between intact and sympathectomized preparations. This implied that the source of ATP was sensory neurons. Holton was the first to suggest, based on these studies, that ATP was acting as a neurotransmitter.

Since Holton's initial studies on ATP, results from in vitro experiments have supported her hypothesis that ATP is a neurotransmitter. Burnstock (1980) and Su (1983) have been the main proponents of neurally-mediated, purine vascular regulation. Burnstock has put forth the "purinergic hypothesis" which states that ATP is released from (1) nonadrenergic, noncholinergic nerves (Burnstock, 1980) and (2) as a co-transmitter with norepinephrine in

sympathetic nerves (Burnstock and Kennedy, 1986).

Nonadrenergic, Noncholinergic Nerves

In several species, there is evidence for a third mediator of autonomic nervous systems function. This is particularly evident in the GI tract, but also the lung, trachea, retractor penis, bladder, esophagus, eye (Burnstock, 1980). In some of these tissues ATP has been implicated as the active substance released and the nerves have been termed purinergic (Burnstock, 1980). When an intrinsic or extrinsic nerve is stimulated, noncholinergic, nonadrenergic nerves are identified when the response is resistant to blockade by either atropine or adrenergic blocking agents (Burnstock, 1980). The neural source for the response is demonstrated by inhibition of the response with tetrodotoxin.

In the vasculature, there is histological evidence for purinergic neurons in the rabbit portal vein and blood vessels from the guinea pig and rat (Bevan and Brayden, 1987). Quinacrine histofluorescence is considered indicative of purinergic nerves (Bevan and Brayden, 1987) and has been observed around the blood vessels of these animals (Burnstock, 1980; Su, 1985).

Neurons containing ganglia have been demonstrated in the walls of vascular smooth muscle two weeks after sympathectomy (Honig and Frierson, 1976; see Neural Mechanisms section of dissertation). These nonadrenergic cells were thought to be the cells that release the vasodilator mediator of exercise (Shepherd, 1983). The identity of the mediator is unknown, but ATP has been suggested (Schrader et al.,

1982).

Stimulation by electrodes of a specific region of the hypothalamus, termed the defense area, elicits skeletal muscle vasodilation. This neurally-mediated vasodilation is a component of an animal's reaction to fright called the defense reaction. In some species, such as cat and dog, this vasodilation is mediated by sympathetic cholinergic neurons (Shepherd, 1983; Neural Mechanisms section of dissertation). In the rabbit this response is nonadrenergic, noncholinergic and may be mediated by purinergic neurons (Shimada and Stitt, 1984).

Data supporting the hypothesis that purinergic neurons exist in the vasculature are scarce. More work is needed to clarify the initial studies. There are more substantial data supporting a role for ATP as a cotransmitter.

Cotransmitter Role

Cotransmission is believed to occur in blood vessels. Cotransmission involves the 2 major autonomic neurotransmitters, acetylcholine and norepinephrine, together with peptides or ATP (Burnstock and Kennedy, 1985). There is evidence that ATP is a cotransmitter with norepinephrine in adrenergic nerve terminals in rabbit ear and pig basilar artery, rabbit aorta and portal vein, guinea pig portal vein, and rat caudal artery (Burnstock and Kennedy, 1986; Su, 1985; Westfall et al., 1987). ATP and norepinephrine are stored and released together in varying ratios in sympathetic perivascular neurons. The action of ATP could be one of a neuromodulator, acting

with norepinephrine to stimulate post synaptic receptors and/or to inhibit norepinephrine release via receptors for ATP or adenosine on the pre-synaptic nerve terminal (Rand et al., 1987).

Burnstock and Kennedy (1986) pointed out that electrophysiological experiments are confirming the co-existance of norepinephrine and ATP in perivascular sympathetic nerves. In general, perivascular nerve stimulation induces excitatory junction potentials (e.j.p.) in vascular smooth muscle that are not sensitive to alpha adrenergic blockade (Burnstock and Kennedy, 1986). It is not clear as to whether or not ATP is responsible for the neurogenic produced contraction as well as the e.j.p. Burnstock and Kennedy (1986) have suggested that this is due to different stimulation parameters used by investigators. In the rabbit central ear artery, long trains of stimulation produced contractions that were mostly sensitive to alpha-adrenoceptor blockade, whereas contractions to a short, 1-second train of stimulation were more resistant to prazosin blockade (Kennedy et al., 1986). The prazosin-resistant contractions were reduced by desensitization of P_2-purinoceptors, indicating that the electrically-stimulated contractions may be mediated by ATP. Thus, ATP and norepinephrine appear to be released together, as cotransmitters from sympathetic perivascular nerves in the rabbit ear artery. Their relative contribution depends on the pattern of stimulation (Kennedy et al., 1986).

Muramatsu (1981) applied ATP to helical strips of the dog basilar artery, in vitro. Exogenous ATP produced responses similar to sympathetic nerve stimulation. An initial contraction response was followed by a transient relaxation or another late contraction.

Transmural stimulation of this artery evoked release predominatly of 3H-purine, as compared to norepinephrine, from the sympathetic nerves. Both contractile and relaxing responses were observed. In the rat caudal artery the amount of ATP released exceeded the amount of norepinephrine released in response to electrical field stimulation (Westfall et al., 1987). In this study, the source of ATP was both neural and nonneural. The authors postulated that the nonneural source of ATP could be derived from smooth muscle or blood vessels as a result of muscle contraction.

The physiological significance of cotransmission has not been determined. One possibility is a temporal separation of events at the smooth muscle neuroeffector junction. In the rabbit ear artery (Kennedy et al., 1986) and rat tail artery (Sneddon and Burnstock, 1984) the cotransmitters mediate distinct responses that are dependent on the stimulation parameters utilized. In terms of exercise, a functional role for cotransmission has not been tested.

Other Neural ATP Interactions

Sympathetic nerve stimulation causes release of adenine nucleotides as a result of norepinephrine acting post-junctionally in the rabbit heart (Fredholm et al., 1982). It was determined that less than 1% of the total purines released could have been released together with norepinephrine. This suggests that sources of ATP include a post-junctional site in the sympathetic nervous system.

ATP release has been demonstrated in other nerve preparations. Silinsky and Hubbard (1973) demonstrated that ATP was released from motor nerve terminals in the rat phrenic nerve-hemidiaphragm. In

further experiments, Silinsky (1975) showed that in response to nerve stimulation, ATP release was possible in the presence of curare, but not hemicholinium-3. Silinsky concluded that ATP was released from motor nerve endings and it may be derived from cholinergic motor nerve terminals. In the electric organ of the Torpedo, Israel et al. (1976) detected post-synaptic ATP release. In these studies, ATP release was reduced by curare and enhanced by eserine, suggesting that acetylcholine may stimulate post-synaptic ATP release. They speculated that ATP may potentiate the acetylcholine response of post-synaptic membranes. In the same organ, the simultaneous release of acetylcholine and ATP was demonstrated in cholinergic synaptosomes (Morel and Meunier, 1981). Similar to the conclusions with norepinephrine, it appears that ATP can be released from both pre- and post-junctional sites in association with acetylcholine. Schrader et al. (1982) demonstrated intra-coronary infusion of acetylcholine elicited release of ATP and purines from guinea pig heart. The source of ATP was not determined in this study. It is apparent that ATP is involved in cholinergic as well as adrenergic responses.

The findings from these experiments strongly support the hypothesis that ATP is involved in neural regulation of the vasculature. ATP interacts with both the cholinergic and adrenergic nervous system, as a cotransmitter in adrenergic nervous system and possibly as a neurotransmitter separate from these systems.

ATP as an Autacoid

When applied directly to vascular smooth muscle ATP produces

depolarization, which can lead to smooth muscle contraction and vasoconstriction (Burnstock and Kennedy, 1985; Burnstock and Kennedy, 1986). When ATP is infused or injected intra-arterially into a preparation in situ, the response is vasodilation (Folkow, 1949; Thompson et al., 1986). This seeming paradox was explained when Furchgott and Zawadzki (1980) discovered that endothelial cells were obligatory for vascular smooth muscle relaxation in response to acetylcholine, and later this was discovered to be true for ATP (DeMey et al., 1982; Burnstock and Kennedy, 1986). Endothelium-dependent vasodilator agonists act on endothelial cell receptors to stimulate formation of a humoral substance by endothelial cells. This relaxing agent, termed endothelium-derived relaxing factor (EDRF) is released by endothelial cells and results in relaxation of vascular smooth muscle (Furchgott, 1983). Thus when given intra-arterially, ATP likely stimulates receptors on endothelial cells that results in EDRF formation and vascular smooth muscle relaxation.

Endothelium-dependent vasodilation has been observed mainly in large arteries, under in vitro conditions (Furchgott, 1983) but also in vivo, in large arteries (Kaiser et al., 1986) and in resistance vessels (Pohl et al., 1987). Under in vitro conditions arteries are cut into segments, placed in a muscle bath containing physiological salt solution and mounted on a force transducer. Muscle contractions or relaxations are measured as changes in tension development. When the isolated arterial segments are preconstricted and the endothelium is intact, addition of ATP to the bath solution produces relaxation (DeMey et al., 1982; Kennedy et al., 1985; White et al., 1985). When the endothelium is removed, ATP vasodilation is abolished and/or ATP

elicits vasoconstriction (Burnstock and Kennedy, 1985; Kennedy et al., 1985; White et al., 1985). Also, if the arterial segments are not pre-constricted ATP produces contraction as the dominant response (DeMey et al., 1982; Kennedy et al., 1985; White et al., 1985). It has been concluded that ATP has a dual effect in the vasculature: relaxation mediated by endothelial receptors and contraction mediated by smooth muscle receptors (Burnstock and Kennedy, 1986).

In almost every tissue tested, the relaxation response to ATP has been demonstrated to be endothelial cell-dependent (Burnstock and Kennedy, 1986). This includes the rabbit and pig aorta, rabbit central ear, basilar, intralobular, femoral and lingual arteries, rat femoral artery, canine femoral, circumflex coronary, splenic arteries, and saphenous vein (Furchgott, 1982; Gordon and Martin, 1983; Martin et al., 1985; Frank and Bevan, 1983; Kennedy and Burnstock, 1985; DeMey and Vanhoutte, 1982; DeMey et al., 1982; Kennedy et al., 1985; Cassis et al., 1987; Burnstock and Kennedy, 1986). The one exception is the rabbit mesentary where ATP acted directly on vascular smooth muscle to produce relaxation via P_2y receptors (Mathieson and Burnstock, 1985). In the rabbit aorta, relaxation by ATP is the result of both a direct action on the smooth muscle and stimulation of endothelial cell receptors, with the endothelial component being much stronger (Furchgott, 1981).

Mechanism of ATP-Induced Endothelium-Dependent Relaxation

Initial investigations into the mechanisms of relaxation produced by endothelium-dependent agents focused on the involvement of

arachidonic acid and its metabolites. Inhibitors of various steps in the metabolism of arachidonic acid also inhibit endothelium-dependent vasodilation although there is variation among species, tissues, vasodilators and inhibitors. Arachidonic acid is formed from unsaturated fatty acid precursors (membrane phospholipids) via the calcium requiring enzyme phospholipase A_2 (Vanhoutte et al., 1986). Arachidonic acid is metabolized intracellularly via 3 major enzymatic pathways: (1) cytochrome p-450-dependent monooxygenase to epoxides and monohydroxy metabolites (Pinto et al., 1986), (2) lipoxygenase to leukotrienes (Samuelsson, 1980) and (3) cyclooxygenase to prostaglandins (Smith, 1986). Metabolites from each of these pathways have been hypothesized to be EDRF (Vanhoutte et al., 1986).

Prostacyclin (PGI_2) is the major prostanoid synthesized in freshly isolated endothelial cells (Smith, 1986) and PGI_2 synthase is localized primarily in the endothelium (Pinto et al., 1986). Despite this suggestive evidence that PGI_2 might be EDRF, many investigations have demonstrated that indomethacin, an inhibitor of cyclooxygenase, does not attenuate endothelium-dependent vasodilation in vitro or in vivo (Kaiser et al., 1986; Furchgott, 1983; Vanhoutte et al., 1986). Thus, EDRF has been hypothesized to be a non-prostaglandin metabolite of arachidonic acid (Furchgott, 1983).

In the canine femoral artery, relaxation to ATP and acetylcholine is endothelium-dependent in vitro (DeMey and Vanhoutte, 1981). Acetylcholine is considered the prototypical endothelium-dependent vasodilator and 5,8,11,14-eicosatetraynoic acid (ETYA) inhibits its relaxation response in many tissues in vitro (Furchgott, 1983; Rapoport and Murad, 1983; Vanhoutte et al., 1986) as well as in vivo (Kaiser et

al., 1986). ETYA is an arachidonic acid analog that inhibits the metabolism of arachidonic acid by all three enzymatic pathways (Pinto et al., 1986). Since ETYA was effective in blocking the endothelium-dependent relaxation but indomethacin was not, a lipoxygenase product of arachidonic acid was thought to be the EDRF for acetylcholine (DeMey et al., 1982). Monooxygenase metabolites were not mentioned as potential EDRF's in this study, perhaps because the existance of this pathway was not well recognized at that time.

In the canine femoral artery endothelium-dependent relaxation produced by ATP was not inhibited by ETYA although ETYA inhibited acetylcholine-induced relaxation (DeMey et al., 1982). The difference in susceptability of ATP vs. acetylcholine to ETYA suggests that either ATP is producing the same EDRF by a different pathway or that a different EDRF is being produced by ATP in this tissue. This is unique for the canine femoral artery because ETYA inhibits ATP and acetylcholine relaxation in rabbit (Furchgott, 1981) and rat (Rapoport et al., 1984) aorta.

EDRF was generally believed to be a product of lipoxygenase because ETYA and other lipoxygenase inhibitors block endothelium-dependent vasodilation. However, it was demonstrated that inhibitors of lipoxygenase do not prevent the relaxation response to acetylcholine unless they also possess anti-oxidant properties (Vanhoutte et al., 1986). Anti-oxidants and non-specific free radical scavengers inhibit endothelium dependent relaxation, as does catecholamines and anoxia (Rubanyi, 1988; Vanhoutte et al., 1986). Oxygen derived free radicals are probably not the source of EDRF (Rubanyi, 1988; Vanhoutte et al., 1986). Possible sources of

nonoxygen-derived free radicals include arachidonic acid metabolites (Rubanyi, 1988).

The action of EDRF is mimicked by nitrovasodilators, such as glyceryl trinitrate (Ignarro et al, 1984; Martin et al, 1985; Rapoport and Murad, 1983). Nitrovasodilators are not dependent on endothelial cells to elicit vascular smooth muscle relaxation (Ignarro, 1984). Nitrovasodilators apparently relax vascular smooth muscle by producing nitric oxide (NO) within the cells which then activates guanylate cyclase and stimulates formation of cyclic guanosine 3',5'-monophosphate (cGMP). A cGMP-dependent protein kinase is activated and this leads to dephosphorylation of myosin light chain and relaxation (Ignarro and Kadowitz, 1985; Rapoport et al., 1984). Relaxation by a number of endothelium dependent vasodilators, including ATP, has been shown to be associated with an increase in vascular smooth muscle cGMP. This increase in cGMP is both time-and concentration-dependent (Furchgott et al., 1984; Ignarro et al., 1984; Peach et al., 1985 and Rapoport et al., 1984). Inhibitors of endothelium-dependent relaxation, ETYA and quinacrine (a phospholipase inhibitor), also inhibited c-GMP formation in response to the endothelium-dependent relaxants, acetylcholine and histamine in the rat aorta (Rapoport and Murad, 1983). Methylene blue, a vital stain and oxidising agent, is well known for its action to inhibit guanylate cyclase activitation by nitrovasodilators and nitric oxide and its ability to antagonize the relaxation produced by these agents (Gruetter et al., 1981; Ignarro and Kadowitz, 1985). Methylene blue is specific for nitric oxide containing vasodilators because relaxation to catecholamines, isoproterenol, PGI_2 or calcium antagonists was not

antagonized by methylene blue (Ignarro and Kadowitz, 1985; Martin et al., 1985; Ignarro et al., 1984). Methylene blue also inhibits relaxation and reduces c-GMP formation by endothelium-dependent vasodilators (Ignarro et al., 1984; Martin et al., 1985; Kaiser et al., 1986). MB is capable of producing superoxide anions in vitro (McCord and Fridovich, 1970) and superoxide anions can inactive EDRF (Rubanyi, 1988). Ignarro et al. (1986) demonstrated in the bovine intrapulmonary artery in vitro, MB did not interfere with the formation, release, or chemical stability of EDRF, but worked by inhibiting guanylate cyclase. Griffith et al. (1985) studied the effects of MB on EDRF in the rabbit aorta. In order to bioassay EDRF they used a system whereby denuded coronary artery was perfused with effluent from an endothelium intact aorta. MB had no greater effect when it was perfused in the aorta than when perfused only in the coronary artery. This indicates that MB is not inactivating EDRF. Thus, MB inhibits endothelium-dependent vasodilation and the relaxation response to nitrovasodilators through its action to inhibit guanylate cyclase. It appears that both endothelium-dependent vasodilators and nitrovasodilators have pfroduction of cGMP as a final common pathway for eliciting vascular smooth muscle relaxation. EDRF stimulates an increase in vascular smooth muscle cGMP and nitrovasdilators produce nitric oxide intracellularly that elevates cGMP. Because of the obvious similarities between nitrovasodilators and endothelium-dependent vasodilators, Furchgott proposed that EDRF may be NO (Furchgott, in press and Vanhoutte, 1987).

Palmer and co-workers (1987) measured NO release from cultured pig aortic endothelial cells, in response to stimulation with the

endothelium-dependent vasodilator, bradykinin. They found that the biological activity of exogenous NO and EDRF were identical. Both EDRF and NO are labile substances with a short half life (approximately 30 seconds) and are degraded readily by superoxide anions (Palmer et al., 1987). The results of Palmer et al. (1987) that NO and EDRF behave similarly in vascular smooth muscle have been confirmed by several different investigators (Ignarro et al., 1987; Ignarro et al., 1988 and Shikano et al., 1987). Pyrogallol, an agent used to generate superoxide anions, inhibits both acetylcholine- and NO-induced relaxations in bovine intrapulmonary artery, whereas isoproterenol and glycerol trinitrate relaxations were unaffected (Ignarro et al., 1988). In the same vessel, oxyhemoglobin inhibited NO-induced relaxations and endothelium-dependent relaxations to acetylcholine and bradykinin (Ignarro et al., 1987). Furthermore, oxyhemoglobin inhibited the accumulation of vascular cGMP caused by both endothelium-dependent relaxants and by NO. These results suggest that EDRF is probably nitric oxide or a chemically related radical species. However, this can only be concluded for the conditions specific to these studies, as there may be more than one EDRF.

The relaxation response to ATP was associated with a significant increase in cyclic GMP content of vascular smooth muscle (Rapoport et al., 1984). The rise in cGMP and the relaxation response were endothelial cell-dependent and both were inhibited by: (1) ETYA, (2) nordihydroguaiaretic acid (NDGA), a lipoxygenase inhibitor and (3) bromophenacyl bromide, a phospholipase A_2 inhibitor (Rapoport et al., 1984). The endothelium-dependent relaxation response to ATP and acetylcholine was inhibited by ETYA and quinacrine in the rabbit

thoracic aorta (Furchgott, 1981). Thus, there is some evidence that EDRF for ATP has similar properties to the proposed EDRF, nitric oxide, for acetylcholine and bradykinin in the bovine pulmonary artery and bradykinin in porcine aortic endothelial cells. However, as mentioned previously, there is some discrepancy between acetylcholine and ATP relaxations in the canine femoral artery (DeMey et al., 1982). If ATP produces nitric oxide as its EDRF then methylene blue, the nitric oxide antagonist, should inhibit ATP-induced relaxations and cGMP accumulation, but this has not been tested. Furthermore, other antagonists of nitric oxide, such as hemoglobin and superoxide anions should also antagonize ATP relaxations (Gruetter et al., 1981; Ignarro et al., 1987; Ignarro et al., 1988 and Martin et al., 1985).

ATP Receptors: Agonists and Antagonists

In 1978, Burnstock proposed that purinoceptors be divided into 2 categories, P1 and P2, based on several criteria. At P1-purinoceptors adenosine (ADO) was the most potent agonist and the agonist order of potency for this receptor was ADO>AMP>ADP>ATP. Antagonists of P1-purinoceptors include methylxanthines, such as theophylline and activation of these receptors was proposed to alter intracellular cyclic adenosine 5'-monophosphate (cAMP) levels (Burnstock, 1980; Burnstock and Kennedy, 1985 and Burnstock and Kennedy, 1986). In contrast, at P2-purinoceptors the agonist potency order was ATP>ADP>AMP>ADO. Burnstock suggested methylxanthines were not antagonists of P2-purinoceptors and occupancy of these receptors did not lead to changes in intracellular cAMP levels (Burnstock and Kennedy, 1985 and Burnstock and Kennedy, 1986). Subsequently,

P1-purinoceptors were subdivided into A_1 (Ri) or A_2 (Ra) receptors. More recently P2-purinoceptors have been subdivided into P_{2x}- and P_{2y}-purinoceptors based upon differences in location, action on target organ, potency of agonists and effects of antagonists (Burnstock and Kennedy, 1985 and Burnstock and Kennedy, 1986).

The discovery that ATP mediates relaxation through an endothelium-dependent mechanism and contraction by acting directly on vascular smooth muscle provided a functional basis for the separation of P2-purinoceptors. ATP binding to P_{2y} receptors on the endothelium elicits relaxation of vascular smooth muscle and ATP binding to P_{2x} receptors on vascular smooth muscle elicits contraction (Burnstock and Kennedy, 1986). P_{2y} receptors are pharmacologically, as well as physiologically different from P_{2x} receptors. At the P_{2y}-purinoceptor, the potency order of ATP agonists mediating relaxation is 2-methylthioATP>>ATP>alpha,beta-methylene ATP. At the P_{2x}-purinoceptor, the potency order for ATP agonists mediating contraction is alpha,beta-methyleneATP>ATP=2 methylthioATP (Burnstock, 1985; Burnstock and Warland, 1987 and Houston et al., 1987). ATP is a more potent agonist at P_{2y} receptors than P_{2x} receptors (Burnstock and Kennedy, 1985). 2-methylthioATP is the most potent P_{2y} agonist while alpha,beta-methylene ATP is the most potent P_{2x} agonist and has very little P_{2y} receptor activity (Burnstock and Kennedy, 1985).

Reported pharmacologic antagonists of P_{2y}-purinoceptors include antazoline, receptor desensitization with ATP, and reactive blue (Burnstock and Kennedy, 1985; Burnstock and Warland, 1987; Shimada and Stitt, 1984).

In the rabbit hindlimb the dilator responses to ATP and to

hypothalamic stimulation (50-500 uA, 77 Hz for 20 sec, 1-2 mS) were inhibited by antazoline. Hypothalamic stimulation at these parameters reproduced the skeletal muscle hyperemia observed during the "defense reaction". It was not demonstrated in this study whether ATP vasodilation was endothelial-cell dependent (Shimada and Stitt, 1984). Antazoline was nonspecific in its action as it reduced vasodilation to histamine and adenosine, as well as ATP. Shimada and Stitt provided additional pharmacological data and rationale to suggest that antazoline's nonspecific actions were probably not relevant to its P-2 inhibitory action: aminophylline, an adenosine antagonist, and antihistamines had no effect on hypothalamic stimulation-induced vasodilation, and antazoline did not reduce vasodilation to acetylcholine. This study suggested that antazoline has potential use a P_{2y} receptor antagonist.

In 1980 Hogaboom provided evidence that arylazido aminopropionyl ATP ($ANAPP_3$) specifically inhibited ATP receptors in guinea pig vas deferens (Hogaboom et al., 1980). $ANAPP_3$ is a photoaffinity labelled ATP molecule that is activated in the presence of ultraviolet or visible light to bind covalently with ATP receptors (Fedan et al., 1985). Nonphotolyzed $ANAPP_3$ also inhibits ATP responses. In guinea pig smooth muscle, the contractile response to ATP but not KCL, norepinephrine, histamine or acetylcholine was inhibited by nonphotolyzed $ANAPP_3$. This effect is reversible if $ANAPP_3$ is removed (Hogaboom et al., 1980). Nonphotolyzed $ANAPP_3$ is also a reversible agonist-antagonist in situ, when administered intraarterially to the cat urinary bladder (Theabold, 1983). An initial contraction (agonist) response is followed by ATP antagonism when $ANAPP_3$ is given in situ,

without photolysis (Fedan et al., 1985). Fedan et al., (1985) and Hogaboom et al., (1980) have suggested that nonphotolyzed $ANAPP_3$ is either a competitive antagonist of P2 receptors or that $ANAPP_3$ produces receptor desensitization similar to ATP. Fedan suggested that at least part of the antagonism is competitive because nonphotolyzed $ANAPP_3$ is more effective than ATP in reducing ATP responses and the agonistic potency of the two are equal.

In the rabbit femoral artery in vitro, nonphotolyzed $ANAPP_3$ inhibited ATP relaxations and not relaxations due to methacholine (Cassis et al., 1987). ATP relaxation was endothelium-dependent and associated with an increase in vascular smooth muscle cGMP. The increase in cGMP and the relaxation response were both inhibited by $ANAPP_3$ and ETYA. The selectivity of $ANAPP_3$ for P2-purinoceptors in the vasculature was tested in the rat aorta (White et al., 1985). Photolyzed $ANAPP_3$ had no effect on relaxations produced by ATP. $ANAPP_3$ inhibited the contractile response to alpha,beta-methylene ATP in aortic strips denuded of endothelium, therefore antagonizing P_{2x}-purinoceptors. These results suggest that photolyzed $ANAPP_3$ was selective for P_{2x}-purinoceptors and P_{2x}-mediated contractile responses. Nonphotolyzed $ANAPP_3$ inhibited P_{2y}-purinoceptors on endothelial cells and inhibited ATP-induced relaxtion (Cassis et al., 1987).

