ADENOSINE AND EXERCISE HYPEREMIA

By

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ABSTRACT

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I measured adenosine release into venous plasma as an index of interstitial adenosine concentration during free flow exercise hyperemia. Isolated, blood-perfused dog calf muscles were stimulated at 6 Hz for ten minutes with free flow. Plasma samples were collected before, during, and after the exercise period for analysis of plasma adenosine concentration ([ADO]) by HPLC. Adenosine release (R_{ADO}) was calculated as plasma flow times venous-arterial [ADO] difference. R_{ADO} (nmole/min/100g) went from -0.1 ± 0.1 at rest to 6.1 ± 4.2 during 6 Hz exercise. Isoproterenol infusion, which caused an increase in blood flow equvalent to 6 Hz exercise, did not result in increased R_{ADO}. Infusion of the 5'-nucleotidase inhibitor, α, β, methylene adenosine 5'-diphoshate (AOPCP) did not prevent the increase in R_{ADO} during exercise. These results support the hypothesis that interstitial adenosine concentration increases during sustained free-flow exercise and that this results in increased release of adenosine into venous plasma.

Dedication

To my father

and to the loving memory

of my mother

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TABLE OF CONTENTS

LIST OF TABLES

I. LITERATURE REVIEW

A. Introduction

Whenever a skeletal muscle exercises, an increase in blood flow occurs which correlates with the increase in muscle metabolism. With graded exercise intensities, one observes proportional increases in blood flow. This phenomenon is known as exercise hyperemia. The search for the cause of exercise hyperemia dates back to 1877. During the last 100 years, many hypothetical mechanisms have been proposed to explain the phenomenon, yet at present the cause of steady state exercise hyperemia remains a mystery. One hypothesis, which has received much attention, and has not been definitively rejected is the adenosine (ADO) hypothesis. It is the purpose of this thesis to critically test the adenosine hypothesis.

This thesis is directed at the cause of steady state, twitch induced exercise hyperemia, and thus we are not searching for a factor to explain all of the dynamics of exercise hyperemia under all circumstances. It is now well established that the vasodilatory mechanism(s) which are brought into play by the muscle depend(s) on a) the exercise pattern, i.e. twitch vs. tetanic contractions, b) the duration of the exercise bout, c) the relationship between supply and demand of oxygen and d) the muscle fiber type.

The most common pattern of exercise (in the laboratory) is a sustained train of twitch contractions. Such exercise patterns can be maintained for hours in skeletal muscle with high oxidative metabolism, and the increase in blood flow remains sustained. The

mechanism of this sustained increase in flow over many minutes to hours is the subject of this introduction.

In 1877, Gaskell demonstrated that exercise hyperemia could occur normally without the presence of extrinsic nerves. Since then, investigators have searched for local chemical or physical factors to explain exercise hyperemia. First I will review the evidence for the physical factors, and then I will address the chemical factors.

B. The Myogenic Hypothesis

Isolated blood vessels will relax when subjected to a quick decrease in initial length, and they will contract if quickly stretched (Johannson and Mellander, 1975; Sparks and Bohr, 1962). This is known as the Bayliss response, named after its first observer (Bayliss, 1902), and is the basis for the myogenic hypothesis of blood flow regulation. There is no doubt that during certain conditions blood vessels in vivo are exposed to an analogous stimulus for initiation of the myogenic response i.e. changes in transmural pressure. For instance during muscle exercise, extravascular pressure will increase due to muscle tension development, and intravascular pressure will decrease due to the extravascular compressive forces restricting arterial inflow and hastening venous outflow. The net result of these changes is that vascular transmural pressure will fall and in fact it will be reversed. This would then reduce tension in the vessel wall and initiate myogenic relaxation. Experimental evidence lends support for this hypothesis. In a resting muscle exposed to large increases in tissue pressure by external muscle compression, the increased vascular conductance changes elicited can account for one third to one half of the vasodilation

occuring during tetanic exercise (Mohrman and Sparks, 1974). The myogenic response, however, does not appear to play an important role in the exercise hyperemia associated with twitch contractions. Bacchus et al. (1981) found that when they exposed a passive muscle to intramuscular pressure changes that were recorded during twitch contractions (up to 50 mm Hg), vascular conductance failed to increase. Thus the myogenic response may play a significant role in the hyperemia associated with tetanic contractions, however, evidence for its involvement in steady state twitch exercise hyperemia is lacking.