Desensitization of ATP receptors is another method used to inhibit ATP responses. When ATP is administered to vascular or nonvascular tissue it produces tachyphylaxis, i.e., there is a period of time following ATP administration that the tissue is refractory to another ATP stimulus (Burnstock and Kennedy, 1985; Hogaboom et al., 1980). Desensitization of ATP relaxation by repeated administration of ATP has

been demonstrated in nonvascular smooth muscle, but not in vascular smooth muscle (Burnstock and Kennedy, 1985). In the rabbit portal vein, the relaxation response to ATP was not inhibited by repeated administration of alpha,beta-methyleneATP (Burnstock and Kennedy, 1985). Since alpha,beta-methyleneATP is not an agonist of the receptors that mediate relaxation, P_{2y}-purinoceptors, this result is not surprising. No one has apparently attempted to inhibit ATP relaxations in the vasculature through desensitization with specific P_{2y}-purinoceptors agents.

Another ATP analogue, reactive blue, antagonized P_{2y}-purinoceptors in the rabbit mesenteric artery (Burnstock and Warland, 1987). However, this is the one exception where P_{2y}-purinoceptors are located on the smooth muscle and not the endothelium. In addition, reactive blue was not a selective ATP antagonist because it antagonized relaxation to acetylcholine and adenosine as well. In the canine coronary artery, reactive blue competitively antagonized the relaxation response to ADP and 2-methylthio-ATP, although this was nonspecific because acetylcholine relaxation was also inhibited (Houston et al., 1987).

Desensitization by alpha, beta-methyleneATP has inhibited P_{2x}-purinoceptor-mediated contractions in rat femoral (Kennedy et al., 1985) and rabbit central ear arteries (Burnstock and Kennedy, 1985). Desensitization has been used extensively to investigate the cotransmitter role of ATP with norepinephrine in the vasculature. Both $ANAPP_3$ and desensitization are potentially useful tools to investigate the physiological significance of vascular P2x receptors.

Dual Function of ATP in Vasculature

Burnstock and Kennedy (1986) have proposed that the existance of two subtypes of ATP receptors in the vasculature having opposite functions suggests that ATP has a dual effect: vasoconstrictor and vasodilator. Intuitively, it seems reasonable that the proposed actions of endogenous ATP could be consistant with ATP acting both as a vasoconstrictor and vasodilator. ATP could be released from platlets, endothelial cells, or intramural neurons to elicit vasodilation via endothelial cells. Indeed, increased levels of ATP have been measured in the vasculature during hypoxia, ischemia, and exercise. If the endothelium has been damaged by artherosclerosis, hypertension, or some other pathological process, then intravascular ATP might act directly on smooth muscle, producing vasoconstriction. This could be beneficial if there is hemorrhage occurring, or it could be harmful if blood supply to skeletal or myocardial muscle is compromised by vasoconstriction.

The physiological relevance of ATP having two opposing functions in the vasculature is mainly speculative. Simply measuring elevated venous concentrations of ATP during muscle contraction concommittant with hyperemia seems even less convincing now that we know ATP can elicit vasoconstriction as well as vasodilation. There is a definite need to determine the physiological relevance of these two potential responses to endogenous ATP and the best available techniques to accomplish this are pharmacological.

Significance of Resistance Vessels

"Resistance vessels" is a term used to describe the location of greatest flow impediment within the vasculature. Large arterioles and small arteries, approximately 100um or less, are considered to be the sites of major resistance to blood flow (Renkin, 1984 and Segal and Duling, 1986). The dual effect of ATP in the vasculature and endothelium-dependent ATP vasodilation have been studied in vitro, with blood vessels larger than 100um. One major criticism of these studies is that we are experimenting with vessels that do not contribute to resistance function in vivo. More recently, investigators have attempted to determine if endothelium-dependent vasodilation occurs in situ, in resistance vessels.

In the hamster cheek pouch, arterioles 50-80um were studied by intravital microscopy for endothelium-dependent relaxation. Neither methylene blue or hemoglobin attenuated the dose-dependent dilations to acetylcholine, suggesting that acetylcholine does not produce dilation by an EDRF pathway in these vessels (Rivers and Duling, 1986). This particular result has not been confirmed in other studies. Furchgott et al. (1987) studied resistance vessels of rabbit and rat mesentary arteries. Collagenase and hemoglobin were effective in reducing acetylcholine vasodilations, but nonspecific effects of these inhibitors were not ruled out. In arterial segments of human skin biopsies, acetylcholine relaxation was endothelial cell dependent and the vasodilation was greater in smaller sized vessels (Aalkjaer et al., 1987). In the rabbit ear artery, the endothelium-dependency of the action of acetycholine in resistance vessels was demonstrated by

mechanical removal of the endothelial layer and loss of relaxation response (Owen and Bevan, 1985). Owen and Bevan (1985) also found that acetylcholine dilation was proportionally greater in smaller vessels. Therefore, there is some evidence for endothelium-dependent vasodilation in resistance vessels and, in fact, the effect in resistance vessels may be greater than in larger arteries. Owen and Bevan (1985) attributed the greater response of smaller vessels to a thinner internal elastic lamina and greater intrinsic tone.

The flow response to the endothelium-dependent vasodilators ATP and acetylcholine was studied in situ, in resistance vessels of the rabbit hindlimb (Pohl et al., 1987). Alterations in blood flow due to resistance vessel function can be evaluated by utilizing an intact hindlimb preparation. Gossypol was used to inhibit endothelium-dependent responses. The mechanism of action of gossypol was not known, although it was suggested that it may inhibit lipoxygenase or act as an antioxidant. Gossypol reduced the vasodilator effects of ATP and acetylcholine, but did not effect the vasodilation to adenosine or nitroglycerin. Gossypol selectively reduced vasodilator responses to substances known to be endothelial-cell dependent and had no effect on nonendothelial cell-dependent vasodilators. These results are consistant with the in vitro studies, except in the hamster, for the existance of endothelium-dependent vasodilation and for ATP mediated endothelium-dependent vasodilation in resistance vessels.

The Contribution of Adenosine 5'-Triphosphate To Exercise Hyperemia

Introduction

It is generally believed that local mechanisms mediate the increase in skeletal muscle blood flow during exercise. During exercise muscle metabolism increases, vasoactive metabolites accumulate in the extracellular fluid and elicit vasodilation (Haddy and Scott, 1968). Despite years of investigation, the identity of a metabolite(s) that accounts for the sustained hyperemia observed during steady state, free flow exercise has not been fully determined.

Adenosine 5'-triphosphate (ATP) has been proposed as a possible mediator of exercise hyperemia (Forrester, 1981). It is a potent vasodilator (Folkow, 1949 and Thompson et al., 1986) and elevated levels of ATP sufficient to account for the hyperemic response (Forrester, 1972) have been measured in the venous effluent of exercising muscle (Forrester, 1966; Forrester and Lind, 1969; Forrester, 1975). ATP is a more potent vasodilator than adenosine and an antagonist of adenosine vasodilation did not reduce exercise hyperemia or ATP vasodilations (Thompson et al., 1986).
These findings raise the possibility that ATP could be a mediator of sustained free flow exercise hyperemia.

If ATP mediates a significant component of exercise hyperemia then antagonists of ATP vasodilator receptors should also reduce exercise hyperemia. Testing of ATP's role in blood flow regulation has been difficult due to lack of selective ATP receptor antagonists. In part, this is due to the complex nature of purine receptors. Purine receptors can be classified into 2 groups, P_1 and P_2-purinoceptors

(Burnstock, 1978). The potency order of agonists for P_1-purinoceptors is adenosine>adenosine 5'-monophosphate (AMP)>adenosine 5'-diphosphate (ADP)>ATP and these receptors are antagonized by methylxanthines (Burnstock, 1978). P_2-purinoceptors are not antagonized by methylxanthines and have an agonist potency order of ATP>ADP>AMP>adenosine (Burnstock, 1978). P_2-purinoceptors have recently been subclassified into P_{2x} receptors and P_{2y} receptors (Burnstock and Kennedy, 1985). In the vasculature ATP acts on both subgroups of P_2-purinoceptors; P_{2x} receptors on vascular smooth muscle which mediate contraction and P_{2y} receptors on the endothelium which mediate vascular smooth muscle relaxation (Burnstock and Kennedy, 1986). Several selective P2-purinoceptor antagonists have recently become available. In this study, we used these agents to inhibit ATP-induced vasodilation in the blood-perfused canine hindlimb.

Experiments were designed to test the hypotheses that (1) ATP-induced vasodilation contributes significantly to sustained free flow exercise hyperemia and (2) ATP contributes significantly to reactive hyperemia following 30 seconds of arterial occlusion. We used three inhibitors of P_2-purinoceptors, arylazido aminopropionyl ATP ($ANAPP_3$), desensitization, and antazoline, to test these hypothesis.

A photoaffinity labelled ATP molecule, $ANAPP_3$ has been used to inhibit P_2-purinoceptors in vascular (Cassis et al., 1987; White et al., 1985) and non-vascular (Hogaboom et al., 1980) smooth muscle. $ANAPP_3$, when activated by light, irreversibly antagonizes P_2-purinoceptors (P_{2x}) that mediate smooth muscle contraction. Without light activation, $ANAPP_3$ is a competitive antagonist of ATP receptors (Fedan et al., 1985; Hogaboom et al., 1980). In the current study we

used $ANAPP_3$ to competitively inhibit ATP vasodilator P2-purinoceptors.

Responses to ATP can be reduced by desensitization of ATP receptors (Burnstock and Kennedy, 1985). Desensitization develops following repeated ATP administration rendering the tissue refractory to another ATP stimulus. In this study, we attempted to desensitize P2-purinoceptors by infusing ATP.

Antazoline inhibited P-2 purinergic vasodilation in the skeletal muscle vasculature of the rabbit, although it is also a H_1 histaminergic receptor antagonist (Shimada and Stitt, 1984; Meier, 1948-1950). We attempted to inhibit P2-purinergic vasodilation in our preparation with antazoline.

Our results suggest that $ANAPP_3$ was selective for ATP receptors, but antazoline and desensitization were not selective ATP antagonists. Our results were not consistant with a role for ATP as a mediator of exercise hyperemia. In the presence of $ANAPP_3$, the blood flow response to exercise and reactive hyperemia was increased, while oxygen consumption was decreased during exercise. Therefore, ATP may limit the blood flow response during steady state hyperemia.

Methods

Twenty four mongrel dogs of either sex, weighing 14 to 20 kg were anesthetized with pentobarbital (30 mg/kg, i.v.) and supplemented hourly (50 mg). The dogs were intubated and mechanically respired (Harvard apparatus) with room air, supplemented with 100% O_2. Arterial blood gases were monitored hourly (Corning Model 165/2, blood gas analyzer). Tidal volume and respiratory rate were set to maintain arterial PCO_2 between 30-42 mmHg and PO_2 between 90-110 mmHg.

Intravenous sodium bicarbonate (150mM) was administered to correct for metabolic acidosis. Esophageal temperature was monitored (Yellow Springs Instrument)and maintained at 37-39°C by heating pads.

A schematic of the preparation utilized in these experiments is shown in Figure 1A. The circulation of the gastrocnemius-plantaris muscles of the left hindlimb was surgically prepared as previously described (Stainsby et al., 1956). All branches of the femoral artery and vein not directly supplying the gastrocnemius-plantaris muscles were ligated.

The achilles tendon was severed and the distal end attached to a specially adapted, isometric force transducer (Grass) used to measure tension development. A nail was positioned laterally in the distal femur and clamped to a rigid support to stabilize the joint. The sciatic nerve was sectioned and the distal end of the nerve placed on bipolar platinum electrodes which were fitted in a small, plexiglass container filled with saline. The nerve was insulated with petroleum jelly and the container wrapped with saline soaked gauze to prevent drying.

The animals were anticoagulated with heparin sodium (1000 U/kg, i.v.) and supplemented hourly (250 U/kg). The femoral artery was cannulated 2-3 cm proximal to where this artery enters the popliteal space and the muscle was perfused with blood from the contralateral femoral artery. Perfusion pressure was held constant at 105 mmHg by a servo-controlled pump (Mohrman, 1980) and continuously measured just proximal to the muscle cannula. Systemic arterial pressure was measured by tapping into the perfusion tubing proximal to the servo pump. Muscle blood flow was monitered with an electromagnetic flow

probe (3mm I.D., cannulating, Zepeda Instruments) and SWF-4 flowmeter (Zepeda Instruments). At the end of the experiment the flow probe was calibrated by timed collection of blood.

A shunt consisting of 2 polyethylene cannulas connected by silastic tubing was positioned so that all the venous effluent draining the muscle was directed into the ipsilateral vein, at the level of the femoral triangle. The shunt provided a venous sampling site. A Y-connector situated proximal to the servo pump provided a sampling port for arterial blood. Approximately 10 ml/min of arterial and venous blood were continuously withdrawn (via a roller pump) for measurement of arterial and venous oxygen content difference (A-VOX Systems). The A-VOX calibration was checked against the a-v O_2 content difference calculated from blood gas analysis. Values for pH, pO_2, and hematocrit were obtained and oxygen content calculated by: O_2 content = $\%O_2$ saturation x hemoglobin concentration. Oxygen content measurements ignore the small percentage (0.3%) of oxygen dissolved in blood. The $\%O_2$ saturation was determined by a nomogram from pO_2 and pH (Rossing and Cain, 1966). Hemoglobin was calculated from the hematocrit: Hb = 0.375 x hematocrit - 1.19. Oxygen consumption (VO_2) was calculated by: VO_2 (ml O_2/min/100g) = blood flow (ml/min/100g) x a-v O_2 content (ml O_2/ml blood).

Muscle contraction was produced by square wave pulse stimulation of the sciatic nerve. Optimal muscle length for maximum tension development was determined before each experiment by stretching the muscle until maximum tension development was obtained. Tension was quantified through weight calibration of the force transducer and expressed in terms of kg/g muscle. Stimulus parameters for twitch

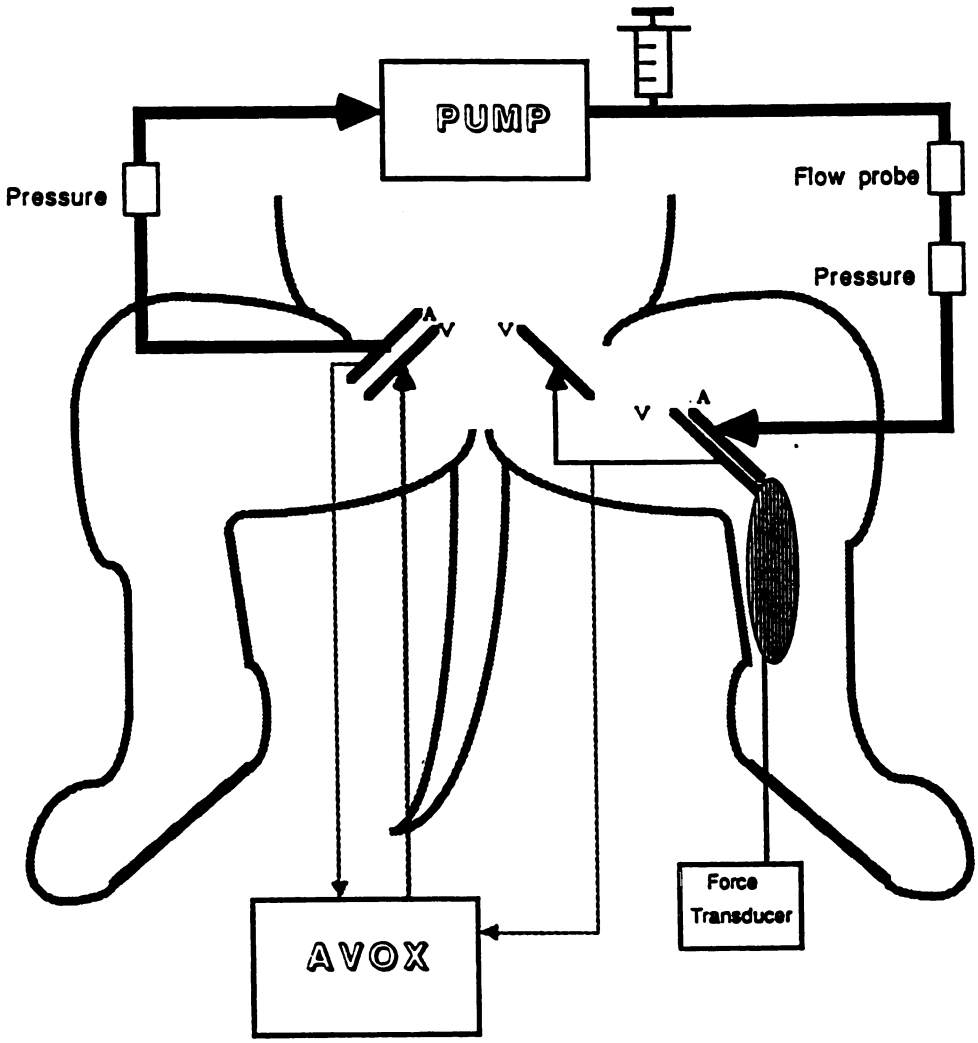

Figure 1A. Schematic representation of the preparation. A= artery, V= vein, Pressure= pressure transducer, Pump= Mohrman pump, Syringe= windkessel.

exercise were 0.2msec, supramaximal voltage (4-6 volts) and either 2.5 Hz or 1.5, 3 and 6 Hz (Grass Stimulator, model S9). Supramaximal voltage was determined by increasing voltage in 1 volt increments until maximum tension development was attained. These parameters are below the threshold for stimulation of sympathetic nerves (Morganroth, et al., 1975; Thompson and Mohrman, 1983).

Systemic arterial pressure, muscle perfusion pressure, blood flow, arterial-venous oxygen content difference, and tension development were continuously recorded on a Grass Polygraph (Model 7).

Experimental Protocols

Prior to the start of the experiment a 3 minute warm-up exercise was performed, at 2 Hz stimulation, followed by approximately 20 minutes of recovery and stabilization. The protocol for $ANAPP_3$ experiments was different from the antazoline and desensitization protocol and therefore will be described separately.

$ANAPP_3$

Following the warm up exercise, dose response relationships to ATP, adenosine (ADO), and acetylcholine (ACH) were obtained, the blood flow response to 30 seconds of arterial occlusion determined and the muscle was then exercised. $ANAPP_3$ or saline vehicle was infused for 15 minutes under basal conditions and then continuously for the remainder of the experiment. The infusion rate was 0.5 ml/min, i.a. Dose response relationships and exercise were repeated in the presence of $ANAPP_3$. During exercise, the rate of $ANAPP_3$ infusion was increased proportionally to the increase in blood flow to maintain a constant

$ANAPP_3$ blood concentration. The blood concentration of $ANAPP_3$ ranged from 20 to 130 uM. This approximate concentration of nonphotolyzed $ANAPP_3$ selectively inhibited ATP responses in vascular smooth muscle, in vitro (Cassis et al., 1987; Hogaboom et al., 1980). Blood concentration was calculated by the equation: blood $ANAPP_3$ [M] = infusion rate $ANAPP_3$ (ml/min) X $ANAPP_3$ infused [M]/ muscle blood flow (ml/min/100g). Doses varied somewhat because of difference in baseline blood flow for each animal.

Dose response curves were obtained by intraarterial bolus injection of 0.3ml of ATP, ADO, and ACH. The maximum vasodilator dose for each agonist represents the maximum dose that could be given without depressing systemic arterial pressure. The difference between baseline (i.e., immediately prior to agonist injection) and peak vasodilator response was measured and this value was compared before and after $ANAPP_3$. The order of vasodilator agonist application was randomized for each experiment. Time was allowed for blood flow to return to baseline value between doses.

Reactive hyperemic response was evaluated by occluding the arterial blood flow to the muscle for 30 seconds. The difference between baseline blood flow and peak vasodilator response was measured and compared before and after $ANAPP_3$. Reactive hyperemia was measured between the first and second dose response curves. Time was allowed for blood flow to return to baseline levels after reactive hyperemia.

Exercise frequencies of 1.5, 3, and 6 Hz were used. These were carried out successively and for a minimum of 3 minutes at each frequency. Blood flow and oxygen consumption were measured at rest and during the steady state exercise at each frequency. The effect of

$ANAPP_3$ on exercise hyperemia was also analyzed by determining the blood flow response at 2 levels of oxygen consumption, 6 and 10 ml O_2/min/100g, and comparing this response before and after $ANAPP_3$. From each experiment, a graph of blood flow vs. oxygen consumption was plotted and the blood flow at each of the 2 oxygen consumptions, before and after $ANAPP_3$ was obtained from this plot by linear interpolation. Because the relationship between flow and metabolism is linear in this range, the interpolation is justified. These 2 levels of oxygen consumption were chosen arbitrarily. During exercise with $ANAPP_3$ we intentionally decreased the blood flow to a level that was equal to the control level at each stimulation frequency by decreasing perfusion pressure. This was done after measuring the steady state values. We did this in order to compare oxygen consumption at equal exercise blood flows, before and after $ANAPP_3$. Arterial blood gases were obtained at rest and at steady state 6 Hz exercise; venous samples were taken at rest and at steady state for each exercise frequency.

Antazoline and Desensitization

The effects of antazoline, ATP desensitization, and vehicle were determined in 3 sets of experiments. The protocol for each experiment consisted of infusion of each agonist for 1 minute, then 5 minutes of exercise. After administration of antazoline (4mg/kg) or desensitization or saline vehicle, agonist infusion and then exercise was repeated. Blood flow and oxygen consumption were obtained at rest and 5 minutes of 2.5Hz exercise. Arterial and venous blood gases were obtained also at rest and 5 minutes of exercise.

ATP, ADO, histamine (HIS), and isoproterenol (ISO) were used to

determine the selectivity of antagonism by antazoline and desensitization. ADO was used to test the specificity of the purinergic receptor blockade. Since antazoline is a H_1 histaminergic receptor antagonist, the effects of antazoline on the HIS response were determined. The effects of these antagonists on beta-adrenergic receptors was assessed by their effects on ISO vasodilation.

The order of agonist infusion was randomized for each experiment and the rate of infusion was 1 ml/min, i.a. The concentration of agonist was chosen such that the flow response at 1 minute would be approximately equivalent to that observed with steady state 2.5 Hz exercise. Baseline blood flows immediately prior to infusion and the 1 minute, steady state values during infusion were measured. Between each infusion or exercise bout, time was allowed for blood flow to return to baseline values. We compared baseline blood flows after antazoline, desensitization or saline;, one minute steady state blood flows after antazoline, desensitization or saline; and the difference between baseline and steady state values after antazoline, desensitization or saline.

In the time control experiments, the saline vehicle was infused for 15 minutes (1 ml/min, i.a.). Antazoline was dissolved in saline and infused in a similar manner. ATP desensitization was achieved by successive infusion of ATP (0.1M, syringe concentration, 1 ml/min, i.a.). Desensitization was assured by periodically testing the vasodilator response to ATP. The criterion for desensitization was an approximate 75% reduction in vasodilator response to ATP. Additional ATP was administered as necessary to maintain desensitization.

Data Analysis

The statistical analysis for paired data was a one-way within subjects analysis of variance (ANOVA) (Linton and Gallo, 1975). In the antazoline and desensitization experiments, some of the variances were not homogenous and results were therefore converted to log numbers to obtain homogeneity. The log values were analyzed statistically in this case. A p value $<.05$ was considered significant.

Drugs

ATP, ACH, HIS, ADO and antazoline were obtained from Sigma and heparin sodium from Elkins-Sinn, Inc.. $ANAPP_3$ was obtained from Dr. Robert J. Theobald, Kirksville, MO. All agonist were dissolved in saline, made fresh daily, stored on ice in the dark and warmed prior to use. $ANAPP_3$ was kept in the dark prior to and during the experiments by covering glassware, syringes and infusion lines with aluminum foil.

Results

$ANAPP_3$ (n=5) ADO, ACH and ATP elicited dose dependent increases in blood flow. The vasodilator response to ATP before and after $ANAPP_3$ is shown in Figure 1. The vasodilator response to ATP at doses between 10^{-8} and 10^{-6}M, was increased in the presence of $ANAPP_3$; there was no effect at 10^{-5} and 10^{-4}M ATP. Vasodilation was reduced by ANAPP3 at 10^{-3}M ATP (Figure 1). There was a crossing of the ATP dose response curves at doses of 10^{-5} to 10^{-4}M ATP which occurred in every experiment. The dose response relationship to ACH before and after $ANAPP_3$ is shown in Figure 2. $ANAPP_3$ increased the vasodilator response to ACH at every dose, except 10^{-4} and 10^{-3}M ($p<.05$). The vasodilator

response to ADO was not affected by $ANAPP_3$ as shown in Figure 3.

The effect of $ANAPP_3$ on exercise hyperemia is shown in Figure 4. $ANAPP_3$ did not affect resting (i.e., immediately prior to exercise) blood flow or oxygen consumption. $ANAPP_3$ decreased oxygen consumption at exercise frequencies of 1.5 and 3 Hz ($p<.05$) but had no significant effect on the blood flow response at these frequencies (Figure 4). $ANAPP_3$ depressed muscle tension development at all 3 stimulation frequencies (Table 1). Blood flow is a linear function of oxygen consumption, therefore we wanted to determine if the primary effect of $ANAPP_3$ was on blood flow or oxygen consumption. In Figure 5 the effect of $ANAPP_3$ on the blood flow response at 2 levels of oxygen consumption, 6 and 10 ml O_2/min/100g is shown. At an oxygen consumption of 6 ml O_2/min/100g, $ANAPP_3$ significantly increased the hyperemic response ($p<.05$). At 10 ml O_2/min/100g, the increase was not statistically significant ($n=4$).

To further determine if the primary effect of $ANAPP_3$ was on metabolism or blood flow, we decreased blood flow during exercise with $ANAPP_3$ to control levels. This is depicted in Figure 6. When blood flows were decreased to control values during $ANAPP_3$, oxygen extraction increased at all three stimulation frequencies. At 1.5 Hz extraction increased from 7.3 to 8.4; at 3 Hz it increased from 9.8 to 10.2; at 6 Hz it increased from 12.0 to 12.3 ml O_2/min/100g (data not shown). Despite the increase in oxygen extraction, oxygen consumption was still below control levels at 1.5 and 6 Hz ($p<.05$), but not significantly at 3 Hz (Figure 6). The peak reactive hyperemic response before and after $ANAPP_3$ is shown in Figure 7. With $ANAPP_3$ treatment, the peak vasodilator response to 30 seconds of arterial occlusion was increased

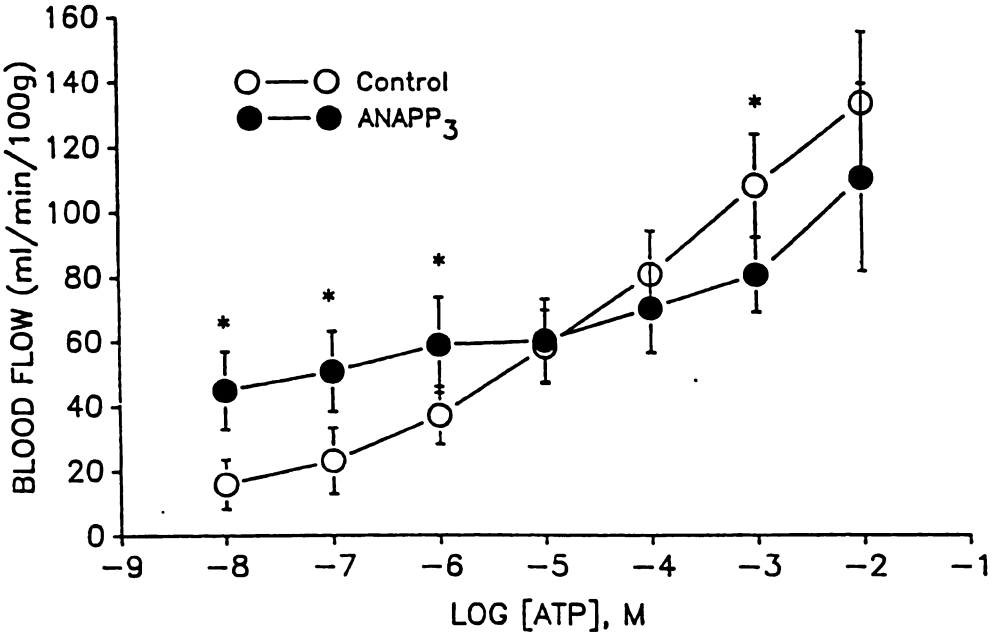

Figure 1. ATP dose response curves before (O) and after (●) ANAPP3. The -LOG M syringe concentration of ATP is shown on the abscissa. ATP was administered in 0.3 ml bolus injections. Data points are means ± S.E. (n=5, except at 10^{-5}M, where n=4). * Significant difference in blood flow after ANAPP3 ($p<.05$).

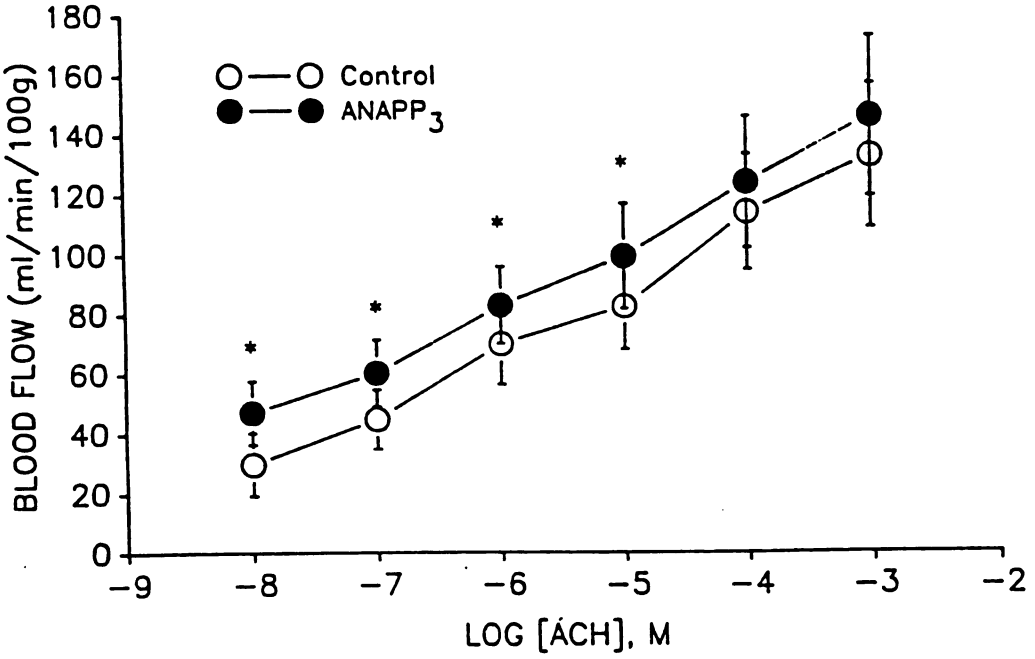

Figure 2. ACH dose response curves before (O) and after (●) ANAPP3. The -LOG M syringe concentration of ACH is shown on the abscissa. ACH was administered in 0.3 ml bolus injections. Data points are means ± S.E. (n=5, except at 10^{-6}M, where n=4). * Significant difference in blood flow after ANAPP3 ($p<.05$).

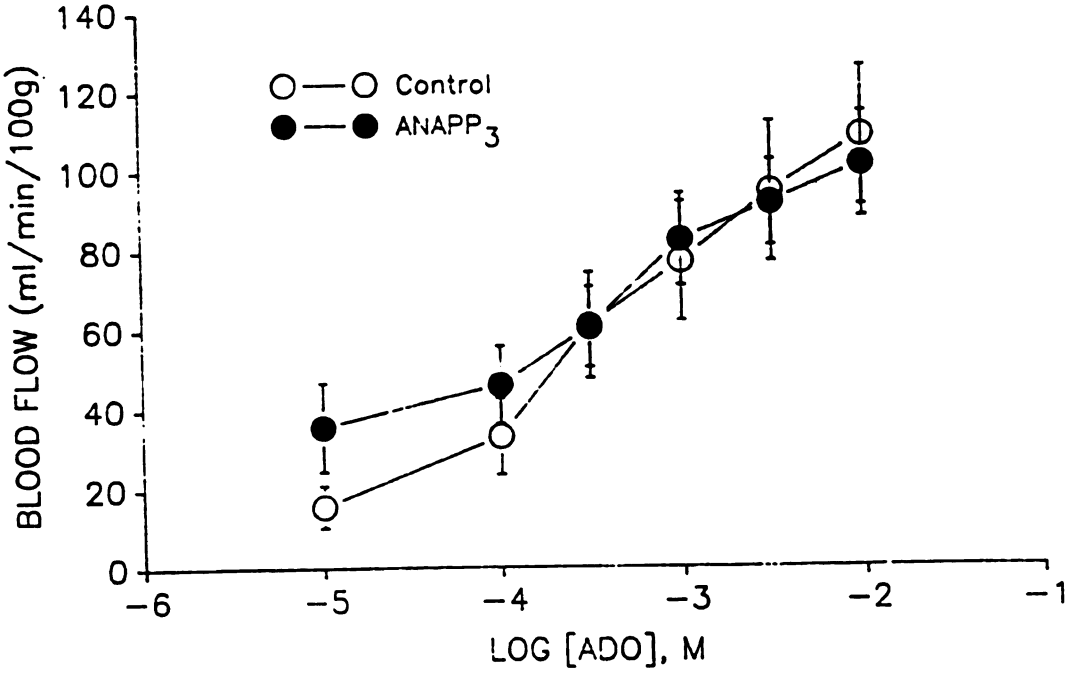

Figure 3. ADO dose response curves before (O) and after (●) ANAPP3. The -LOG M syringe concentration of ADO is shown on the abscissa. ADO was administered in 0.3 ml bolus injections. Data points are means ± S.E. (n=5). No differences in blood flow to ADO were detected after ANAPP3.

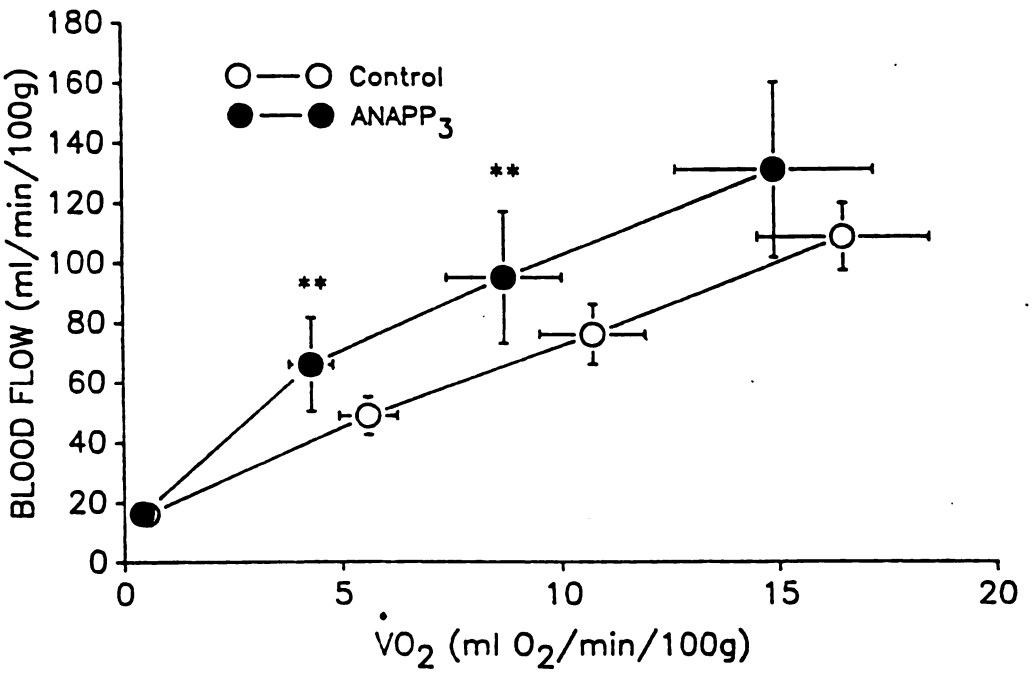

Figure 4. Exercise hyperemia before (O) and and after (●) ANAPP3. The data points are blood flow and oxygen consumption values measured at rest (immediately prior to exercise) and steady state hyperemia at 1.5, 3, and 6 Hz. Values are means ± S.E. (n=5).
**Significant difference in oxygen consumption after ANAPP3 ($p<.05$). No differences in blood flow were detected after ANAPP3.

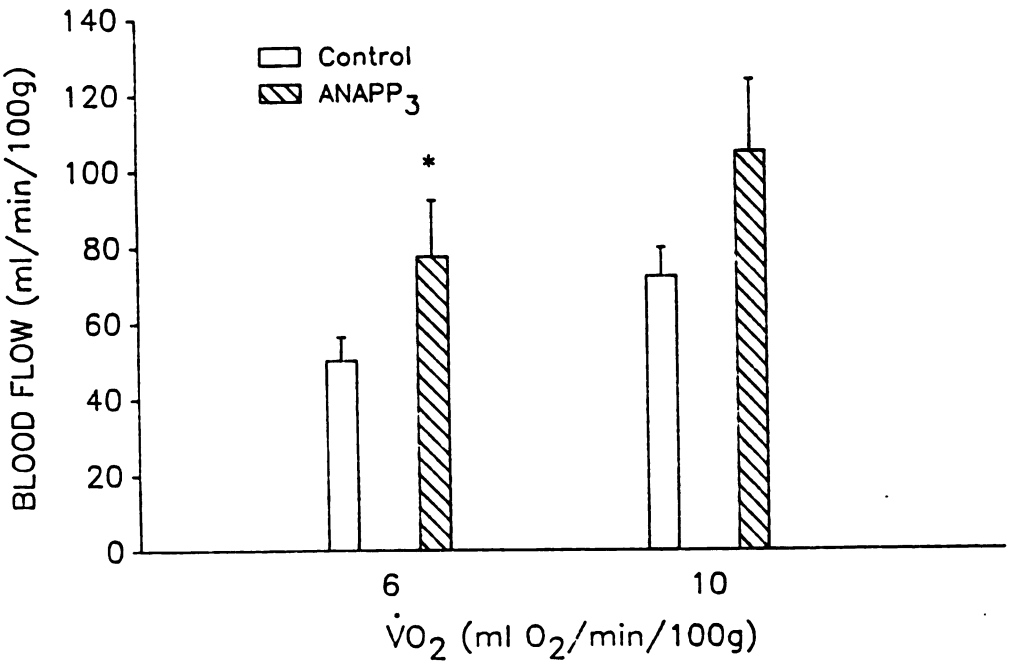

Figure 5. The blood flow response before (open box) and after (hatched box) ANAPP3 at oxygen consumptions of 6 and 10 ml/min/100g. The blood flows were determined from plots of blood flow vs. oxygen consumption by linear interpolation at these 2 values of oxygen consumption. The blood flow values are means ± S.E. (n=5 at V02=6, n=4 at V02=10). * Significant difference in blood flow response after ANAPP3 ($p<.05$).

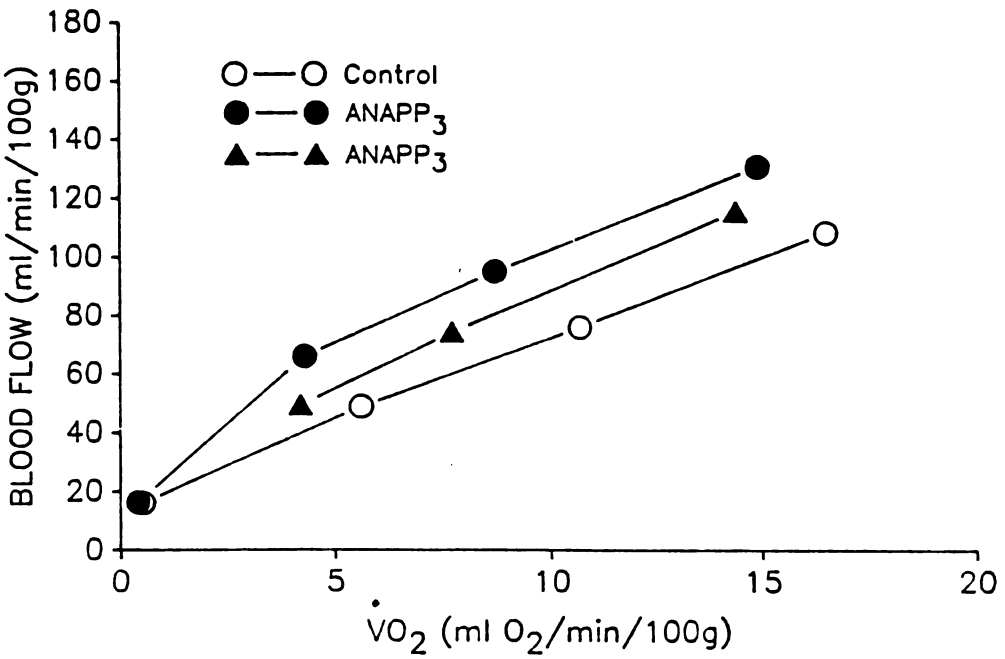

Figure 6. The effect of equalizing blood flows during exercise with ANAPP3. This figure is the same as Figure 4 with the addition of the line with closed triangles that represents the blood flow response and oxygen consumption during exercise with ANAPP3 when blood flow is equalized to the control level.

($p<.05$).

ANAPP$_3$ Time Controls (n=5) Dose response curves for ATP and ACH were done in the presence of saline vehicle and the effect of saline on ATP is depicted in Figure 8. There was no change in the dilator response in the presence of saline vehicle to ATP or ACH (Figure 9), at any dose.

Exercise hyperemia before and after saline vehicle is shown in Figure 10. In response to 1.5, 3, and 6 Hz stimulation, muscle blood flow increased as a function of oxygen consumption and both increased proportionately with the frequency of stimulation. There was no effect of saline vehicle on blood flow or oxygen consumption at rest or any stimulation frequency. However, muscle tension development was attenuated at 1.5 and 3 Hz (Table 1). The blood flow response at oxygen consumptions of 6 and 10 ml 0_2/min/100g was not affected by vehicle saline (Figure 11).

The peak reactive hyperemic response before and after saline is shown in Figure 7. Saline had no effect on this response.

Antazoline (n=5) The blood flow response at baseline and one minute of infusion of ATP, ADO, HIS and ISO is shown in Figure 12. The blood flow response to each of the vasodilators and the baseline blood flows were significantly depressed in presence of antazoline ($p<.05$). When the difference in blood flows between baseline and steady state values were compared, antazoline significantly ($p<.05$) depressed the vasodilator response to ATP (65.1$\pm$19.8 to 39.2$\pm$17.7 ml/min/100g) but not to ADO (42.9$\pm$17.2 to 11.4$\pm$4.9 ml/min/100g), ISO (105.7$\pm$31.5 to

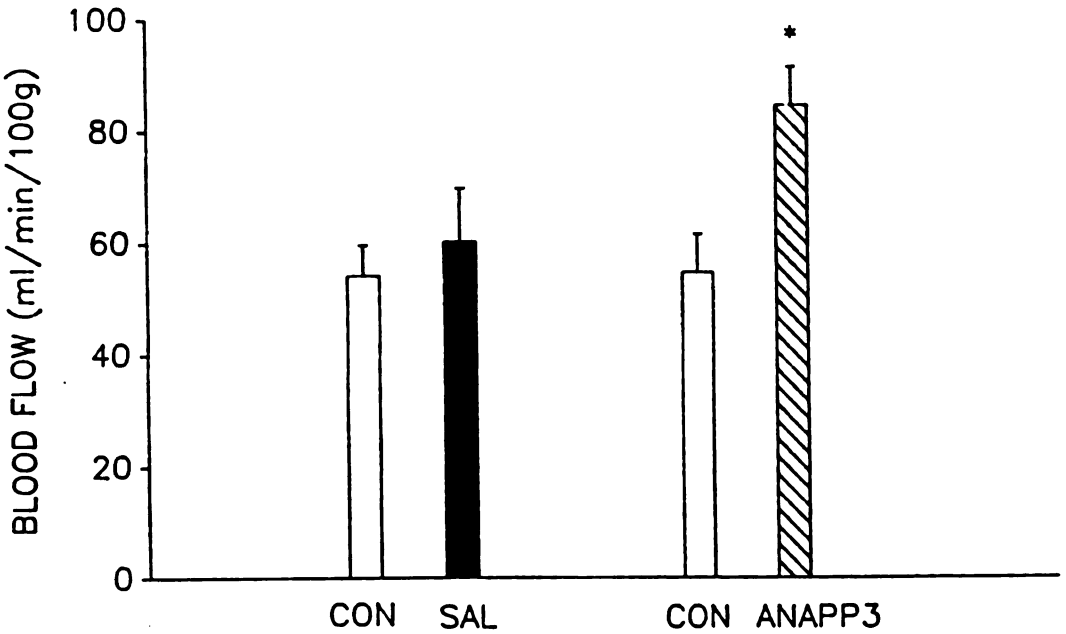

Figure 7. Peak reactive hyperemic response before (control, open boxes) and after saline (filled box) or ANAPP3 (hatched box). The peak vasodilator response to 30 seconds of arterial occlusion was measured. The values are means $\pm$ S.E. (n=5). * Significant difference in hyperemic response after ANAPP3 ($p<.05$), no difference detected after saline.

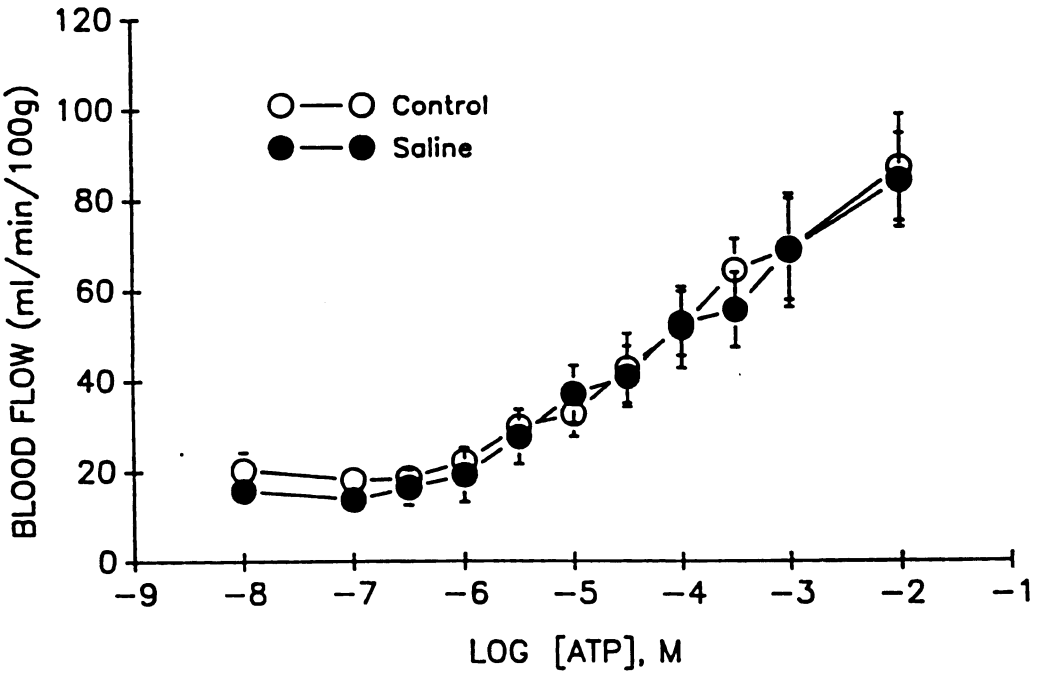

Figure 8. ATP dose response curves before (O) and after (●) saline. The -LOG M syringe concentration of ATP is shown on the abscissa. ATP was administered in 0.3 ml bolus injections. Data points are means ± S.E. (n=5). No differences in blood flows were detected after saline.

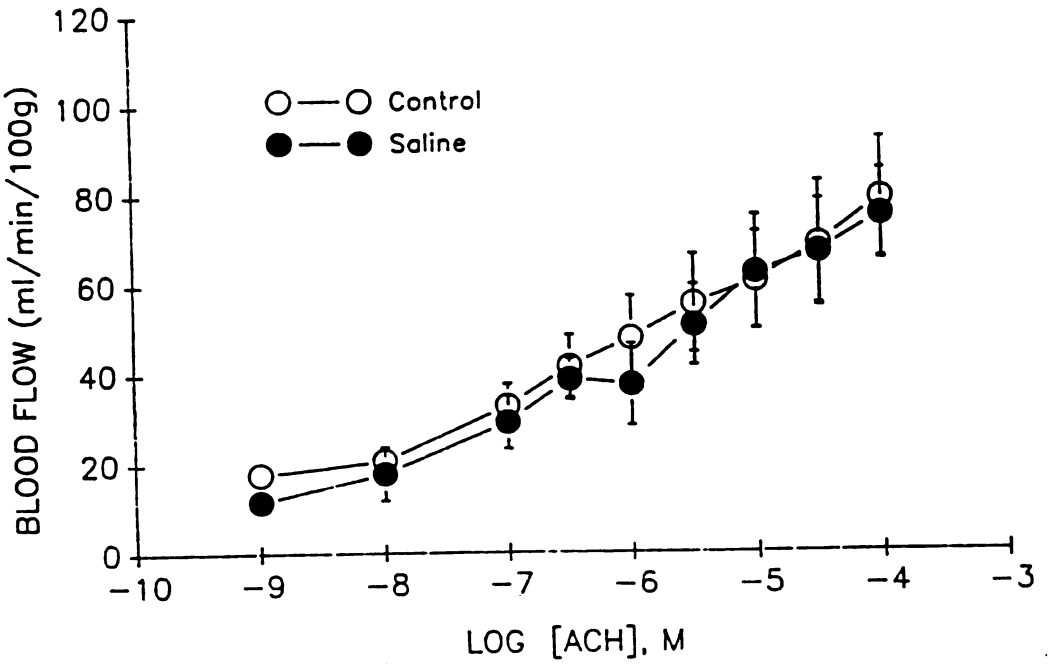

Figure 9. ACH dose response curves before (O) and after (●) saline. The -LOG M syringe concentration of ACH is shown on the abscissa. ACH was administered in 0.3 ml bolus injections. Data points are means ± S.E. (n=5). No differences in blood flow were detected after saline.