C. The Metabolic Hypothesis

It has been known for many years that changes in muscle metabolism are tightly coupled to changes in muscle blood flow, in the steady state. It has been assumed that a factor related to oxidative metabolism is responsible for the changes in vascular conductance associated with increased muscle work (Kramer et al., 1939). It is difficult to imagine that a parallel mechanism unrelated to metabolism could be so precise, such that the tight correlation between VO_2 and blood flow happened to be just a fortuitous relationship. Mohrman and Sparks (1973) provided additional evidence strengthening the relationship between the two variables by observing that the dynamic changes in VO_2 and vascular conductance were very similar in time course during sinusoidal twitch exercise. Thus factors linked directly to oxidative metabolism may indeed cause the steady state vasodilation associated with twitch exercise. This evidence, however, does not rule out the participation of factors not linked directly to oxidative metabolism. This distinction will be discussed later.

1. Direct effect of oxygen

The venous outflow PO_2 of an exercising muscle falls during exercise. It has also been observed that vascular smooth muscle strips relax when exposed to a hypoxic bathing medium (Detar and Bohr, 1968). These findings led to the hypothesis that a decrease in vessel wall PO_2 during exercise causes the steady state increase in vascular conductance (Guyton et al., 1964). In evaluating this hypothesis the important question is: Does arteriolar vascular smooth muscle PO_2 during free flow exercise fall sufficiently to induce smooth muscle relaxation? Duling (1975) has determined that vessel wall PO_2 is primarily influenced by adjacent luminal blood PO_2. Thus, it is not intuitively obvious that vessel wall PO_2 will fall sufficiently, since flow delivery of oxygen increases during free flow exercise. Sparks (1980) adressed this question on theoretical grounds by the use of a computer simulation compartmental model. The model predicts that as muscle work increases the vessel wall PO_2 first falls, but then as flow delivery of O_2 increases, the vessel wall PO_2 rises. This prediction is in general agreement with the experimental observation that cremaster muscle exercise dilation occurs without a change in periarteriolar PO_2 (Gorczynski and Duling, 1978). These results cast strong doubt on the role of vessel wall PO_2 in the mediation of normal exercise hyperemia.

2. Tissue PO_2

If lack of O_2 itself is not the cause for exercise hyperemia, perhaps O_2 is involved indirectly by altering the rate of an oxygen dependent metabolic process within muscle cells, which then results in the release of some vasoactive metabolite into the interstitium. Alternatively, it is conceivable that the mechanism controlling vascular

conductance becomes activated simultaneously with the activation of oxidative metabolism. Both of these pathways for metabolite production would become activated with increased muscle metabolism and therefore it is difficult to define the exact nature of the stimulus responsible for metabolite release. For example: ATP hydrolysis occurs during muscle contraction. This will result in an increased ADP concentration which stimulates oxidative phosphorylation i.e. ADP+Pi ATP. At the same time AMP concentration will increase by mass reaction through the enzyme myokinase, which catalyzes the reaction: 2ADP=ATP+AMP (McGilvery and Murray, 1969). This in turn may result in adenosine formation by the action of 5'nucleotidase. Thus, coincident with the increase in oxygen consumption is the formation of adenosine, a potent vasodilator metabolite. The other possible stimulus mentioned above for the formation of a vasoactive metabolite is through a decrease in tissue PO_2. As VO_2 increases with exercise the concentration of cellular oxygen declines. This is believed to occur since muscle venous blood PO_2, which is in equilibrium with muscle tissue PO_2, falls during exercise (Sparks, 1980). If cell PO_2 falls to a critical level at which oxidative phosphorylation is reduced, the flux of ADP to ATP will decrease. This results in a greater net flux from ATP to ADP, which will then raise the ADP concentration. Once again AMP and adenosine will increase by the reactions previously described. Although it is not known whether this would occur under free flow conditions, this mechanism will become operable whenever there is an imbalance between the oxygen supply and the oxygen use of the tissue. Several investigators have attempted to determine the relative importance of the latter mechanism in exercise hyperemia. Gorezynski and Duling (1978)