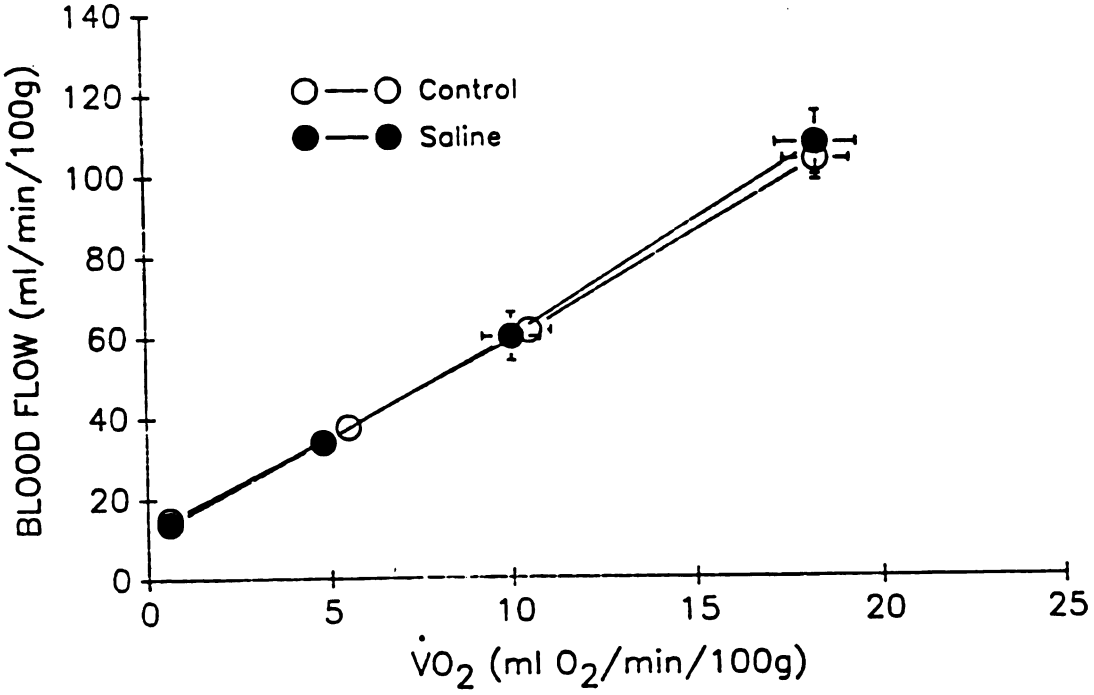

Figure 10. Exercise hyperemia before (O) and after (●) saline. The data points are blood flow and oxygen consumption values measured at rest and steady state hyperemia of 1.5, 3. and 6 Hz. Values are means ± S.E. (n=5). No differences detected in blood flow or oxygen consumption after saline.

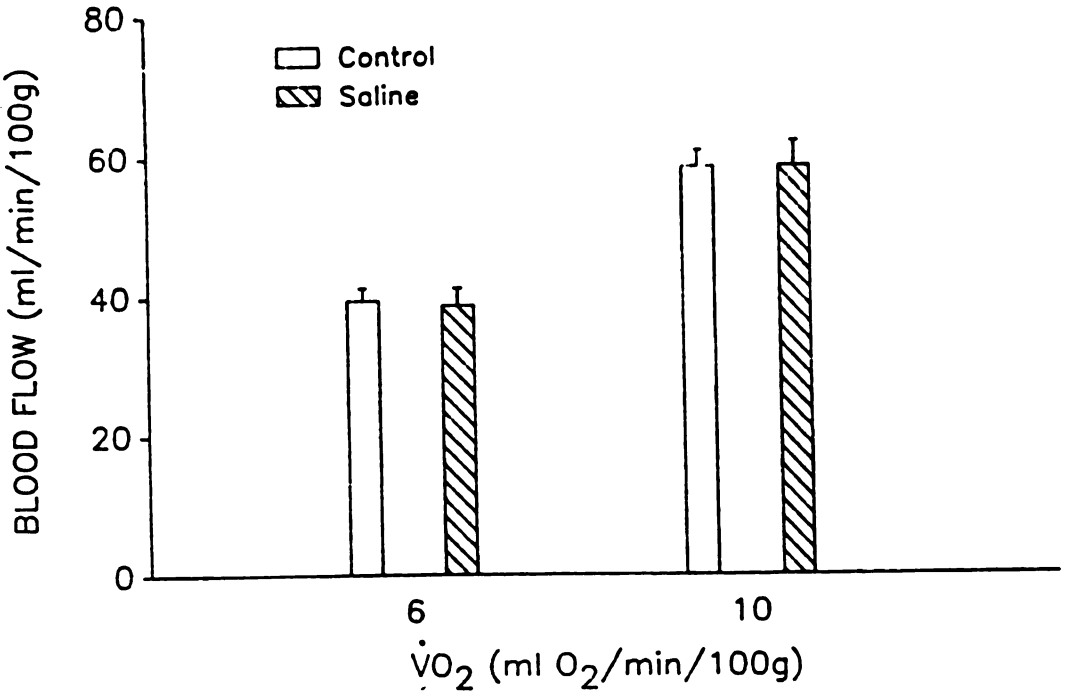

Figure 11. The blood flow response before (open blocks) and after (hatched blocks) saline at oxygen consumptions of 6 and 10 ml/min/100g. The blood flows were determined from plots of blood flow vs. oxygen consumption by linear interpolation at these 2 values of oxygen consumption. The blood flow values are means $\pm$ S.E. (n=5). No differences in blood flow were detected after saline.

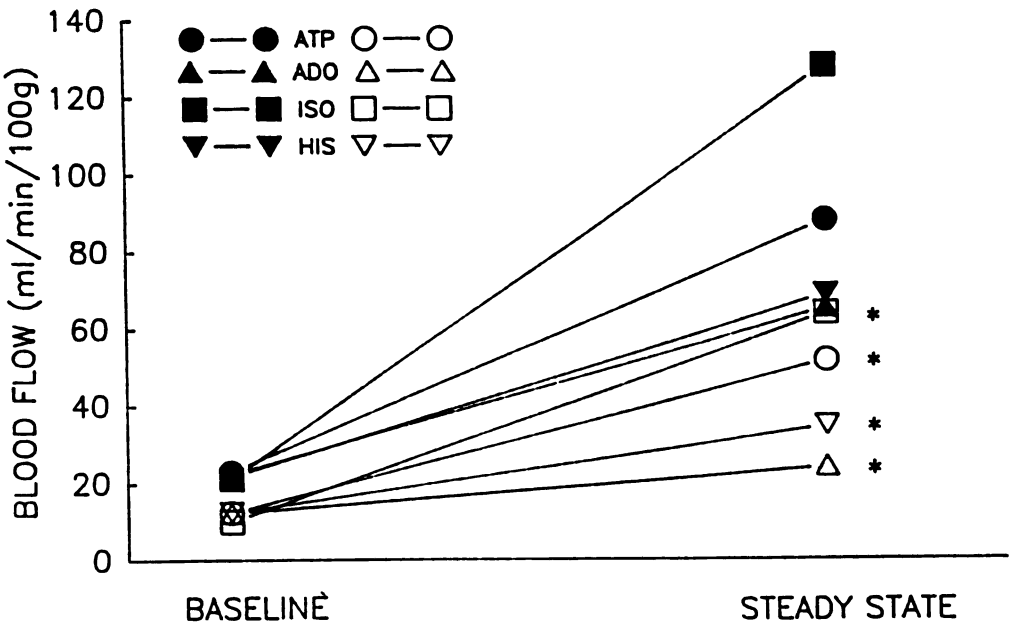

Figure 12. The effect of antazoline on blood flow at baseline (i.e., immediately before agonist infusion) and steady state infusion of ATP, ADO. ISO and HIS. The closed in symbols represent the blood flow before antazoline (control) and the open symbols after antazoline. Values are means $\pm$ S.E. (n=5). * Significant difference in blood flow after antazoline.

52.9±11.3 ml/min/100g), or HIS (47.2±18.6 to 22.3±6.1 ml/min/100g).

Exercise hyperemia is depicted in Figure 13 before and after antazoline. Antazoline had no effect on resting blood flow or oxygen consumption. Antazoline significantly reduced the blood flow response to 2.5 Hz exercise without altering oxygen consumption ($p<.05$). Muscle tension development was not affected by antazoline (Table 1).

ATP Desensitization (n=4) The blood flow response at one minute of infusion of ATP, ADO and ISO before and after desensitization is shown in Figure 14. Desensitization decreased the blood flow response to one minute of infusion of ATP and ADO and decreased baseline blood flows to these agonists ($p<.05$). When the difference in blood flows between baseline and steady state values were compared, desensitization significantly ($p<.05$) depressed the vasodilator response to ATP (39.9±11.0 to 5.2±2.7 ml/min/100g), but not to ADO (31.0±19.7 to 14.1±9.6 ml/min/100g) or HIS (52.4±28.6 to 39.6±28.0 ml/min/100g).

The effect of ATP desensitization on exercise hyperemia is depicted in Figure 15. The blood flow response at rest and 2.5 Hz stimulation was depressed by ATP desensitization ($p<.05$). Oxygen consumption at rest and during stimulation and tension development was not altered by ATP desensitization (Figure 15, Table 1).

Antazoline and Desensitization Time Controls (n=5). Figure 16 shows that baseline and 1 minute steady state blood flows were not significantly altered by saline vehicle, except prior to histamine infusion, where the baseline flow was decreased. When the difference in blood flows between baseline and steady state values were compared,

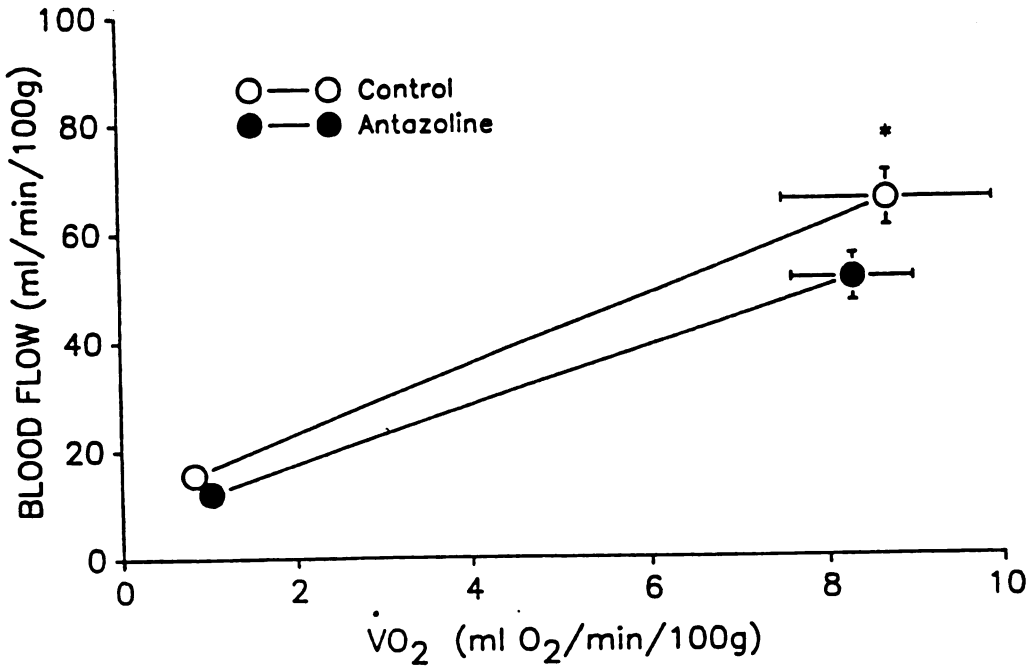

Figure 13. Exercise hyperemia before (O) and after (●) antazoline. The data points are blood flow and oxygen consumption measured at rest and during steady state hyperemia at 2.5 Hz. Values are means ± S.E. (n=5). * Significant difference in blood flow after antazoline ($p<.05$). No difference detected in oxygen consumption after antazoline.

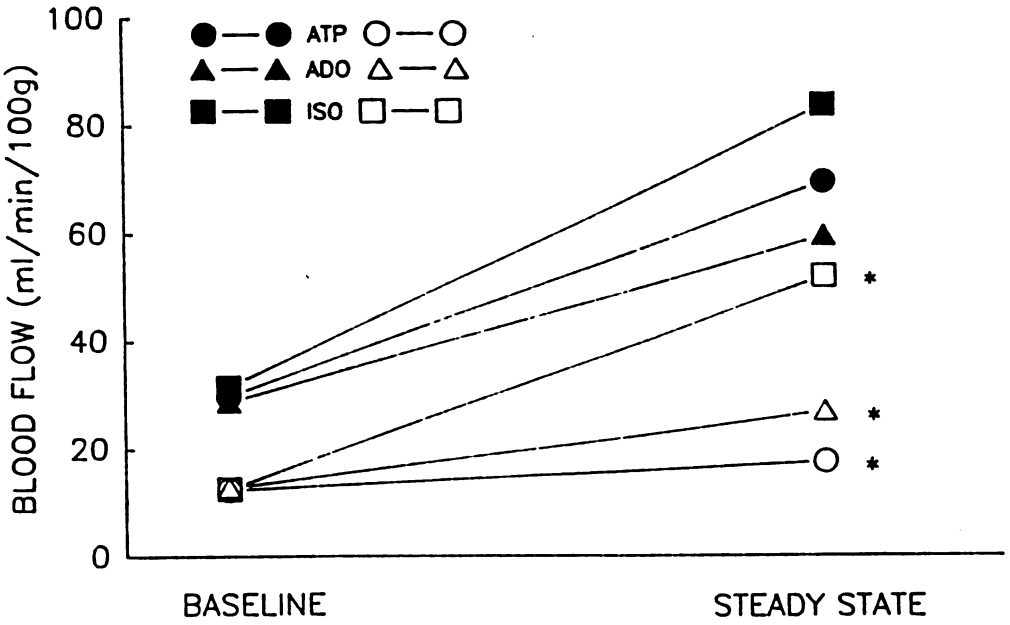

Figure 14. The effect of ATP desensitization on blood flow at baseline (i.e., immediately before agonist infusion) and steady state infusion of ATP, ADO and ISO. The closed symbols represent the blood flows before desensitization (control) and the open symbols after desensitization. Values are means $\pm$ S.E. (n=4 for ATP, n=3 for ADO and ISO). * Significant difference in blood flow after desensitization ($p<.05$).

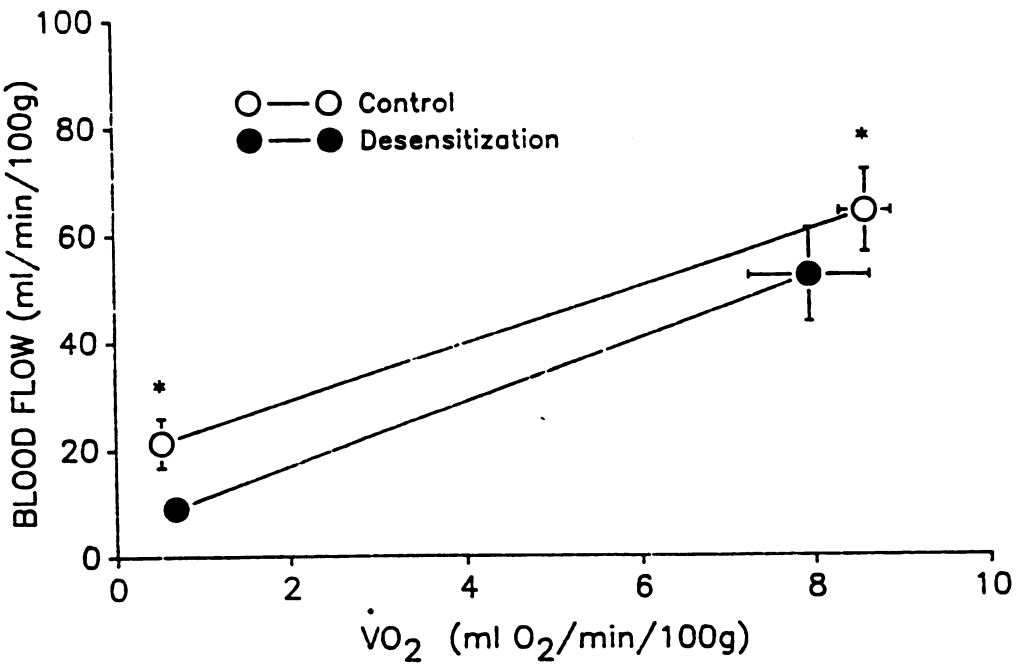

Figure 15. Exercise hyperemia before (O) and after (●) ATP desensitization. The data points are blood flow and oxygen consumption values measured at rest and during steady state hyperemia at 2.5 Hz. Values are means ± S.E. (n=4). * Significant difference in blood flow after ATP desensitization ($p<.05$). No differences detected in oxygen consumption after desensitization.

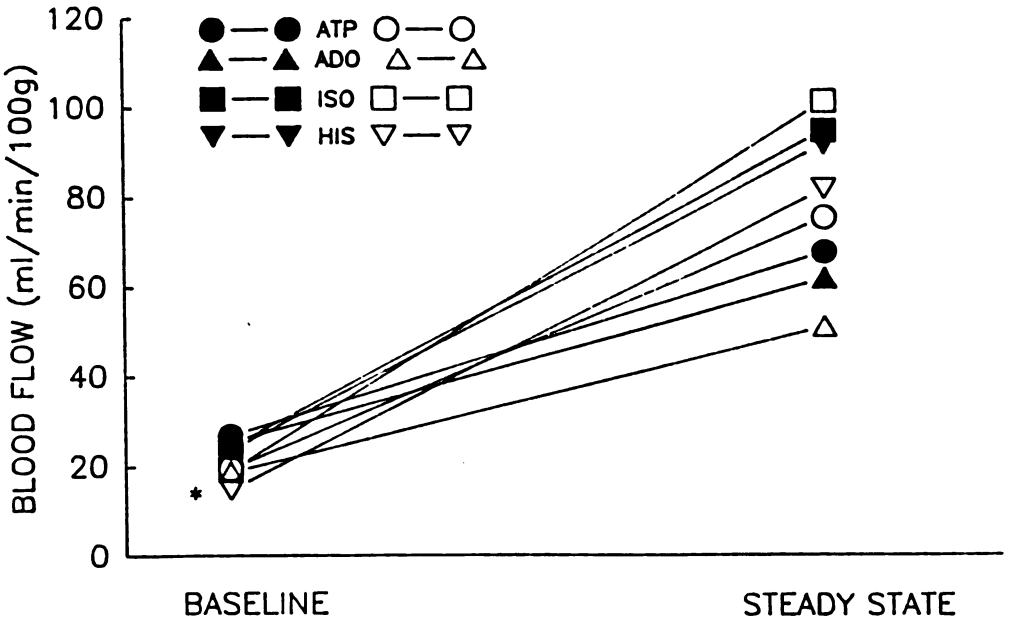

Figure 16. The effect of saline on blood flow at baseline (i.e., immediately before agonist infusion) and steady state infusion of ATP, ADO, ISO, and HIS. The closed symbols respresent the blood flow before saline and the open symbols after saline. Values are means $\pm$ S.E. (n=5). * Significant difference in blood flow after saline ($p<.05$).

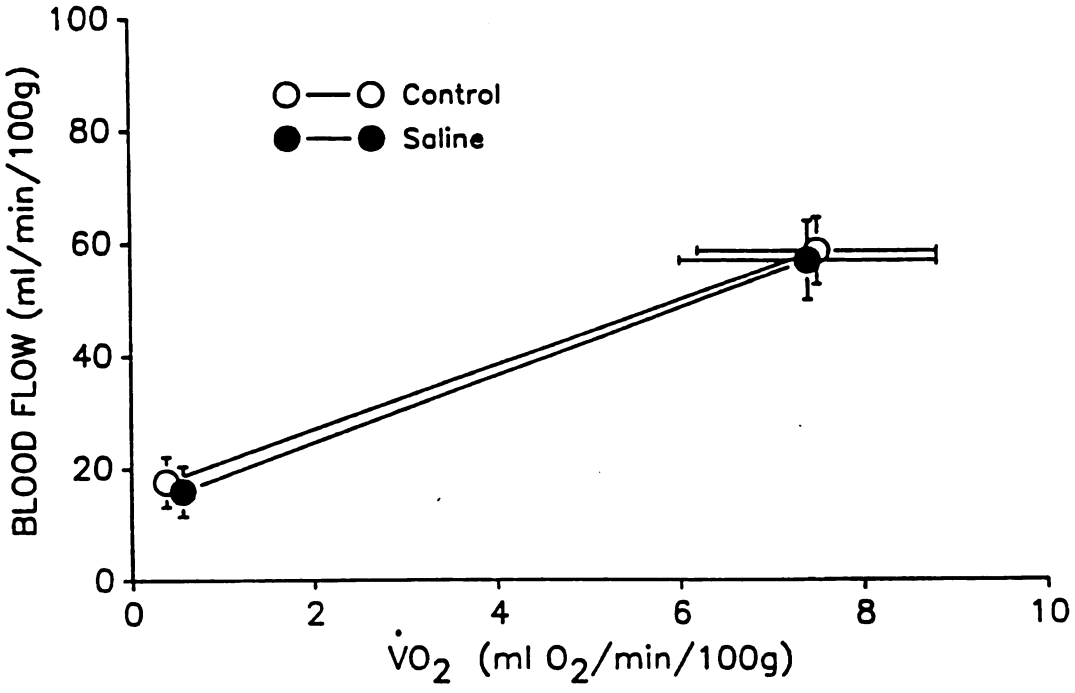

Figure 17. Exercise hyperemia before (O) and after (●) saline. The data points are blood flow and oxygen consumption values measured at rest and during steady state hyperemia at 2.5 Hz. Values are means ± S.E. (n=5). No differences in blood flow or oxygen consumption after saline were detected.

Table 1. Tension development (kg/g) before (i.e., control) and after a treatment.

	Hz			
	1.5	2.5	3.0	6.0
Control	49.5±5.7	54.9±5.1	52.6±5.3	48.9±5.3
Saline	44.1±4.9*	54.1±5.3	49.2±5.4*	45.6±4.4
Control	------	51.9±9.0	------	------
Antazoline	------	51.1±9.5	------	------
Control	------	55.5±16.4	------	------
Desensit.	------	56.9±17.2	------	------
Control	59.2±4.9	------	62.7±5.1	53.8±5.4
ANAPP3	54.3±5.1*	------	55.1±5.8*	44.5±6.7*
Control	58.6±7.1	------	61.6±6.9	54.9±5.0
MB	38.8±7.7*	------	44.3±7.7*	36.8±5.3*
Control	57.3±8.7	------	55.6±6.9	46.5±5.5
Ethanol	50.2±7.7*	------	53.9±7.8	46.9±5.5
Control	59.2±3.4	------	60.2±3.2	51.0±2.7
ETYA	54.7±2.7*	------	57.4±2.8*	47.8±6.0

The control responses are those values obtained prior to administering a treatment or saline vehicle. Dotted lines within the table indicate that tests were not conducted at these frequencies. Values are means ± S.E., n=5. *Significant difference in tension development between control and treatment ($p<.05$).

there was no affect of saline on the vasodilator response to ATP, ADO, ISO or HIS (data not shown).

The effect of saline vehicle on exercise hyperemia is shown in Figure 17. There was no effect of saline vehicle on blood flow or oxygen consumption at rest or during 2.5 Hz muscle stimulation. There was no change in tension development (Table 1).

Discussion

We observed a dose-dependent vasodilation to ATP given intra-arterially to the blood-perfused canine hindlimb. Exogenously administered ATP elicits vasodilation in the skeletal muscle vasculature of other species as well (Shimada and Stitt, 1984; Forrester, 1966; Duff et al., 1954; and Folkow, 1949). In vitro, ATP has a "dual" effect on isolated segments of large arteries: relaxation if the endothelium is intact and contraction if the endothelium is removed (Burnstock and Kennedy, 1986). In almost every blood vessel tested, the relaxation response to ATP has been demonstrated to be endothelium-dependent (Burnstock and Kennedy, 1986). The one exception is the rabbit mesentary where ATP acts directly on smooth muscle to produce relaxation (Mathieson and Burnstock, 1985). ATP endothelium-dependent relaxation occurs in large arteries, in vitro, (DeMey et al., 1982; Kennedy et al., 1986; and White et al., 1985) and in resistance vessels, in vivo (Pohl et al., 1987). It is likely that intraarterial ATP is producing vasodilation in our preparation via an endothelial-dependent pathway since this is true for almost every tissue tested, including the canine femoral artery (DeMey et al., 1982). In addition we have shown that ATP's vasodilator effect in

canine skeletal muscle is antagonized by methylene blue, lending further support to an endothelial cell mediated effect (Chapter III).

We used $ANAPP_3$ to inhibit ATP vasodilation in our preparation. In 1980 Hogaboom provided evidence that $ANAPP_3$ specifically inhibited ATP receptors in guinea pig vas deferens (Hogaboom et al., 1980). $ANAPP_3$ is a photoaffinity labelled ATP molecule that is activated in the presence of ultraviolet or visible light to bind covalently with ATP receptors (Fedan et al., 1985). $ANAPP_3$ that is not photolyzed also inhibits ATP responses. In guinea pig smooth muscle, the contractile response to ATP but not potassium chloride, norepinephrine, HIS or ACH was inhibited by nonphotolyzed $ANAPP_3$. This effect is reversible if $ANAPP_3$ is removed (Hogaboom et al., 1980). Nonphotolyzed $ANAPP_3$ is also a reversible agonist-antagonist in situ, when administered intraarterially to the cat urinary bladder (Theabold, 1983). An initial contraction (agonist) response is followed by ATP antagonism when $ANAPP_3$ is given in situ, without photolysis (Fedan et al., 1985). Fedan et al. (1985) and Hogaboom et al. (1980) have suggested that nonphotolyzed $ANAPP_3$ is either a competitive antagonist of P2 receptors or that $ANAPP_3$ produces receptor desensitization similar to ATP. Fedan et al. (1985) suggested that at least part of the antagonism is competitive because nonphotolyzed $ANAPP_3$ is more effective than ATP in reducing ATP responses and the agonistic potency of the two are equal. Cassis et al. (1987) used nonphotolyzed $ANAPP_3$ to reduce ATP but not methacholine relaxation in the rabbit femoral artery, in vitro. ATP relaxation was shown to be endothelial-cell dependent in this preparation. These results provide evidence that nonphotolyzed $ANAPP_3$ can selectively inhibit endothelial ATP P2-purinoceptors and

ATP-mediated relaxation in the vasculature.

In our preparation, nonphotolyzed ANAPP3 increased the vasodilator response to low doses of ATP. One possible explanation for this result is that $ANAPP_3$ was acting as a partial agonist-antagonist. If it was acting as a partial agonist-antagonist, then the agonist effect (i.e., vasodilation) would be expected to occur under baseline conditions, in addition to during ATP dosing. There was a small, transient vasodilation when $ANAPP_3$ was initially infused but blood flow returned to baseline values within approximately 2 minutes. Therefore, nonphotolyzed $ANAPP_3$ is most likely acting as a competitive ATP antagonist, and not as a partial agonist-antagonist. Another possible explanation for the increase in vasodilator response is that the net effect of ATP is an algebraic sum of an endothelial cell-mediated vasodilation and a vascular smooth muscle-mediated vasoconstriction. $ANAPP_3$ may inhibit the vasoconstriction at low doses of ATP. In the isolated rat aorta, in the presence of endothelial cells, ATP elicited vascular smooth muscle contraction at low doses and relaxation at high doses (White et al., 1985). Therefore, even in the presence of endothelium, ATP can elicit vasoconstriction. This was also true in the isolated rat femoral artery where ATP-induced contraction and relaxation both occurred when the endothelium was present and the vessel was pre-contracted with noradrenaline (Kennedy et al., 1985). Our data suggest that an ATP-induced vasoconstrictor influence may occur simultaneously with the observed vasodilation and that $ANAPP_3$ inhibits the vasoconstriction at low doses of ATP. The differential effect of $ANAPP_3$ at low as compared to higher doses of ATP may reflect a difference in P_{2x} and P_{2y} receptor affinities for $ANAPP_3$. We are not

aware of any studies that have looked at the affinity of nonphotolyzed $ANAPP_3$ for these two receptors. Another possibility for the differential effect at low and high doses of ATP is the availability of ATP to the receptors. When ATP is administered to our blood-perfused muscle, the quantity of ATP reaching the P_{2x} receptors on vascular smooth muscle will be less than the quantity of ATP exposed to endothelial P_{2y} receptors because ATP is rapidly degraded by endothelial ectonucleotidases (Pearson and Gordon, 1985). This may explain why $ANAPP_3$ is a more efficacious antagonist of P_{2x} receptors than P_{2y} receptors at lower doses of ATP. Thus, we think that nonphotolyzed $ANAPP_3$ antagonized both P_{2x}- and P_{2y}-purinoceptors in our preparation. We suggest that exogenous ATP produces a dual effect in blood-perfused, resistance vessels, as described by Burnstock and Kennedy (1986) for larger blood vessels, with vasodilation being the dominant response.

The vasodilator response to 10^{-3}M ATP was reduced in the presence of $ANAPP_3$. The inhibition of vasodilation was selective for ATP. $ANAPP_3$ had no effect on vasodilations produced by ADO and increased the vasodilations produced by ACH. Therefore, it is likely that $ANAPP_3$ inhibited P_{2y} receptor-mediated vasodilation to ATP in our preparation. In one study that tested the effect of $ANAPP_3$ on ATP receptors in the vasculature, photo-activated $ANAPP_3$ failed to inhibit the relaxation response to ATP in rat aortic strips, although it did inhibit contractions induced by the ATP analogue, alpha, beta-methyleneATP (White et al., 1985). There are several differences in our preparation versus the one used by White et al. that might account for this difference in susceptability to $ANAPP_3$ inhibition. For example, we

used nonphotolyzed $ANAPP_3$ and studied blood-perfused canine resistance vessels whereas they used photolyzed $ANAPP_3$ and strips of rat aorta, in vitro. However, our results in vivo are in agreement with those of Cassis et al. (1987) in the rabbit femoral artery, that nonphotolyzed $ANAPP_3$ selectively inhibits P2-purinergic relaxation in the vasculature. However, at low concentrations of ATP the main effect of nonphotolyzed $ANAPP_3$ is to inhibit P2-purinergic contraction in our preparation.

The vasodilator action of ACH was increased in the presence of $ANAPP_3$ in the present study. This suggests some interaction between ACH vasodilation and ATP. Other investigators have observed that ACH can stimulate ATP release. ACH infused into the guinea pig myocardium stimulated release of ATP and an increase in coronary flow (Schrader et al., 1982). ATP release was measured from the post synaptic membrane of the Torpedo electric organ in response to ACH stimulation (Israel et al., 1976). ACH can also initiate release of ATP from isolated myenteric varicosities (White, 1985). There is convincing evidence that ATP is a cotransmitter with norepinephrine in the perivascular sympathetic neurons (Burnstock and Kennedy, 1985; Bevan and Brayden, 1987; Su, 1985). One possible explanation for our results is that exogenous ACH stimulates release of ATP from perivascular adrenergic neurons. When ACH is infused intraarterially, it produces not only the observable vasodilation but also induces ATP release from perivascular nerves, which has a vasoconstrictor influence which is normally masked by ACH-induced vasodilation. In the presence of the P_{2x}-purinoceptor antagonist $ANAPP_3$, ATP vasoconstriction is inhibited and therefore, the vasodilator response to ACH is increased.

In every experiment we observed a tendency for the blood flow response to muscular contraction to be increased in the presence of $ANAPP_3$. Despite increased flow, muscle oxygen consumption was reduced by $ANAPP_3$ at stimulation frequencies of 1.5 and 3 Hz. Since blood flow increased, the lowered oxygen consumption was reflected in a decrease in arterial-venous oxygen content difference. This decrease in oxygen content difference could be due to $ANAPP_3$ adversely affecting the muscle's ability to extract oxygen or that the vasodilator effect of $ANAPP_3$ increases oxygen delivery so that less oxygen is extracted. To determine if $ANAPP_3$ was affecting the muscle's ability to remove oxygen from blood, we intentionally decreased blood flow during exercise with $ANAPP_3$ infusion to levels observed during control exercise. In response to the lowering of blood flow to control levels, oxygen extraction increased, which indicates that part of the depression of consumption was due to the elevated blood flow. However, oxygen consumption was still depressed at 1.5 and 6 Hz after equalizing blood flows. This indicates that in the presence of $ANAPP_3$, muscle oxygen consumption was directly inhibited. These results suggest that $ANAPP_3$ decreased oxygen consumption by two mechanisms, a vasodilator effect and a direct inhibitory effect on muscle oxygen consumption.

Tension development was significantly depressed in the presence of $ANAPP_3$ at all three stimulation frequencies; this is compatable with a direct effect of $ANAPP_3$ on muscle oxygen consumption. The decrease in tension development may not be a completely reliable indicator of $ANAPP_3$'s effect because tension development was also decreased at 1.5 and 3 Hz by saline vehicle. However, $ANAPP_3$ decreased tension at all 3 frequencies and decreased oxygen extraction and consumption, while

saline did not have these effects. Thus, we believe that $ANAPP_3$ decreased muscle oxygen consumption by a direct action on the muscle.

Since there is a linear relationship between oxidative metabolism and blood flow (Haddy and Scott, 1968; Barcroft, 1972) and oxygen consumption was decreased at 1.5 and 3 Hz, comparing blood flows before and after $ANAPP_3$ at 1.5 and 3 Hz is not a complete test of the effect of $ANAPP_3$ on exercise hyperemia. Therefore, we assumed a linear relationship between blood flow and oxygen consumption and calculated blood flow at 2 levels of oxygen consumption before and after $ANAPP_3$. The blood flow response in the presence of $ANAPP_3$ was significantly increased at an oxygen consumption of 6 ml O_2/min/100g. This result suggests that $ANAPP_3$ blocks vasoconstrictor receptors during exercise, most likely the P_{2x}-purinoceptors. Thus, endogenous release of ATP during exercise may limit the blood flow response. Most studies have linked ATP with a vasodilator action during exercise. For example, the venous concentration of ATP and blood flow both increased during human forearm exercise at 10% maximum voluntary contraction (mvc) (Forrester and Lind, 1969; Lind and McNicol, 1967). However, during 20% mvc and during the post exercise period for both stimulus levels, blood flow and ATP levels were inversely related. The results from these experiments suggest that elevated venous concentration of ATP may correlate better with a fall in blood flow, not an increase. Thus, other studies may actually support our contention that ATP acts predominately as a vasoconstrictor during exercise.

A transient increase in muscle blood flow occurs after the blood supply to an organ has been occluded; this is termed reactive hyperemia (Barcroft, 1972). A myogenic mechanism, vasodilator metabolites, and

arterial wall hypoxia are thought to contribute to the peak hyperemic response (Barcroft, 1972; Johnson and Henrich, 1975; Sparks, 1978). Prostaglandin release is responsible for some of the vasodilation (Beaty and Donald, 1979; Kilbom and Wennmalm, 1976), but other metabolites have not been identified. Since ATP is a potent vasodilator, we considered it a candidate for mediating peak reactive hyperemia. In the presence of an ATP receptor antagonist $ANAPP_3$, we found that peak reactive hyperemia was increased. No ATP was measurable in the venous plasma of the human forearm after 4 minutes of arterial occlusion (Forrester, 1972) or in the venous plasma from canine hindlimb after 5 minutes of arterial occlusion (Dobson et al., 1971). Intracoronary infusions of ATP did not produce a peak blood flow response equivalent to 20 seconds of reactive hyperemia in the canine myocardium, without also affecting heart rate and contractility (Moir and Downs, 1972). Therefore, it seems that ATP does not mediate the peak hyperemic response and possibly decreases or limits reactive hyperemia.

Antazoline at a dose of 5 mg/kg inhibited vasodilation in response to ATP in rabbit skeletal muscle, although it also inhibited vasodilation to HIS and ADO (Shimada and Stitt, 1984). In our preparation, antazoline at a dose of 4 mg/kg reduced the vasodilator response to HIS, ISO, ADO and ATP. Higher doses of antazoline depressed tension development and lower doses were less efficacious in reducing ATP vasodilation. When the differences in blood flow between baseline and steady state levels were compared, antazoline significantly decreased the vasodilator response to ATP, but not to ADO, HIS or ISO. However, there was a definite tendency for depression

of the dilator response to these agonists with antazoline treatment. Antazoline is not therefore, a specific P-2 purinergic antagonist in our preparation or the rabbit hindlimb.

At 2.5 Hz exercise antazoline reduced blood flow for a given metabolic rate. Oxygen consumption was not affected and therefore oxygen extraction must have increased. The decrease in blood flow was not due to a depression of muscle contractility because there was no effect on tension development. Thus, antazoline reduced exercise hyperemia to a degree and in a manner that would be consistant with inhibiting a vasodilator pathway(s).

In previous studies it was demonstrated that antihistamines did not affect the decrease in vascular resistance of constant flow exercise (Daniel and Honig, 1980) or the vasodilator response to tetanic contractions (Powell and Brody, 1976). The exercise conditions utilized in our experiments were much different than in these studies. We did not restrict flow during exercise, we used twitch contractions and we measured steady state blood flow values. Ours is the first study in which an antihistamine was tested during free flow steady state exercise. Antazoline significantly reduced exercise hyperemia and the flow response to HIS, thus we cannot rule out HIS as a possible mediator of steady state free flow exercise hyperemia.

Repeated administration of ATP decreased the vasodilator response to ATP and the blood flow response to 2.5 Hz exercise. However, desensitization was nonspecific, as ADO and ISO vasodilator responses were also decreased. Therefore no conclusions can be drawn concerning the role of ATP in exercise from the ATP desensitization experiments.

Summary

We have demonstrated that nonphotolyzed $ANAPP_3$ selectively inhibits ATP vasodilation, as it does not reduce the vasodilator response to ADO or ACH. In the presence of nonphotolyzed $ANAPP_3$, the blood flow response to exogenous ATP is increased at low doses of ATP and decreased at higher doses. We suggest that nonphotolyzed $ANAPP_3$ blocks both P_{2x}- and P_{2y}-purinoceptors in the blood-perfused canine hindlimb. We have demonstrated that exogenous ATP produces a concentration-dependent vasodilation and possibly, a vasoconstriction that is masked by the vasodilation. The increase in the vasodilator response in the presence of $ANAPP_3$ elicited a decrease in oxygen extraction that was partially returned to control levels when blood flows were equalized during exercise. In the presence of $ANAPP_3$ the blood flow response during muscle contraction is increased at an oxygen consumption of 6 ml O_2/min/100g. Since the blood flow response is increased, not decreased in the presence of a selective ATP antagonist, ATP does not mediate vasodilation and may instead, mediate vasoconstriction during exercise. ATP appears to limit the peak vasodilator response following arterial occlusion. Therefore, ATP contributes a vasoconstrictor influence to two important physiological processes, exercise and reactive hyperemia.

From the results of the present study we conclude that both antazoline and ATP receptor desensitization were nonspecific in inhibiting ATP vasodilation, although antazoline inhibited exercise hyperemia. If the mechanism of action of antazoline to inhibit exercise hyperemia was determined, we might better understand what mediates the increase in blood flow.

III. The Contribution of Endothelium-Dependent Vasodilation To Exercise Hyperemia

Introduction

The relaxation response to a number of vasodilators has been demonstrated to depend on the presence of an intact endothelium (Vanhoutte et al., 1986; Furchgott, 1983). Endothelium-dependent relaxation has been observed mainly in large arteries, under in vitro conditions (Furchgott, 1983; Vanhoutte et al., 1986). In most vascular beds, arteries <100 um in diameter are thought to provide the greatest resistance to blood flow (Renkin, 1984; Fronek and Zweifach, 1975). Recently, investigators have studied endothelium-dependent vasodilation in resistance vessels. Pohl et al. (1987) showed that in the rabbit hindlimb, the vasodilator response to 3 doses of acetylcholine (ACH) and 1 dose of adenosine 5'-triphosphate (ATP) was depressed after administration of gossypol, which inhibits endothelium-dependent relaxation. In the rabbit ear artery in vitro, there was an inverse relationship between arterial diameter and endothelium-dependent relaxation to ACH in arteries ranging from 75 to 300 um in diameter (Owen and Bevan, 1985). In rat cremaster arterioles (Kaley et al., 1986) and in pial arterioles of the mouse brain (Watanable et al., 1988) endothelium-dependent vasodilation was demonstrated in vivo by microscopy. Endothelium-dependent vasodilation to ACH has also been demonstrated in human subcutaneous (Aalkjaer et al., 1987), rabbit mesenteric (Furchgott et al., 1987) and rat hindlimb (Kaley et al., 1986) resistance vessels. Endothelium-dependent relaxation was demonstrated in vivo to ACH in the blood perfused canine

femoral artery (Cocks and Angus, 1983; Kaiser et al., 1986). Thus, there is sufficient data to suggest that this process occurs in resistance vessels and in vivo.

The functional significance of endothelium-dependent vasodilation has not yet been fully demonstrated. If endothelium-dependent vasodilation occurs in resistance vessels, then this process could contribute to physiological regulation of blood flow. Vasodilation in resistance vessels contributes significantly to increased blood flow during exercise (Mohrman and Sparks, 1973; Barcroft, 1972; Haddy and Scott, 1968; Granger et al., 1976). The decrease in resistance in response to exercise is thought to be mediated locally, by a signal that links metabolism to blood flow: vasodilator metabolites (Shepherd, 1983; Haddy and Scott, 1968; Sparks, 1978). No single vasodilator has been found to account for the steady state hyperemia that occurs with free flow exercise (Shepherd, 1983; Sparks, 1978). Therefore, it seems likely that free flow exercise hyperemia depends on several vasodilators. It is possible that some vasodilator metabolites elicit vascular smooth relaxation through a final common pathway such as endothelium-dependent vasodilation. If endothelium-dependent vasodilation is a final common pathway for vasodilator metabolites that contribute to exercise hyperemia, then inhibition of this pathway should reduce exercise hyperemia.

We tested the hypotheses that (1) endothelium-dependent vasodilation occurs in blood perfused resistance vessels of the canine hindlimb in vivo, (2) endothelium-dependent vasodilation contributes significantly to steady state free flow exercise hyperemia (3) endothelium-dependent vasodilation contributes to peak reactive

hyperemia in the cainine hindlimb. Since endothelial cells of resistance vessels cannot be mechanically removed in our preparation, we inhibited endothelial-cell dependent vasodilation pharmacologically, with methylene blue (MB) and 5,8,11,14-eicosatetraynoic acid (ETYA). These two agents inhibit ACH-induced endothelium-dependent relaxation in the blood perfused canine femoral artery (Kaiser et al., 1986) and endothelium-dependent relaxation in vitro (Holzman, 1982; Gruetter and Lemke, 1986; Ignarro et al., 1986; Ignarro et al., 1984; DeMey et al., 1982; Furchgott, 1981; Furchgott, 1983; Rapoport and Murad, 1983; Peach et al., 1983; Martin et al., 1985) and in vivo (Kaley et al., 1986).

Methods

Twenty mongrel dogs of either sex, weighing 14.1 to 37.3 kg were anesthetized with pentobarbital (30 mg/kg, i.v.) and supplemented hourly (50 mg). The dogs were intubated and mechanically respired (Harvard apparatus) with room air, supplemented with 100% O_2. Arterial blood gases were monitored hourly (Corning Model 165/2, blood gas analyzer). Tidal volume and respiratory rate were set to maintain arterial PCO_2 between 30-42 mmHg and PO_2 between 90-110 mmHg. Intravenous sodium bicarbonate (150mM) was administered to correct for metabolic acidosis. Esophageal temperature was monitored (Yellow Springs Instrument)and maintained at 37-39°C by heating pads.

The circulation of the gastrocnemius-plantaris muscles of the left hindlimb was surgically prepared as previously described (Stainsby et al., 1956). All branches of the femoral artery and vein not directly supplying the gastrocnemius-plantaris muscles were ligated.

The achilles tendon was severed and the distal end attached to a

specially adapted, isometric force transducer (Grass) used to measure tension development. A nail was positioned laterally in the distal femur and clamped to a rigid support to stabilize the joint. The sciatic nerve was sectioned and the distal end of the nerve placed on bipolar platinum electrodes which were fitted in a small, plexiglass container filled with saline. The nerve was insulted with petroleum jelly and the container wrapped with saline soaked gauze to prevent drying.

The animals were anticoagulated with heparin sodium (1000 U/kg, i.v.) and supplemented hourly (250 U/kg). The femoral artery was cannulated 2-3 cm proximal to where this artery enters the popliteal space and the muscle was perfused with blood from the contralateral femoral artery. Perfusion pressure was held constant at 105 mmHg by a servo-controlled pump (Mohrman, 1980) and continuously measured just proximal to the muscle cannula. Systemic arterial pressure was measured by tapping into the perfusion tubing proximal to the servo pump. Muscle blood flow was monitered with an electromagnetic flow probe (3mm I.D., cannulating, Zepeda Instruments) and SWF-4 flowmeter (Zepeda Instruments). At the end of the experiment the flow probe was calibrated by timed collection of blood.

A shunt consisting of 2 polyethylene cannulas connected by silastic tubing was positioned so that all the venous effluent draining the muscle was directed into the ipsilateral vein, at the level of the femoral triangle. The shunt provided a venous sampling site. A Y-connector situated proximal to the servo pump provided a sampling port for arterial blood. Approximately 10 ml/min of arterial and venous blood were continuously withdrawn (via a roller pump) for

measurement of arterial and venous oxygen content difference (A-VOX Systems). The A-VOX calibration was checked against the a-v O_2 content difference calculated from blood gas analysis. Values for pH, pO_2, and hematocrit were obtained and oxygen content calculated by: O_2 content = $\%O_2$ saturation x hemoglobin concentration. Oxygen content measurements ignore the small percentage (0.3%) of oxygen dissolved in blood. The $\%O_2$ saturation was determined by a nomogram from pO_2 and pH (Rossing and Cain, 1966). Hemoglobin was calculated from the hematocrit: Hb = 0.375 x hematocrit - 1.19. Oxygen consumption (VO_2) was calculated by: VO_2 (ml O_2/min/100g) = blood flow (ml/min/100g) x a-v O_2 content (ml O_2/ml blood).

Muscle contraction was produced by square wave pulse stimulation of the sciatic nerve. Optimal muscle length for maximum tension development was determined before each experiment and tension development was monitered. Stimulus parameters for twitch exercise were 0.2msec, supramaximal voltage (4-6 volts) and frequencies of 1.5, 3 and 6 Hz. These parameters are below the threshold for stimulation of sympathetic nerves (Morganroth, et al., 1975; Thompson and Mohrman, 1983).

Systemic arterial pressure, muscle perfusion pressure, blood flow, arterial-venous oxygen content difference, and tension development were continuously recorded on a Grass Polygraph (Model 7).

Experimental Protocol

Prior to the start of the experiment a 3 minute warm-up exercise was performed, at 2 Hz stimulation, followed by approximately 20 minutes of recovery and stabilization.

Following the warm up exercise, dose response curves to ATP, ACH and adenosine (ADO) in ETYA experiments or isoproterenol (ISO) in MB experiments were obtained, reactive hyperemic response measured and the muscle was then exercised. MB (2 mg/kg, except in 1 experiment where 2.5 mg/kg was given) or saline vehicle was administered by intraarterial (i.a.) infusion. The infusion rate was 1 ml/min for a period of 15 minutes. The calculated arterial blood concentration of MB ranged from 0.5-1.3 mM (n=5). ETYA was dissolved in ethanol (95%) and given intraperitoneally (i.p.). An initial dose of 45 mg/kg ETYA (dissolved in approximately 25 ml ethanol) was given and a supplemental dose of 22.5 mg/kg i.p. (approximately 12 ml ethanol) was administered 1 hour after the initial dose. ETYA administered in a similar manner and dose inhibited flow-dependent dilation and endothelium-dependent relaxation to ACH in canine femoral artery, in vivo (Kaiser et al., 1986). A period of 40 minutes following the initial ETYA dose was allowed for ETYA to take effect. The ethanol vehicle was administered in a similar manner in separate experiments. Dose response curves, reactive hyperemia and exercise were repeated in the presence of either MB (n=5), saline vehicle (n=5), ETYA (n=5) or ethanol vehicle (n=5).

Dose response curves were obtained by intraarterial bolus injections of 0.3ml of ATP, ACH, ADO or ISO. The maximum vasodilator dose for each agonist represents the maximum dose that could be given without decreasing systemic arterial pressure. The difference between baseline and peak vasodilator response was measured and this value was compared before and after a treatment. The order of vasodilator agonist application was randomized. Time was allowed for blood flow to return to baseline value (i.e., the blood flow immediately prior to

vasodilator injection) between doses.

Peak reactive hyperemic response was evaluated by occluding the arterial blood flow to the muscle for 30 seconds. The difference between baseline blood flow and peak vasodilator response was measured before and after MB or ETYA. Time was allowed for blood flow to return to baseline following reactive hyperemia.

Exercise frequencies of 1.5, 3 and 6 Hz were used. These were carried out successively, for a minimum of 3 minutes at each frequency. Blood flow and oxygen consumption were measured at rest and during steady state exercise at each frequency and compared before and after a treatment. The effect of MB or ETYA on exercise hyperemia was also analyzed by determining their effects on the blood flow response at 2 levels of oxygen consumption, 6 and 10 ml O_2/min/100g. From each experiment, a graph of blood flow vs. oxygen consumption was plotted and the blood flow at 6 and 10 ml O_2/min/100g before and after MB or ETYA, was obtained from this plot by linear interpolation. The relationship between flow and metabolism is linear in this range and interpolation is therefore justified. These 2 levels of oxygen consumption were chosen arbitrarily. During 1.5 and 6 Hz exercise with MB or ETYA, we intentionally altered the blood flow to a level that was equal to the control blood flow at 1.5 and 6 Hz. This was done after obtaining the steady state values, by decreasing perfusion pressure. We did this in order to compare oxygen extraction at equal exercise blood flows, before and after MB or ETYA. Arterial blood gases were obtained at rest and at steady state 6 Hz exercise; venous samples were taken at rest and steady state for each exercise frequency.

Data Analysis

The statistical analysis for paired data was a one-way within subjects analysis of variance (ANOVA), (Linton and Gallo, 1975). For the ACH dose response curves with MB, at doses of 3×10^{-7}, 3×10^{-6} and 10^{-5}M the variances were not homogeneous and results were therefore converted to log numbers to obtain homogeneity. The log values were analyzed statistically in this case. A p value of $<.05$ was considered significant.

Drugs

ATP, ACH, ADO, and ISO were obtained from Sigma, heparin sodium from Elkins-Sinn, Inc., MB from Merck. ETYA (RO3-1428) was a gift from Hoffman La Roche. All agents, except the ETYA, were dissolved in saline, made fresh daily, stored on ice in the dark and warmed prior to use. ETYA was dissolved in (95%) ethanol by vigorous stirring immediately prior to use.

Results

MB (n=5) ACH, ATP and ISO produced dose-dependent vasodilations. The vasodilator response to ACH before and after MB is shown in Figure 18. MB shifted the dose response curve to ACH to the right of control at every dose except 3×10^{-5}M ($p<.05$). The effect of MB on the dose response curve to ATP is shown in Figure 19. MB depressed the vasodilator response to ATP at all doses except 10^{-5}, 10^{-3}, and 10^{-2}M ($p<.05$). The ISO dose response curve (Figure 20) was not affected by MB.