have shown in the suffused cremaster preparation, that if the fall in tissue PO_2 during exercise is prevented by increasing suffusate PO_2, exercise vasodilation is reduced but not abolished. Examination of the off response to exercise reveals that the dynamics for the restoration of vascular conductance is much slower than the return of tissue PO_2. This indicates that the PO_2 decrease associated with muscle exercise determines only a portion of the overall vascular response, and that there exists another mechanism controlling vascular conductance that is unrelated to the level of tissue oxygenation. Mohrman and Sparks (1973) reached a similar conclusion in their experiments in the dog hindlimb. They observed that when sinusoidal twitch exercise was performed with excessive blood flow to the muscle, vascular conductance dynamics were quicker than the oxygen consumption dynamics. Under these conditions it appears that a control system unrelated to VO_2 became operable.

3. PCO_2, pH, and lactate

Increases in tissue PCO_2, [H+], and [lactic acid] all accompany muscle exercise. Since each agent can cause smooth muscle relaxation in vivo and in vitro, all three agents have been proposed to be involved in exercise hyperemia. Based on two lines of evidence, however, only a relatively minor role can be attributed to these agents. The first is that skeletal muscle resistance vessels are not sensitive enough to changes in the concentration of these chemicals that physiologically occur during muscle exercise. Emerson (1974) demonstrated this point by raising the [H+] by increasing [lactic acid] of the blood perfusing a resting muscle, to levels of H+ measured in the venous blood of exercising muscle. Radawski (1975), and Stowe (1975) both present evidence on the relative inability of high PCO_2 and thus increased [H+]

as well, to significantly alter the vascular conductance of resting muscle. The other line of evidence which addresses only H+ and lactic acid is the observation that in patients with monoiodoacetate poisoning or Mcardles syndrome, who are unable to produce lactic acid, exercise hyperemia still occurs (Tobin, 1965). In view of the recent finding that H+ potentiates the vasoactive potency of adenosine (Merrill, 1978), H+ and thus CO_2 and lactic acid, may play a role in sustaining exercise hyperemia through potentiating other vasodilator systems.

4. Osmolarity

In 1967, Mellander and co-workers first proposed a role for tissue osmolarity in exercise hyperemia. Evidence suggests, however, that the relative contribution of this proposed mediator is species specific. For instance the potential for the release of osmotically active particles is greater in cats than in dogs (Scott et al., 1970). Humans are probably somewhere in between (Lundvall et al., 1969). Absolute increases of osmolarity in dog skeletal muscle are relatively small in comparison to the changes that occur in cat muscle at the same work intensity (Scott et al., 1970). In addition, when these venous osmolarity changes are reproduced in a resting muscle with infusions of hypertonic solutions, the vascular conductance changes are significant in cat muscle particularly at the start of the infusion, but trivial in dog muscle (Scott and Radawski, 1971). Another argument against tissue osmolarity playing a significant role in steady state exercise hyperemia in dog and human skeletal muscle is the fact that the initial increases in venous osmolarity at the start of exercise wane after a few minutes while vascular conductance remains elevated (Morganroth et al., 1975; Stowe et al., 1975). Thus tissue osmolarity may be important in cat

muscle, but does not appear to play a significant role in dog or human skeletal muscle. In addition, if tissue osmolarity plays a role in exercise hyperemia, it probably occurs during the first few minutes of exercise but not during the steady state.