The effect of MB on exercise hyperemia is depicted in Figure 21.

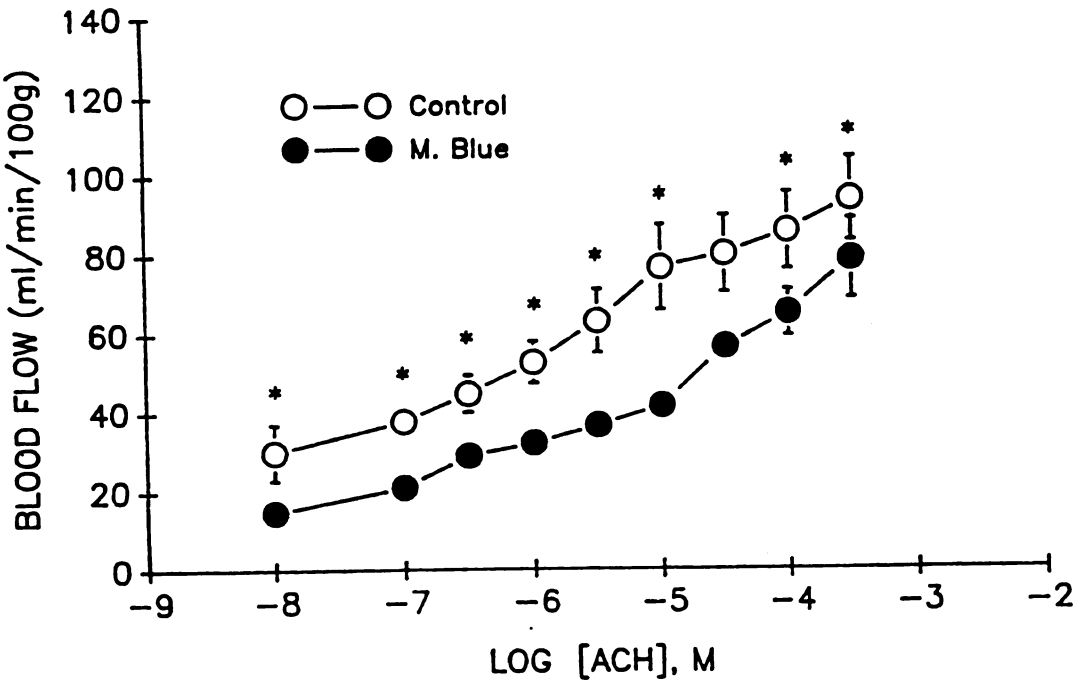

Figure 18. ACH dose response curves before (O) and after (●) methylene blue (MB). The -LOG M syringe concentration of ACH is shown on the abscissa. ACH was administered in 0.3 ml bolus injections. Data points are means ± S.E. (n=5). * Significant difference in blood flow after MB.

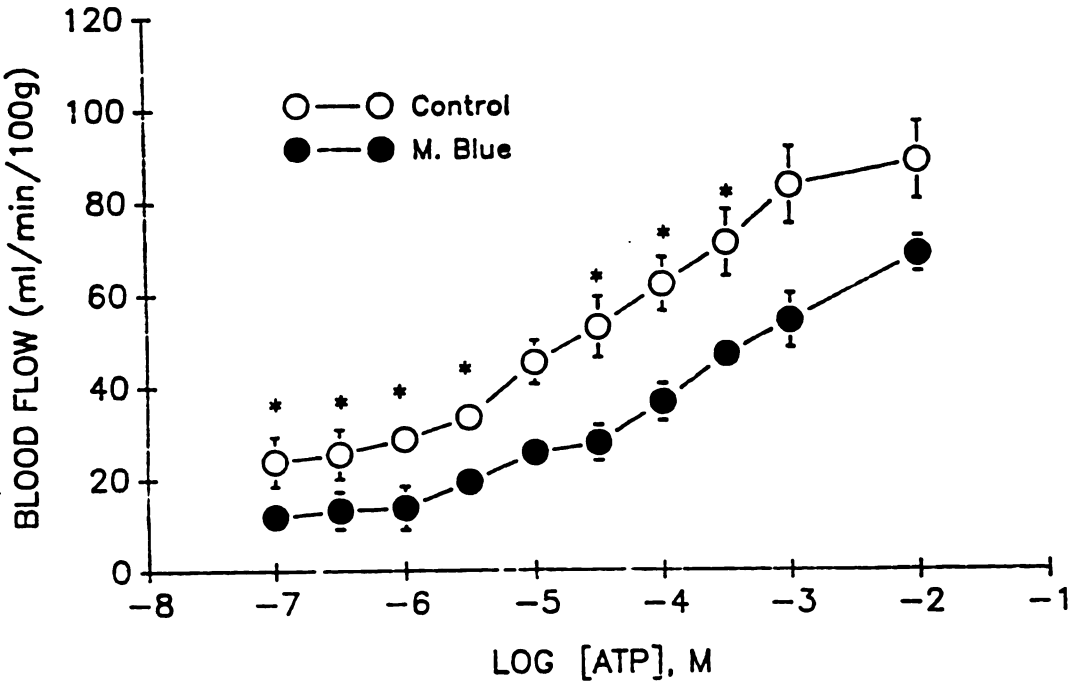

Figure 19. ATP dose response curves before (O) and after (●) methylene blue (MB). The -LOG M syringe concentration of ATP is shown on the abscissa. ATP was administered in 0.3 ml bolus injections. Data points are means ± S.E. (n=5). * Significant difference in blood flow after MB ($p<.05$).

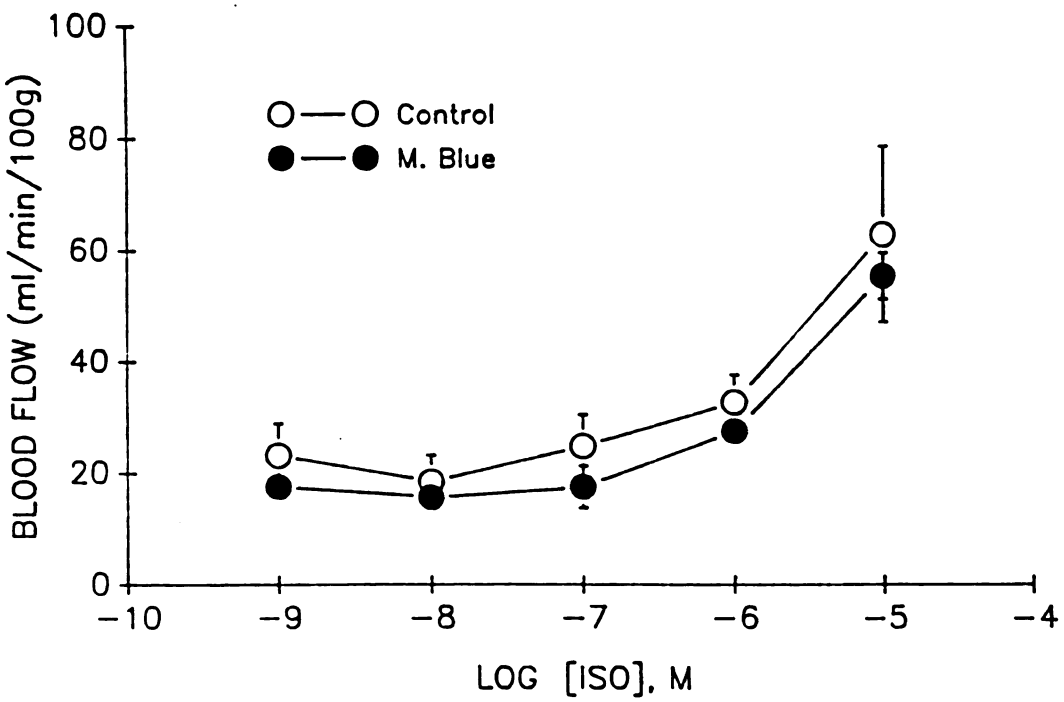

Figure 20. ISO dose response curve before (O) and after (●) methylene blue (MB). The -LOG M syringe concentration of ISO is shown on the abscissa. ISO was administered in 0.3 ml bolus injections. Data points are means ± S.E. (n=5). No differences detected in blood flow after MB.

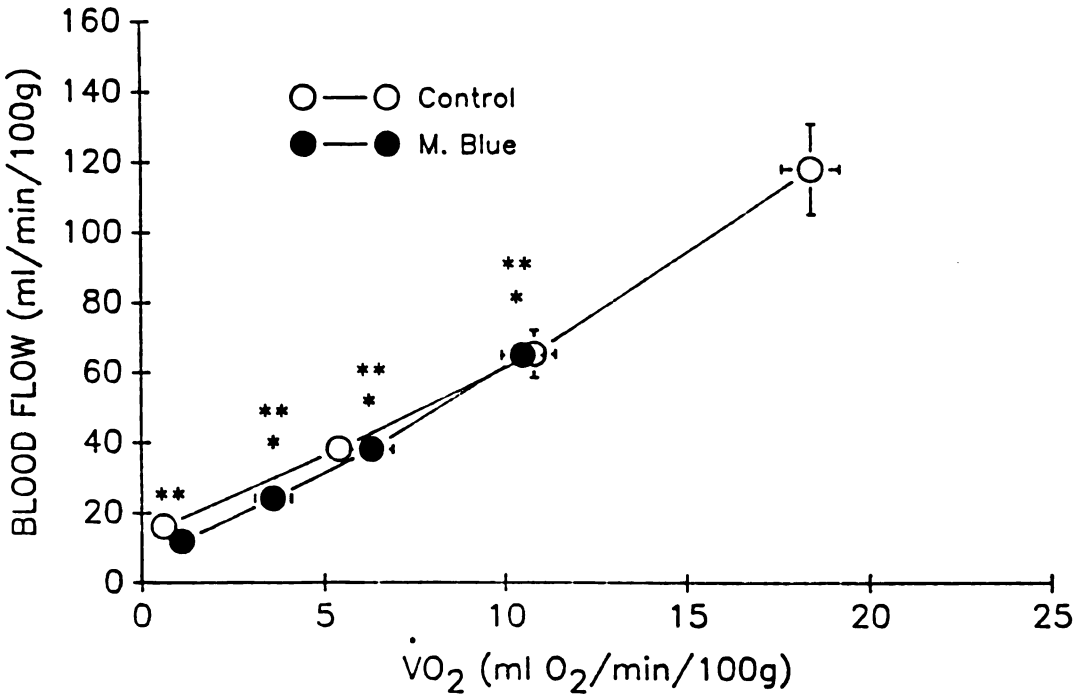

Figure 21. Exercise hyperemia before (O) and after (●) methylene blue (MB). The data points are blood flow and oxygen consumption values measured at rest (immediately prior to exercise) and steady state hyperemia at 1.5, 3 and 6 Hz. Values are means ± S.E. (n=5).
*Significant difference in blood flow after MB ($\overline{p}<.05$).
** Significant difference in oxygen consumption after MB ($p<.05$).

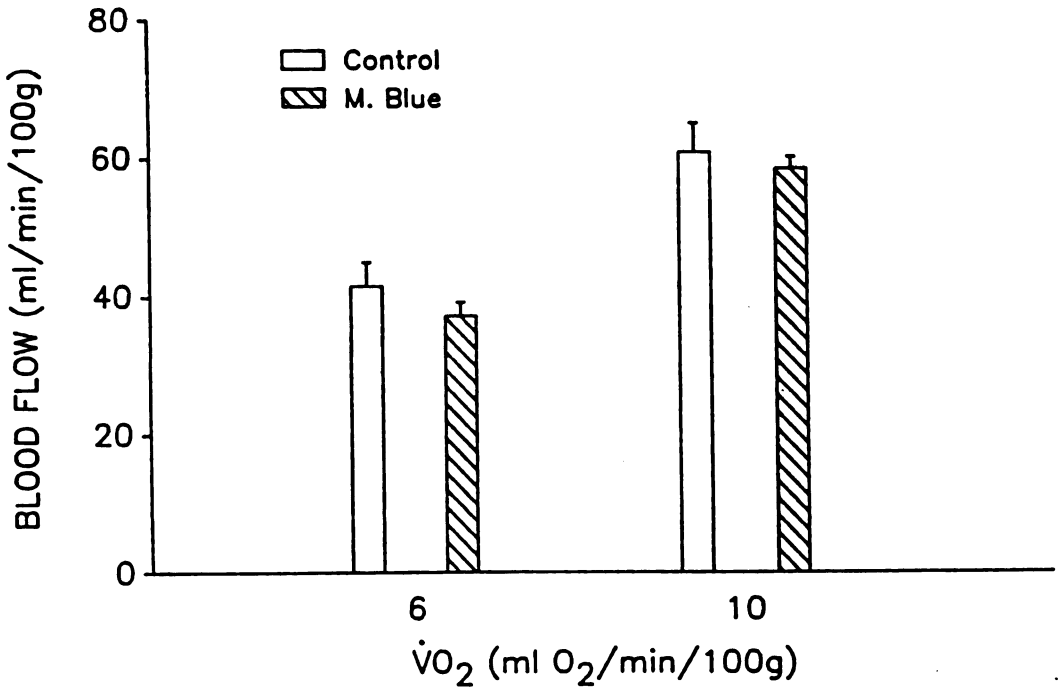

Figure 22. The blood flow response before (open boxes) and after (hatched boxes) methylene blue (MB) at oxygen consumptions of 6 and 10 ml/min/100g. The blood flows were determined from plots of blood flow vs. oxygen consumption by linear interpolation at these 2 values of oxygen consumption. The blood flow values are means $\pm$ S.E. (n=5). No differences were detected in blood flows after MB.

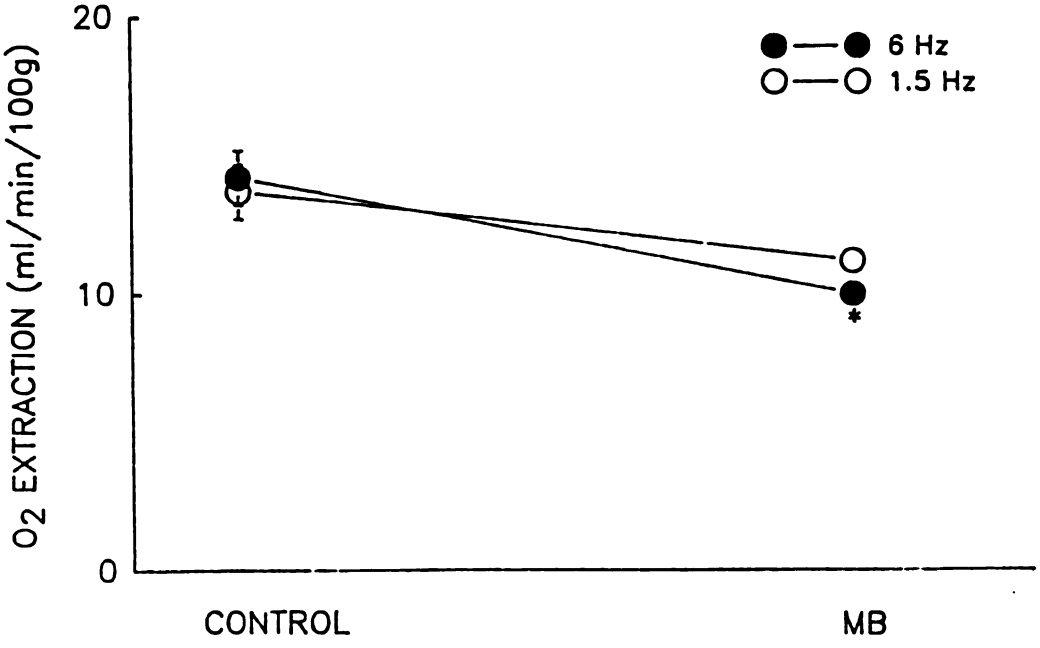

Figure 23. The arterial-venous oxygen content difference (oxygen extraction) before (control) and after methylene blue (MB) when blood flows are equalized during 1.5 and 6 Hz. The open circles represent 1.5 Hz values and the closed circles 6 Hz values. * Significant difference in oxygen extraction after MB ($p<.05$).

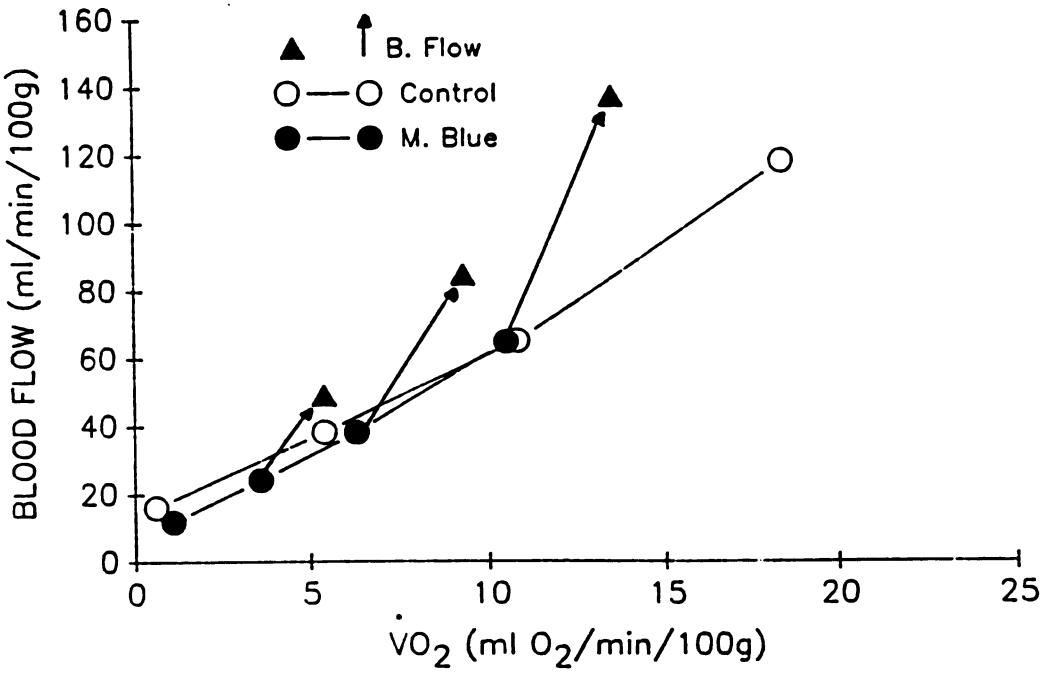

Figure 24. The effect of equalizing blood flow during MB. This figure is the same as figure 21 with the addition of closed triangles that represent the oxygen consumption and blood flow values measured when blood flows are equalized during 1.5, 3, and 6 Hz. The lines with an arrow indicate the increase in blood flow and oxygen consumption that occur when blood flow is increased to a level equal to (1.5 Hz) or greater than (3 and 6 Hz) the blood flow measured during exercise with MB (closed circles).

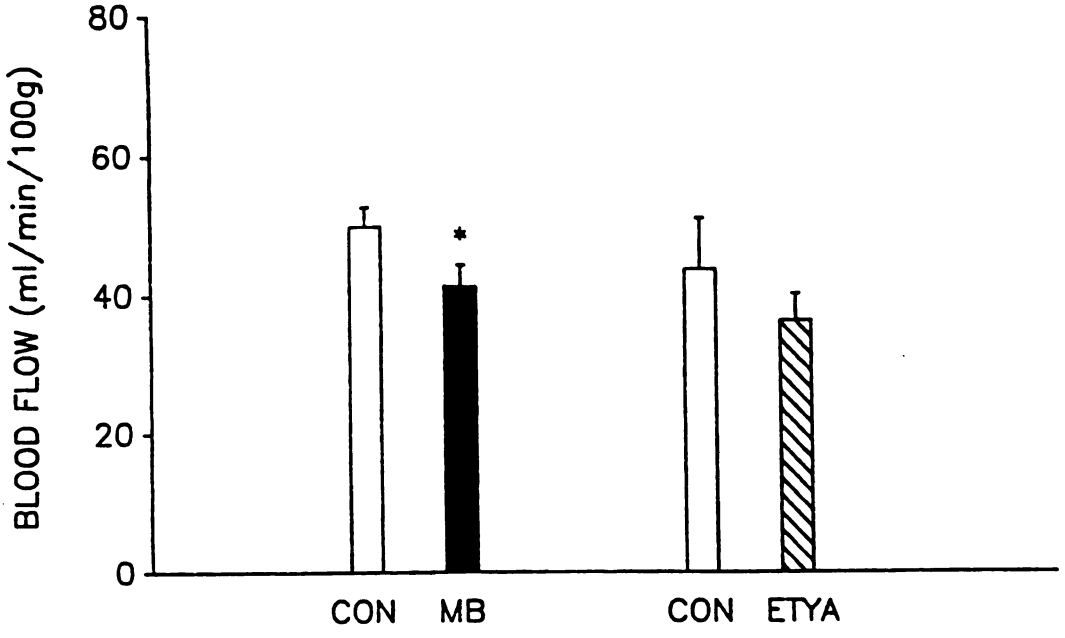

Figure 25. Peak reactive hyperemic response before (control, open boxes) and after methylene blue (MB, filled in box) or ETYA (hatched box). The peak vasodilator response to 30 seconds of arterial occlusion was measured. The values are means $\pm$ S.E. (n=5). * Significant difference in hyperemic response after MB ($p<.05$), no differences detected for ETYA.

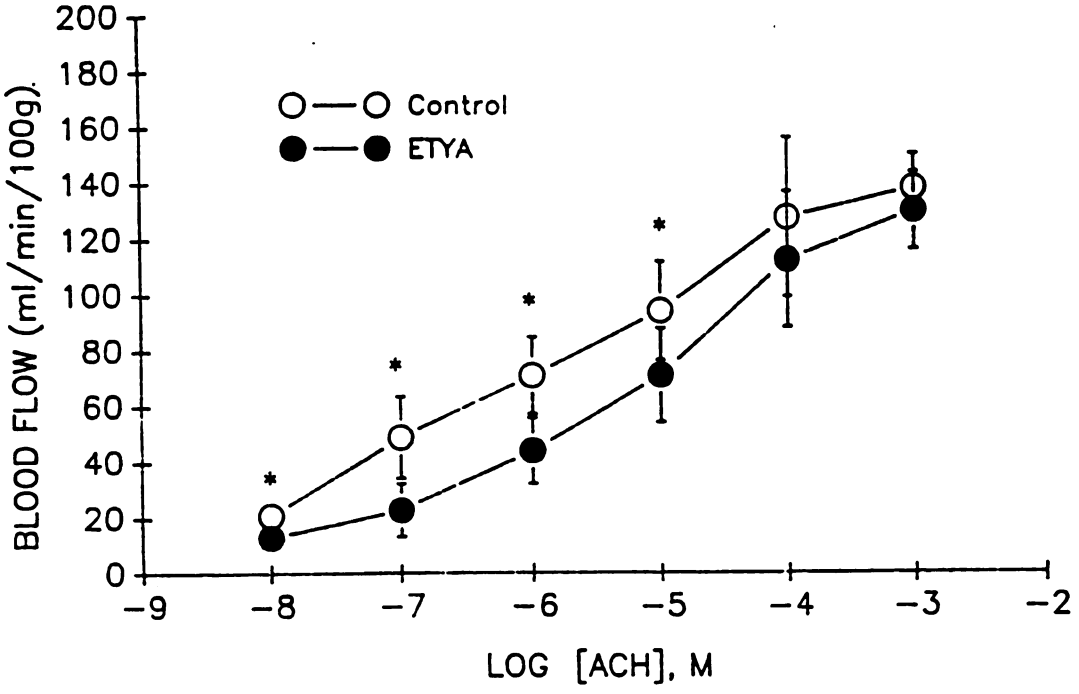

Figure 26. ACH dose response curves before (O) and after (●) ETYA. The -LOG M syringe concentration of ACH is shown on the abscissa. ACH was administered in 0.3ml bolus injections. Data points are means ± S.E. (n=5, except at 10^{-7} and 10^{-5} where n=4). * Significant difference in blood flow after ETYA ($p<.05$).

MB did not effect resting blood flow (i.e., immediately prior to exercise); however, MB decreased the blood flow response to 1.5, 3, and 6 Hz muscle contractions ($p<.05$). MB increased oxygen consumption at rest and decreased oxygen consumption at all stimulation frequencies and and decreased tension development (Table 1) at all 3 stimulation frequencies ($p<.05$). Since MB depressed both metabolism and steady state blood flow at all 3 stimulation frequencies and blood flow is function of metabolism, we wanted to determine if MB was primarily depressing metabolism or blood flow. Therefore, we calculated blood flows before and after MB at 2 levels of oxygen consumption as shown in Figure 22. At oxygen consumptions of 6 and 10 ml O_2/min/100g, MB had no effect on the calculated blood flow response. To determine if MB was directly affecting oxygen consumption, we increased blood flow during exercise with MB, to the control level. Figure 24 depicts the blood flow response and oxygen consumption when blood flows were equalized during exercise with MB. Oxygen consumption (Figure 24) and extraction (Figure 23) after MB were not significantly depressed at 1.5 Hz when blood flows were equalized. The effect of increasing blood flow to control levels is depicted in Figure 24. When blood flow was increased to a level equal to control blood flow at 1.5 Hz (no significant difference), oxygen consumption was no different than control. When blood flow was increased to levels even greater than control at 3 and 6 Hz, oxygen consumption increases but does not reach control levels (Figure 24). During MB administration, oxygen consumption went from 6.3 to 9.3 when blood flow was increased during 3 Hz exercise and from 10.5 to 13.5 ml O_2/min/100g when blood flow was increased during 6 Hz exercise. However, oxygen consumption was still

depressed compared to control at 3 and 6 Hz ($p<.05$, Figure 24) and extraction was significantly lower than control levels at 6 Hz (Figure 23, n=4, $p<.05$). There was a significant decrease in the peak reactive hyperemic response to 30 seconds of arterial occlusion in the presence of MB (Figure 25, $p<.05$).

ETYA (n=5) The ACH dose response curve before and after ETYA is shown in Figure 26. ETYA shifted the ACH dose response to the right of control at all doses except 10^{-4} and 10^{-3}M ($p<.05$). The effect of ETYA on the ATP dose response curve is shown in Figure 27. ETYA had no effect on the dose response curve to ATP, except at 10^{-5}M, where the vasodilator response was decreased ($p<.05$). The ADO dose response curve is shown in Figure 28. ETYA had no effect on ADO vasodilation at any dose.

Exercise hyperemia before and after ETYA is depicted in Figure 29. Compared to the control response, ETYA decreased blood flow at rest and 1.5 Hz and decreased oxygen consumption at 1.5 Hz, but not at rest or any other stimulation frequency ($p<.05$). ETYA depressed tension development at 1.5 and 3 Hz (Table 1). The blood flow response at 2 levels of oxygen consumption before and after ETYA is shown in Figure 30. ETYA had no effect on the blood flow response at 6 or 10 ml O_2/min/100g. When blood flows were equalized during 1.5 Hz stimulation, there was no difference in oxygen extraction (Figure 32) or oxygen consumption (Figure 31) after ETYA. ETYA had no effect on the peak reactive hypermic response to arterial occlusion (Figure 25).

Saline Vehicle (n=5) Experiments were performed in a similar

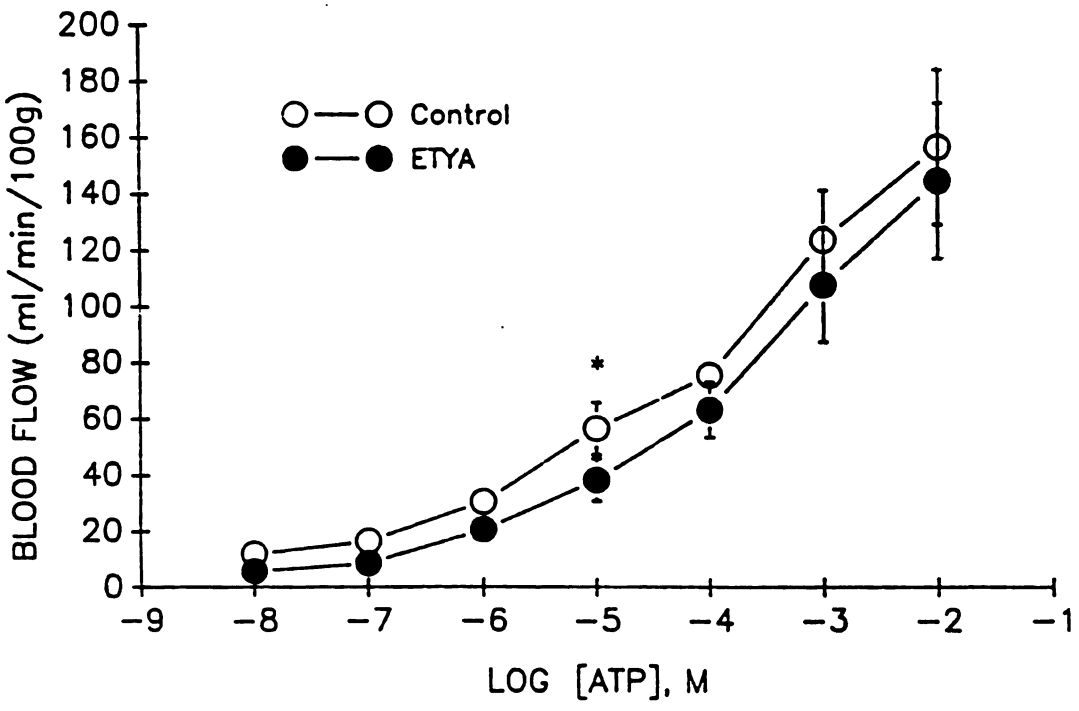

Figure 27. ATP dose response curves before (O) and after (●) ETYA. The -LOG M syringe concentration of ATP is shown on the abscissa. ATP was administered in 0.3 ml bolus injections. Data points are means ± S.E. (n=5, except at 10^{-4} and 10^{-2}). * Significant difference in blood flow after ETYA ($p<.05$).

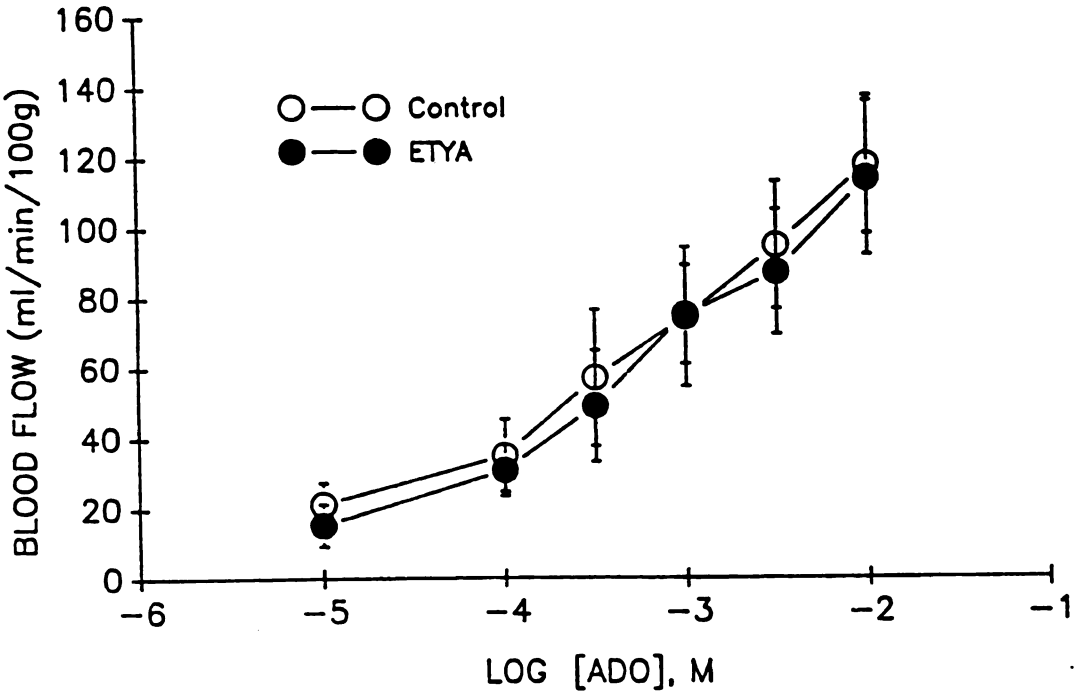

Figure 28. ADO dose response curves before (O) and after (●) ETYA. The -LOG M syringe concentration of ADO is shown on the abscissa. ADO was administered in 0.3 ml bolus injections. Data points are means ± S.E. (n=5 except at $3X10^{-4}$ where n=4). No difference in blood flows were detected after ETYA.

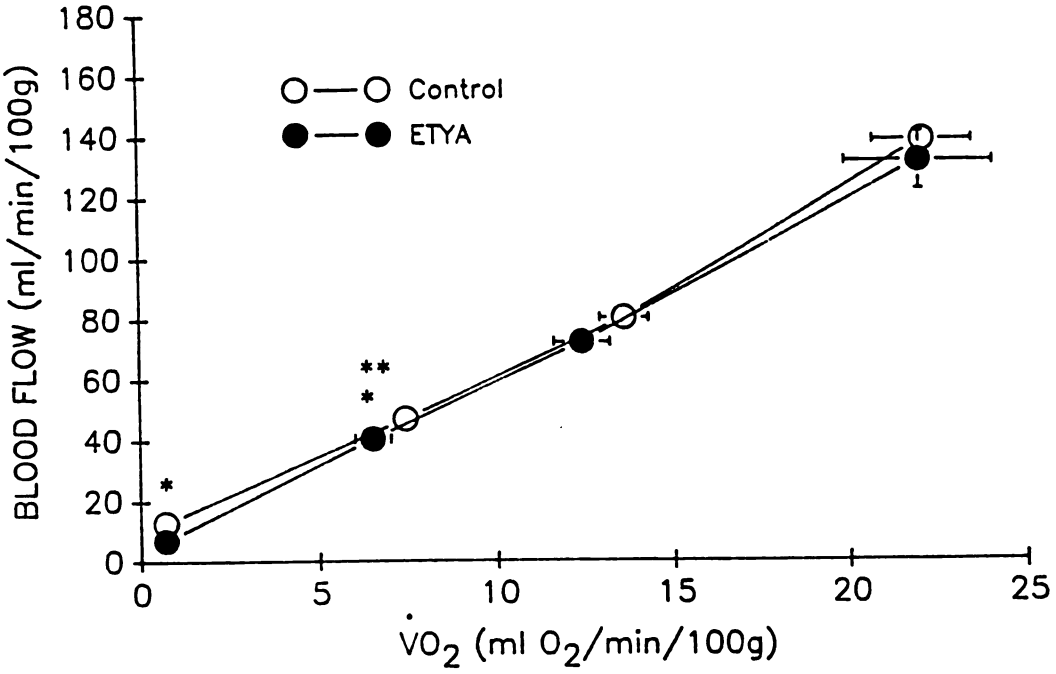

Figure 29. Exercise hyperemia before (O) and after (●) ETYA. The data points are blood flow and oxygen consumption values measured at rest and steady state hyperemia at 1.5, 3 and 6 Hz. Values are means ± S.E. (n=5). * Significant difference in blood flow after ETYA ($p<.05$). ** Significant difference in oxygen consumption after ETYA ($p<.05$).

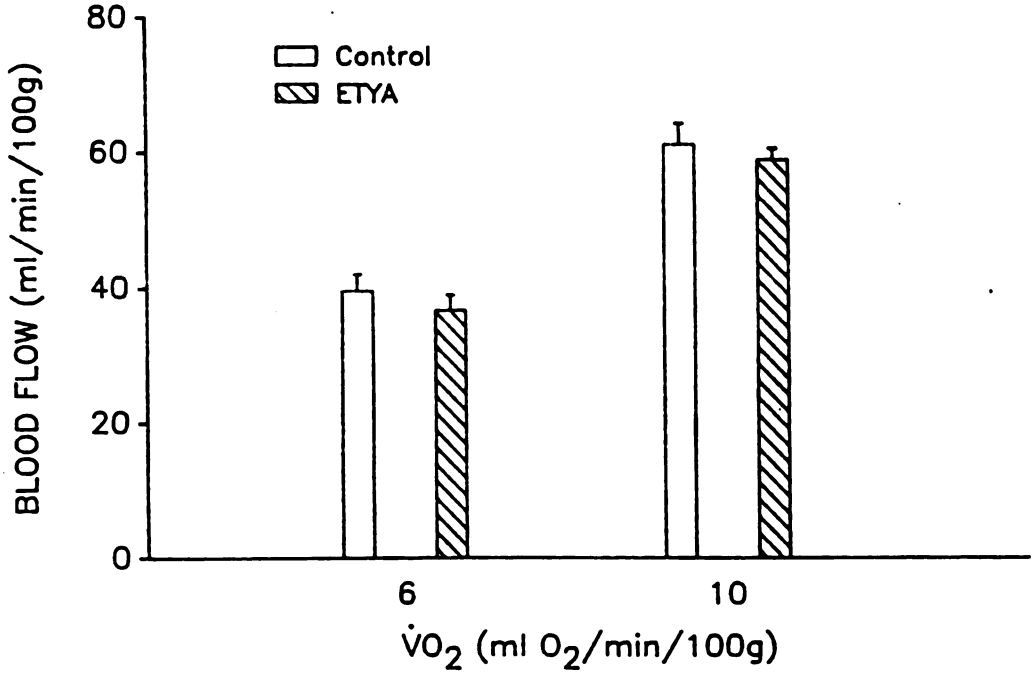

Figure 30. The blood flow response before (open boxes) and after (hatched boxes) ETYA at oxygen consumptions of 6 and 10 ml/min/100g. The blood flows were determined from plots of blood flow vs. oxygen consumption by linear interpolation at these 2 values of oxygen consumption. The blood flow values are means $\pm$ S.E. (n=5). No differences were detected after ETYA.

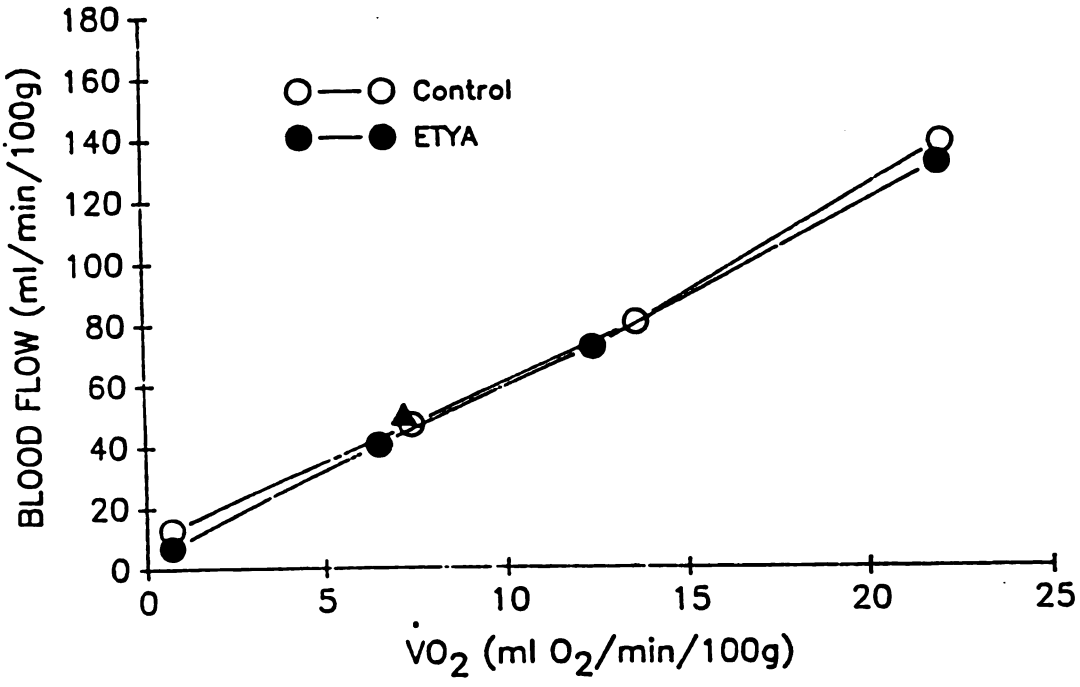

Figure 31. Exercise hyperemia during ETYA when blood flow is equalized to the control level during 1.5 Hz exercise. This graph is the same as Figure 29 with the addition of a closed triangle that represents the blood flow response and oxygen consumption when blood flows are equalized during 1.5 Hz exercise.

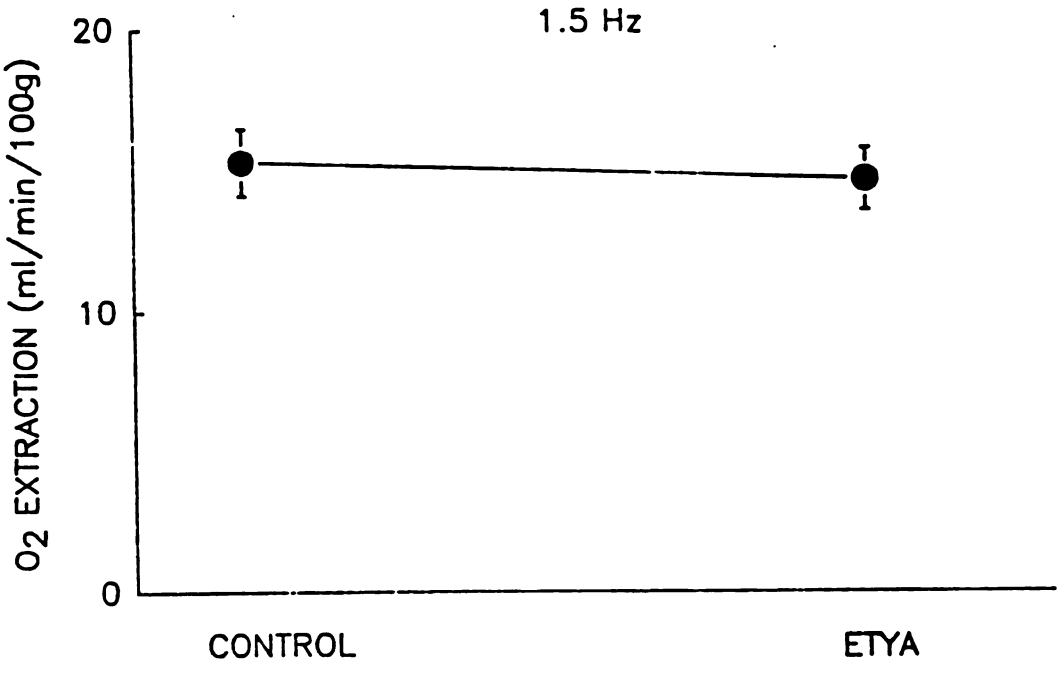

Figure 32. The arterial-venous oxygen content difference (oxygen extraction) before (control) and after ETYA when blood flows are equalized during 1.5 Hz exercise. The extraction values are means $\pm$ S.E. (n=5). No difference in oxygen extraction was detected after ETYA.

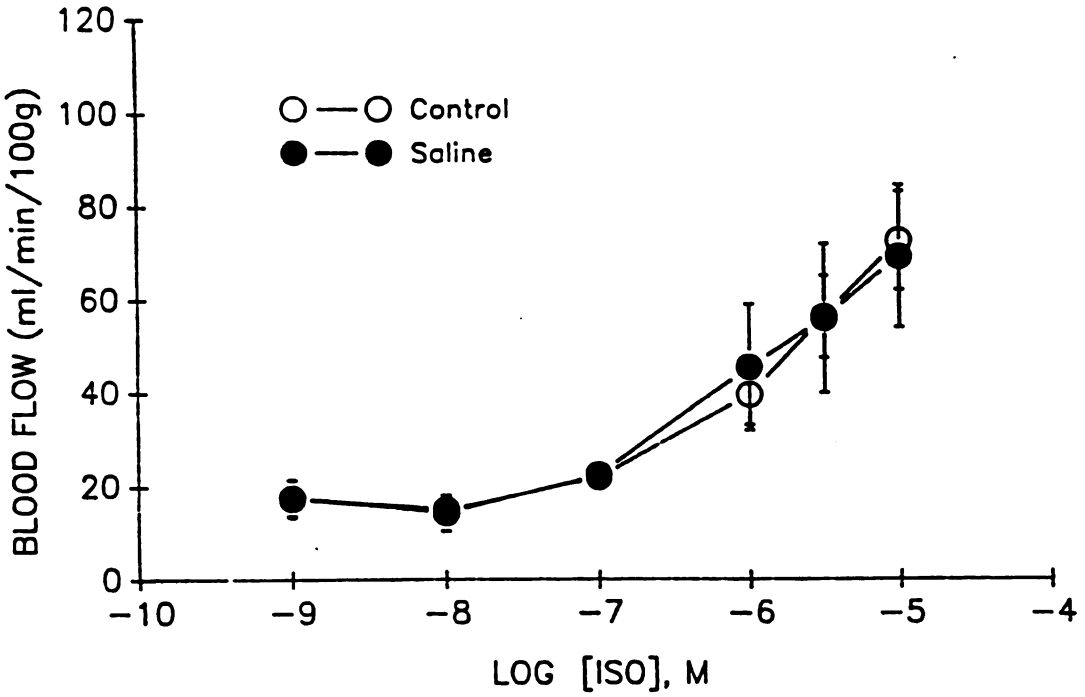

Figure 33. ISO dose response curve before (O) and after (●) saline. The -LOG M syringe concentration of ISO is shown on the abscissa. ISO was administered in 0.3 ml bolus injections. Data points are means ± S.E. (n=5). No differences in blood flow were detected after MB.

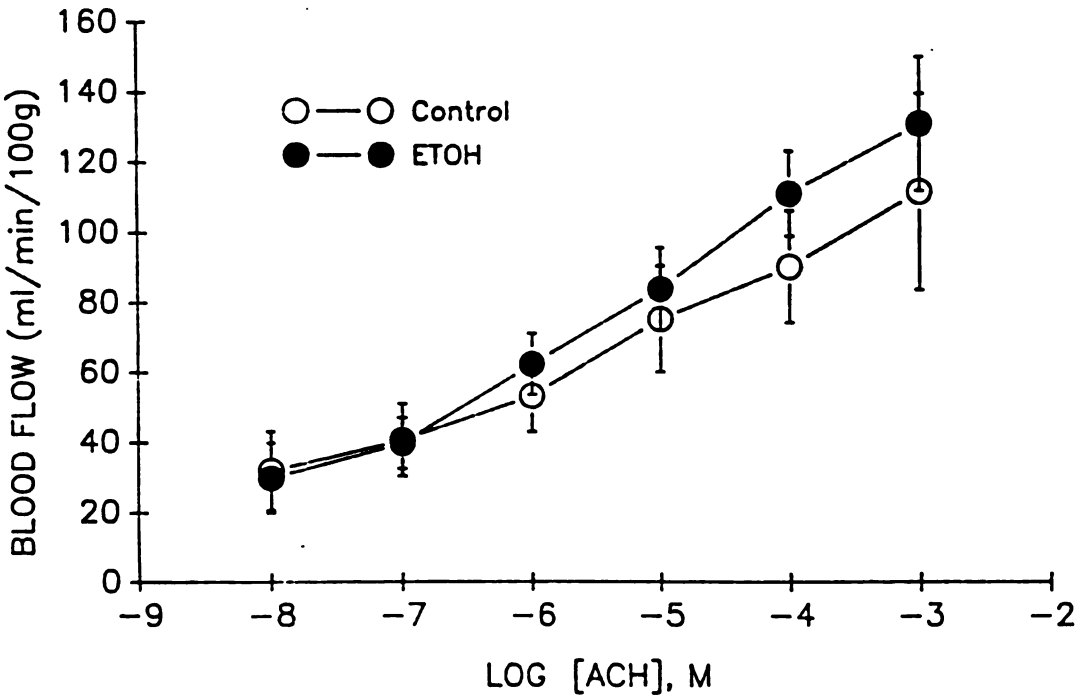

Figure 34. ACH dose response curves before (O) and after (●) ethanol. The -LOG M syringe concentration of ACH is shown on the abscissa. ACH was administered in 0.3 ml bolus injections. Data points are means ± S.E. (n=5, except at 10^{-3} where n=4). No differences were detected after ethanol.

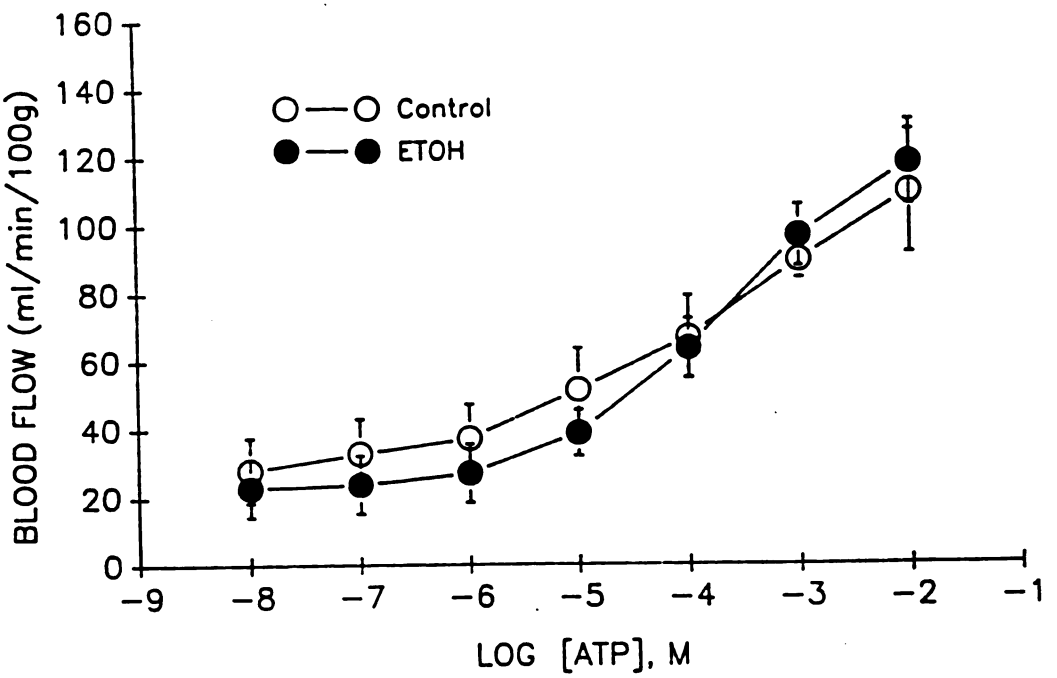

Figure 35. ATP dose response curves before (O) and after (●) ethanol. The -LOG M syringe concentration of ATP is shown on the abscissa. ATP was administered in 0.3 ml bolus injections. Data points are means ± S.E. (n=5). No differences in blood flow were detected after ethanol.

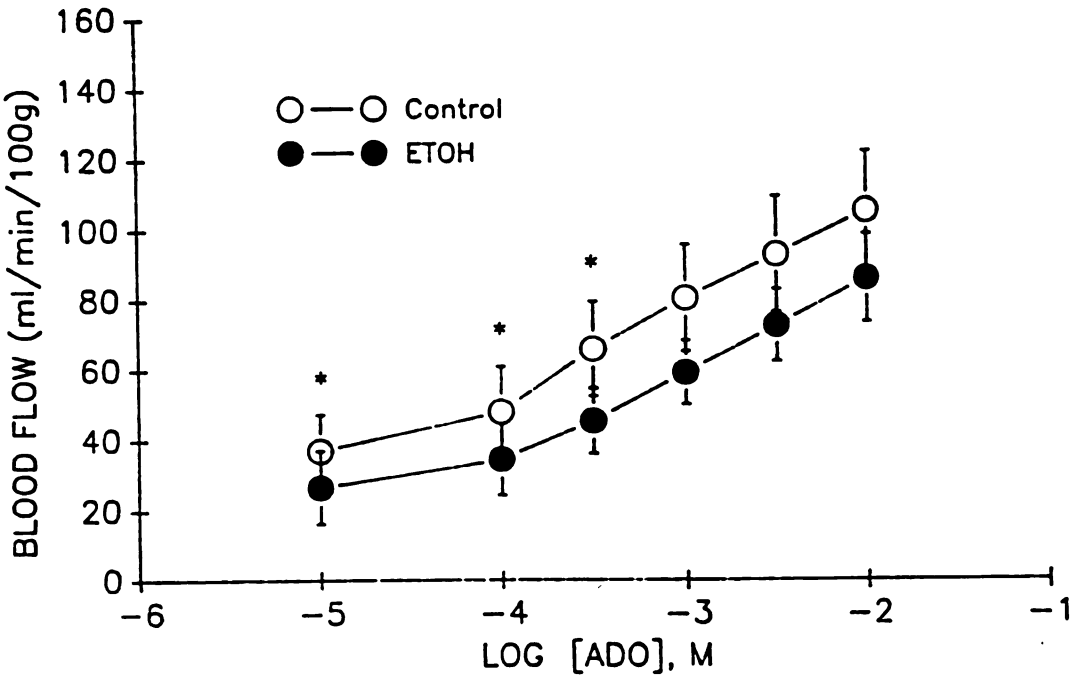

Figure 36. ADO dose response curves before (O) and after (●) ethanol. The -LOG M syringe concentration of ADO is shown on the abscissa. ADO was administered in 0.3 ml bolus injections. Data points are means ± S.E. (n=5 except at 10^{-4} where n=4). * Significant difference in blood flow after ethanol.

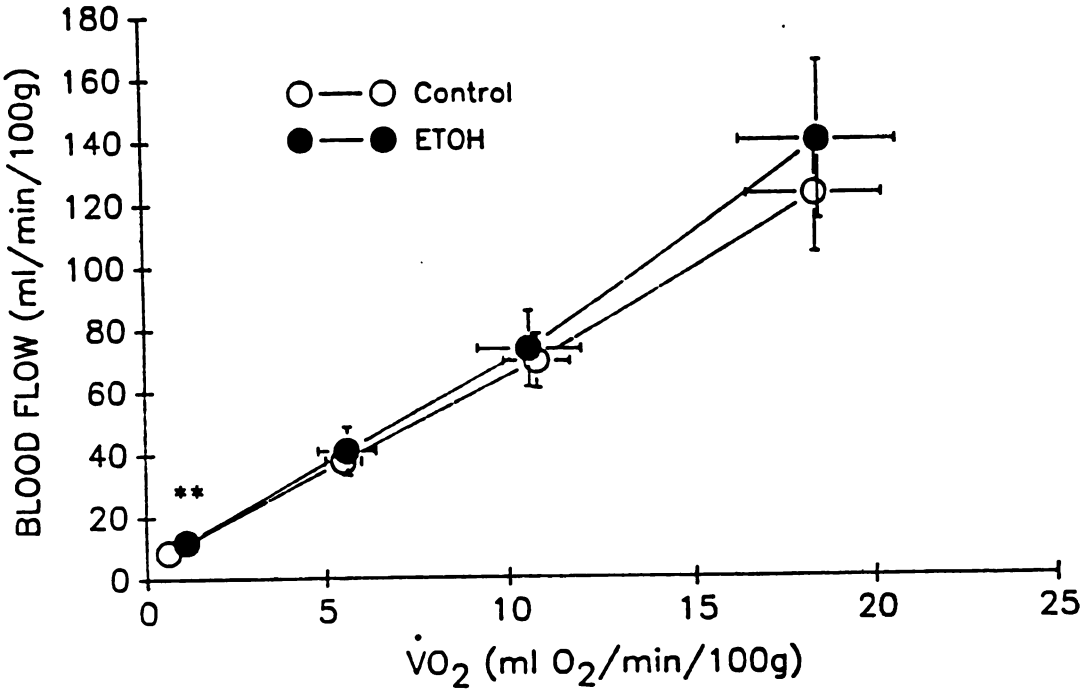

Figure 37. Exercise hyperemia before (O) and after (●) ethanol. The data points are blood flow and oxygen consumption values measured at rest and steady state hyperemia at 1.5, 3, and 6 Hz. Values are means ± S.E. (n=5). **Significant difference in oxygen consumption after ethanol. No differences in blood flow were detected after ethanol.

manner to the MB experiments except the saline vehicle was given instead of MB. Saline vehicle had no effect on the vasodilator response to ACH (Figure 9), ISO (Figure 33), or ATP (Figure 8).

Figure 10 depicts the effect of saline vehicle on exercise hyperemia. Saline had no effect on either blood flow or oxygen consumption at rest or any stimulation frequency. However, saline depressed muscle tension development at 1.5 and 3 Hz ($p<.05$, table 1). In addition, saline had no effects on the calculated blood flow response at oxygen consumptions of 6 or 10 ml O_2/min/100g (Figure 11) and no effect on oxygen extraction when blood flows were equalized at 1.5 and 6 Hz stimulation frequencies (data not shown). Saline had no effect on the peak hyperemic response to arterial occlusion (Figure 7).

Ethanol Vehicle (n=5) In these experiments, ethanol (95%), the vehicle used for ETYA, was given in the same manner as ETYA and the experimental protocol was similarly carried out. Ethanol had no effects on the ACH dose response curve, except at 10^{-3}M, where the blood flow response was decreased (Figure 34) or the ATP dose response curve (Figure 35). The vasodilator response to ADO is shown in Figure 36. Ethanol depressed the vasodilator response to ADO at doses of 10^{-5}, 10^{-4} and 3×10^{-4}M ($p<.05$).

As shown in Figure 37, ethanol had no effects on blood flow at rest or during exercise (n=5). Ethanol increased resting oxygen consumption ($p<.05$) but did not affect oxygen consumption during exercise. Tension development was depressed at 1.5 Hz (Table 1). Ethanol did not change the blood flow response calculated for the 2 levels of oxygen consumption and did not effect the peak reactive

hyperemic response (data not shown).

Discussion

ACH and ATP elicited dose-dependent vasodilations in the present study. It has been demonstrated in vitro for ATP (DeMey et al., 1982; Kennedy et al., 1985; Rapoport et al., 1984; Cassis et al., 1987) and ACH (Furchgott and Zawadzki, 1980; Rapoport and Murad, 1983; Griffith et al., 1984; Martin et al., 1985, Rubanyi and Vanhoutte, 1985) and in vivo for ATP and ACH (Pohl et al., 1987; Cocks and Angus, 1983; Kaiser et al., 1983; Busse and Pohl, 1988; Furchgott et al., 1987; Kaley et al., 1986; Watanabe et al., 1988) that these two vasodilators are dependent on the endothelium. ATP and ACH bind to purinergic and muscarinic receptors, respectively, on the endothelial cell and elicit formation and/or release of a substance termed endothelium-derived relaxing factor (EDRF) (Burnstock and Kennedy, 1986; Cassis et al., 1987; Furchgott, 1981). EDRF stimulates guanylate cyclase activity in vascular smooth muscle which results in a rise in cyclic guanosine monophosphate (cGMP) production and relaxation (Holzmann, 1982; Furchgott, 1983; Ignarro et al., 1984, Ignarro et al., 1984a; Ignarro and Kadowitz, 1985; Martin et al., 1985; Rapoport and Murad, 1983; Rapoport et al., 1984). Nitrovasodilators, i.e., those that can form nitric oxide-free radical, also produce relaxation by increasing vascular smooth muscle cGMP, although their action is directly on the smooth muscle and not endothelium-dependent (Ignarro et al., 1984; Ignarro and Kadowitz, 1985; Murad, 1986). Therefore, there is a final common pathway, cGMP production, for endothelium-dependent vasodilators and nitrovasodilators to elicit vascular smooth muscle relaxation

(Martin et al., 1985). MB inhibits the accumulation of cGMP in vascular smooth muscle by inactivating the enzyme, guanylate cyclase. MB inhibits the relaxation response to both nitric-oxide containing vasodilators and endothelium-dependent vasodilators, through inhibition of guanylate cyclase (Gruetter et al., 1981; Ignarro et al., 1984a; Ignarro et al., 1984; Ignarro and Kadowitz, 1985; Martin et al., 1985; Ignarro et al., 1986a;) and not through a direct effect on EDRF (Ignarro et al., 1986b; Griffith et al., 1985).

Our results in vivo confirm results from in vitro studies that dose-dependent vasodilations to ACH and ATP are reduced by inhibitors of endothelium-dependent relaxation, MB and ETYA. MB depressed the vasodilator response to both ACH and ATP in our preparation. ACH and ATP produce dose- and time-dependent increases in vascular smooth muscle cGMP (Holzman, 1982; Rapoport and Murad, 1983; Ignarro et al., 1984; Ingnarro et al., 1984a; Ignarro and Kadowitz, 1985; Rapoport et al., 1984; Martin et al., 1985). In vivo, MB reduced the relaxation response to ACH in the blood perfused canine femoral artery (Kaiser et al., 1986), rabbit hindlimb (Kaley et al., 1986) and mouse pial arterioles (Watanabe et al., 1988), although it did not attenuate the vasodilator response to ACH in the hamster cheek pouch (Rivers and Duling, 1986). MB reduced the vasodilator response to ATP in the rabbit hindlimb (Kaley et al., 1986). We tested the specificity of MB for endothelium-dependent vasodilation by determining its effects on ISO vasodilations, which are not endothelium-dependent, not associated with an increase in vascular smooth muscle cGMP, not inhibited by MB in rabbit aorta (Martin et al., 1985) and not affected by mechanical removal of endothelial cells (DeMey and Vanhoutte, 1982; Ignarro et

al., 1984; Furchgott, 1981a; Furchgott, 1981b;), except in the canine coronary artery (Rubanyi and Vanhoutte, 1985). We found no effect of MB on ISO vasodilator responses. Thus, our results in the blood perfused canine hindlimb resistance vessels are consistant with data in vitro that ACH and ATP relaxations are inhibited by MB while ISO is not affected. This is strong evidence supporting the hypothesis that endothelium-dependent vasodilation occurs in this preparation.

In the present study, ETYA shifted the ACH dose response curve to the right of control except at the 2 highest doses, 10^{-3} and 10^{-2}M. Thus, ETYA is an effective inhibitor of ACH vasodilation in canine hindlimb resistance vessels.

We found that ETYA did not inhibit the vasodilator response to ATP, except at 10^{-5}M ATP. Thus, ETYA does not inhibit endothelium-dependent vasodilation to ATP in a manner comparable to MB in our preparation. We expected that ETYA would inhibit ATP vasodilation in our preparation based on the findings that (1) ETYA reduces or inhibits the relaxation response and the accumulation of cGMP in vascular smooth muscle in response to ACH and ATP in vitro in the rat thoracic aorta (Rapoport and Murad, 1983) and rabbit thoracic aorta (Furchgott, 1981) and (2) ETYA administered in the same dose and manner to the blood perfused canine artery, in vivo depressed the endothelium-dependent increase in femoral artery diameter in response to ACH (Kaiser et al., 1986).

In the canine femoral artery in vitro, ETYA did not reduce the endothelium-dependent relaxation response to ATP, although it depressed the response to ACH (DeMey et al., 1982). In the rat hindlimb, ETYA did not reduce vasodilation to ATP, although MB (Kaley et al., 1986)

and gossypol (Pohl et al., 1987) were effective. ETYA is an arachidonic acid analog that blocks 2 and possibly 3, enzymatic pathways of arachidonic acid metabolism, cyclooxygenase, lipoxygenase, and possibly cytochrome P-450-dependent monooxygenase (Flower, 1974; Pinto et al., 1986). Since indomethacin, a blocker of the enzyme cyclooxygenase, does not reduce endothelium-dependent relaxation (Furchgott and Zawadzki, 1980; DeMey et al., 1982; Hull et al., 1986; Rapoport et al., 1984) the mechanism of action of ETYA to inhibit endothelium-dependent vasodilation is not through inhibition of cyclooxygenase pathway. ETYA may alternatively work as an antioxidant or free radical scavenger to inhibit this process (Rubanyi, 1988; Vanhoutte et al., 1986; Griffith et al., 1984). ATP and ACH may have different mechanisms for producing EDRF. ATP and ACH have separate receptors on endothelial cells for producing vasodilation; muscarinic, atropine sensitive receptor for ACH (Furchgott et al., 1981; Ignarro et al., 1984 paper) and P_{2y}-purinergic receptor for ATP (Burnstock and Kennedy, 1986). Our results suggest that ATP and ACH elicit endothelium-dependent vasodilation through separate pathways or different EDRF(s) in the canine hindlimb.

The vasodilator response to ADO was not inhibited by ETYA in our preparation or in the rabbit thoracic aorta (Furchgott et al., 1981). We hypothesized that the relaxation response to ADO would not be inhibited by ETYA based on results from the canine femoral, pulmonary, saphenous and splenic arteries and veins that ADO relaxation was independent of the endothelium (DeMey and Vanhoutte, 1982; DeMey and Vanhoutte, 1981). In addition the relaxation response to ADO is not dependent on endothelial cells in the rat femoral artery (Kennedy and

Burnstock, 1985) and the rabbit aorta (Furchgott, 1981). However, in the pig aorta and canine coronary artery, the relaxation response to ADO was reduced when the endothelium was removed (Gordon and Martin, 1983; Rubanyi and Vanhoutte, 1985). ADO relaxation was also partly dependent on the endothelium in rabbit basilar, intrapulmonary (Frank and Bevan, 1983) and central ear artery (Kennedy and Burnstock, 1985). Therefore, the relaxation response to ADO can be partly dependent on the endothelium in some arteries. However, in the canine femoral artery, ADO is thought to act directly on vascular smooth muscle P1 purinoceptors to elicit vasodilation (DeMey and Vanhoutte, 1982; Burnstock, 1978). We believe the lack of effect of ETYA on ADO vasodilation supports the contention that ETYA is acting specifically on endothelial cell-mediated processes in inhibiting ACH vasodilation in our preparation.

MB significantly depressed blood flow, oxygen consumption and tension development during exercise. Since the blood flow response during steady state exercise is a linear function of muscle metabolism (Haddy and Scott, 1968; Mohrman and Sparks, 1973) we wanted to determine if MB was primarily affecting metabolism or blood flow. Therefore, we compared (1) the blood flow response before and after MB at 2 levels of oxygen consumption and (2) oxygen extraction at equivalent blood flows before and after MB. At oxygen consumptions of 6 and 10 ml O_2/min/100g, the blood flow response during MB is not different than control. This suggests that MB does not decrease the blood flow response for a given metabolic rate up to 10 ml O_2/min/100g. However, during 1.5 Hz exercise with MB there was no difference in oxygen consumption when blood flow was increased to control levels by

increasing muscle perfusion pressure. This suggests that MB is inhibiting a vasodilator pathway that contributes to 1.5 Hz exercise hyperemia. Therefore, it is likely that endothelium-dependent vasodilation is contributing to 1.5 Hz exercise hyperemia. When blood flow was increased to a level equivalent to or above control during 3 and 6 Hz exercise, oxygen consumption increased but still remained less than control. The increased oxygen consumption which accompanied the artificial increase in flow at 3 and 6 Hz in the presence of MB suggests that oxygen consumption was partly limited by the failure of flow to increase in the presence of MB. However, MB also depressed muscle oxygen extraction at 3 and 6 Hz. These results indicate that MB reduces oxygen consumption during exercise by 2 mechanisms, limitation of blood flow and and a direct effect on muscle to inhibit oxygen extraction. The decreased extraction of oxygen at a blood flow equivalent to or greater than the control value suggests that either (1) oxygen transport from red cells to mitochondria is inhibited or (2) oxidative phosphorylation is inhibited. MB can act as a reducing or oxidizing agent, depending upon its concentration (Goodman and Gilman, 1980). At the low dose used in this study, MB can be used as a reducing agent to treat methemoglobinemia (Goodman and Gilman, 1980; Harvey and Keitt, 1983). The increase in resting oxygen consumption and decrease during exercise suggests that MB may be altering the cycling of reducing equivalents and affecting mitochondrial electron transport.

In the rabbit hindlimb, gossypol, an inhibitor of endothelium-dependent vasodilation, decreased oxygen consumption and muscle PO_2 under resting conditions while not affecting blood flow

(Busse and Pohl, 1988). Since ADO restored tissue PO_2 and oxygen consumption it was concluded that endothelium-dependent vasodilation is important for adequate tissue oxygen supply. Our results point to a direct effect of MB on the muscle; however, we cannot rule out other possible microcirculatory effects, perhaps a redistribution of oxygen delivery at the microvascular level.

ETYA decreased blood flow, oxygen consumption and muscle tension development during 1.5 Hz exercise. When blood flow was equalized to control levels during ETYA, oxygen consumption returned to control values. This indicates that ETYA is inhibiting a vasodilator pathway during 1.5 Hz exercise. This result is consistant with the results of the MB experiments that endothelium-dependent vasodilation contributes to 1.5 Hz exercise hyperemia. There was no difference in the blood flow response or oxygen consumption at 3 or 6 Hz, no effect of ETYA on the blood flow response at 6 or 10 ml O_2/min/100g. Given that ETYA antagonized ACH-induced dilation, we conclude that vasodilation mediated by the EDRF pathway associated with ACH is a significant factor in exercise hyperemia. ETYA, like MB, increased resting oxygen consumption by increasing oxygen extraction. Therefore, in the canine hindlimb, inhibitors of endothelium-dependent vasodilation also increase resting muscle oxygen utilization.

Reactive hyperemia is the transient increase in blood flow that occurs following arterial occlusion (Barcroft, 1972). The increase in blood flow and arterial-venous oxygen difference allows the tissue to recover adequate oxygenation (Granger et al., 1975). MB decreased the peak vasodilator response to 30 seconds of arterial occlusion, suggesting that endothelium-dependent vasodilation contributes to this

hyperemic response. ETYA was not effective in reducing the peak reactive hyperemic response. Therefore, despite the observation that both ETYA and MB decrease ACH-induced vasodilations and increase resting oxygen consumption in this preparation, these two agents do not have similar effects on reactive hyperemia, 3 and 6 Hz exercise hyperemia or ATP-induced vasodilations. This is evidence in favor of the possibility that exercise hyperemia at 3 and 6 Hz, ATP-induced vasodilations and reactive hyperemia are partially caused by a mediator that activates guanylate cyclase, but not the EDRF produced by ACH.

Summary

Our results provide strong evidence that endothelium-dependent vasodilation occurs in resistance vessels, in vivo. We found that MB inhibits the vasodilator response to ACH and ATP, while ETYA inhibits ACH but not ATP vasodilation. Endothelium-dependent vasodilation appears to contributes to exercise hyperemia during 1.5 Hz stimulation as evidenced by the depression in blood flow by MB and ETYA. Endothelium-dependent vasodilation may contribute to a portion of the hyperemic response at 3 and 6 Hz. However, inhibition by MB at these frequencies also directly depresses muscle oxygen consumption. Endothelium-dependent vasodilation contributes to the peak reactive hyperemic response to 30 seconds of occlusion of arterial blood flow.

Summary and Conclusions

ATP produces a dose-dependent vasodilation in skeletal muscle resistance vessels when given intraarterially. In the current study it was determined that ATP-induced vasodilation is endothelium-dependent. This was demonstrated by: (1) the inhibition of ATP vasodilation by the endothelial cell P2-purinoceptor antagonist $ANAPP_3$ (2) the reduction in ATP vasodilation by methylene blue, an agent that inhibits endothelium-dependent vasodilation. In addition, ATP-induced vasodilation is endothelium-dependent in every tissue that has been tested (Burnstock and Kennedy, 1986), except the rabbit mesenteric artery (Mathieson and Burnstock, 1985). Despite the observation that exogenous ATP is a potent vasodilator in this preparation and in other vascular beds, we found that inhibition of ATP vasodilation does not reduce exercise exercise hyperemia. Therefore, we reject the hypothesis that ATP contributes to free flow steady state exercise hyperemia in the canine hindlimb.

The P2-purinoceptor anatagonist nonphotolyzed $ANAPP_3$ inhibited both ATP-mediated dilation and contraction in the canine hindlimb. At low doses of ATP, $ANAPP_3$ increased the vasodilator response to exogenous ATP. This suggests that exogenous ATP has a constrictor influence, although the dominant response is the observed vasodilation. It has been demonstrated in vitro that ATP produces relaxation of isolated strips or rings of arteries and veins through an endothelial cell-mediated process and contraction through receptors located on the vascular smooth muscle (Burnstock and Kennedy, 1986). In the presence of the endothelium, both relaxation and contraction can occur together

in vitro (Kennedy et al., 1985; White et al., 1985). In vitro, ATP has equal access to endothelial and vascular smooth muscle receptors. In vivo, exogenous ATP given intraarterially could reach endothelial cell receptors to produce vasodilation. However, we found evidence to suggest that exogenous ATP also produces vasoconstriction. Therefore, ATP probably traverses the endothelium to reach P2-purinoceptors that mediate contraction on vascular smooth muscle or ATP produces constriction by some other mechanism in this preparation. It is interesting that $ANAPP_3$ also increased the dilator response to ACH, but not to ADO. A tenable explanation for this is that ACH stimulates release of ATP which exerts a constrictor influence on the ACH-mediated dilation. One possible implication of these results is that ATP can produce both dilation and constriction in this preparation.

In the present study, no evidence for ATP mediating the hyperemic response to exercise was found. Instead, it appears that release of ATP during exercise elicits a vasoconstrictor influence on the hyperemia. This is a novel observation; conclusions from previous studies have suggested a vasodilator contribution of ATP. However, this study is the first where a selective antagonist of ATP receptors was used to test the hypothesis that ATP contributes to exercise hyperemia. Results from these experiments suggest that exogenous ATP produces both dilation and constriction of the canine hindlimb vasculature and that endogenous release of ATP during exercise may elicit a constrictor influence, thus limiting the blood flow response to exercise.

Two pharmacological agents that inhibit endothelium-dependent vasodilation in vitro, also inhibited endothelium-dependent

vasodilation in this preparation. MB and ETYA reduced the vasodilator response to ACH and ATP; these two vasodilators produce endothelium-dependent vasodilation in vitro (Furchgott, 1983) and in vivo (Pohl et al., 1987). MB did not reduce the dilator response to ISO, an agent that is not dependent on the endothelium in other tissues. ETYA did not reduce the dilator response to ADO, an agent that does not depend on the endothelium in the most tissues, including the canine femoral artery. When the blood flow response was compared at equivalent oxygen consumptions of 6 and 10 ml O_2/min/100g before and after either MB or ETYA, there was no difference.

MB and ETYA both decreased the blood flow response during 1.5 Hz exercise. This suggests that an EDRF pathway is contributing to the hyperemic response during 1.5 Hz stimulation. Since MB and ETYA also reduced ACH vasodilation, the EDRF produced during exercise may be similar to that associated with ACH-induced vasodilation. MB decreased the dilator response to ATP, but ETYA did not. Since exercise at 1.5 Hz was inhibited by both MB and ETYA, endothelium-dependent vasodilation associated with ATP is probably different from that of 1.5 Hz exercise. These results further suggest that ATP is not a significant mediator of exercise hyperemia.

It was observed that MB and $ANAPP_3$ both reduced oxygen consumption during 3 and 6 Hz exercise. This was partly due to their effects on blood flow. When blood flows were adjusted during exercise with MB or $ANAPP_3$ to levels observed during control exercise, oxygen consumption increased toward, but did not equal, the control values. This suggests that MB and $ANAPP_3$ depress muscle metabolism directly. The mechanism of this depression was not determined in these experiments. Either or

both the transport of oxygen or oxidative phosphorylation by the muscle were probably depressed.

Antazoline and ATP desensitization were effective inhibitors of ATP dilations, but also reduced dilations to ACH, ISO and HIS. Therefore, no conclusions can be drawn from results with these two agents with regard to the role of ATP in exercise hyperemia. Antazoline inhibited the blood flow response to 2.5 Hz stimulation in a manner that is consistant with blocking a vasodilator pathway. Antazoline reduced HIS vasodilations and HIS has not been tested as a mediator of free flow steady state exercise hyperemia. Therefore, we cannot rule out the possibility that HIS is a significant mediator of 2.5 Hz exercise hyperemia.

The results from this study have several possible physiological implications. First, ATP does not contribute to vasodilation during exercise in the canine hindlimb. Administering ATP to the canine hindlimb produces a both dilator and constrictor response, with the dilation being dominant. However, endogenous release of ATP during exercise apparently elicits a net vasoconstrictor effect. This observation could apply to other agents that produce vasodilation when given exogenously. Due to the interaction between endothelial cells and vascular smooth muscle, the overall response to an exogenous vasoactive agent may not be qualitatively similar to the in vivo response. Secondly, resistance vessels of the canine hindlimb exhibit endothelium-dependent vasodilation in vivo and this pathway contributes to 1.5 Hz exercise hyperemia.

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