5. Potassium

Potassium ion in low concentrations is known to be a good vasodilator (Dawes, 1941). Since it is released into the venous blood during muscle exercise, the potassium ion has been proposed to play a role in exercise hyperemia (Sparks, 1980). In light of the transitory nature of potassium induced vasodilation (Kjellmer, 1965; Chen et al., 1972; Duling, 1975; Gellai, 1974), and the observation that with extended periods of exercise, potassium release wanes with time (Morganroth et al., 1975; Stowe et al., 1975; Brace et al., 1974), potassium release can not be very important during steady state exercise hyperemia. Since potassium is released into the ISF even prior to muscle contraction, a role for potassium in the initiation of exercise hyperemia is more convincing. Ouabain, in concentrations which block the vasodilation of exogenously administered potassium, slows the onset of exercise hyperemia but has no effect on the steady state flows (Chen et al., 1972). Hazeyama and Sparks (1979) have provided additional evidence in favor of potassium in the initiation of exercise hyperemia by demonstrationg that in potassium depleted dogs one of the initial phases of exercise vasodilation is abolished. These authors used a simple diffusion model to calculate the time course of ISF $[K^+]$ after the initiation of exercise. The model predicts that $[K^+]$ in the ISF increases fast enough to be responsible for one of the initial phases of exercise vasodilation.

6. Prostaglandins

Some prostaglandins are potent vasodilators (Bevegard and Oro, 1969), and can be synthesized by the arterial smooth muscle (Terragno et al., 1975). It has recently been proposed that these substances play a role in the local control of blood flow. In support of this, Kilbom and Wennmalm (1976) observed that indomethacin, a prostaglandin synthesis blocker, attenuated the hyperemia of exercising human muscle by as much as 25%. Another group also demonstrated that indomethacin administration severely reduced exercise hyperemia in the canine hindlimb (Janczewsk et al., 1974). They also found measurable release of PGE_2 during exercise. On the other hand, Beaty and Donald (1979), found that indomethacin had no effect on steady state exercise hyperemia of the canine hindlimb. Young and Sparks (1979) confirmed this result by demonstrating that indomethacin did not alter the relationship between VO_2 and blood flow, and in addition observed a dissociation between the release of PGE and the change in vascular conductance. Thus it appears that prostaglandins are not involved in normal exercise hyperemia. They may play a role, however, in the control of vascular conductance under resting conditions, and during restricted flow exercise.

7. Phosphate

In 1970, Hilton proposed that inorganic phosphate caused exercise hypermia based on the observation that it was released from white muscle but not red muscle, consistent with the presence and absence of exercise hyperemia in the two muscles respectively. He also demonstrated that exogenous infusion of inorganic phosphate causes vasodilation which mimics exercise hyperemia. There are problems with this hypothesis.

Many other investigators have not been able to reproduce the latter result. Arterial infusions of highly concentrated inorganic phosphate do not raise vascular conductance of resting muscle significantly (Overbeck et al., 1961; Dobson et al., 1971; Barcroft et al., 1971). Also exercise hyperemia is routinely demonstrated in high oxidative muscle, and as mentioned above there is no evidence that inorganic phosphate is released from this muscle type.

8. Local Neurons

Honig and Frierson (1976) recently proposed a local neurogenic mechanism to account for a substantial portion of exercise hyperemia. This proposal was primarily based on the observation that arterial infusions of local anesthetics blocked the vasodilation associated with short exercise periods at 2 Hz. They concluded that the local anesthetics block ganglion cells which are intrinsic to the arteriolar vessel wall. This viewpoint, however, has not been widely accepted. It is debatable whether the effect of the anesthetics can be attributed to a specific blocking action on the intrinsic neurons. Until this specificity can be demonstrated further evaluation of this hypothesis will be difficult. This interesting hypothesis, however, deserves further investigation.

9. ATP

Abood (1962) first demonstrated that depolarized skeletal muscle cells were capable of releasing ATP. It was subsequently demonstrated, both in man (Forrester and Lind, 1969) and dog (Chen et al., 1972), that exercising skeletal muscle released ATP into the venous effluent draining the muscle. Since the vasoactive potency of the adenine nucleotides had been known for some time (Drury and Szent-Gyorgyi,

1929), a role for ATP in exercise vasodilation was proposed. Although many reports have since appeared favoring this proposal, the ATP hypothesis has also received much criticism. It appears that a major concern of many investigators is the unproven fact that a highly charged molecule such as ATP can get out of normal, intact cells. Regardless of whether or not this can be proven, one observation makes the evidence for the ATP hypothesis equivocal. Collingsworth and Selleck (1974) have demonstrated that when large amounts of ATP are infused into a dog gracilis muscle, perfused with Ringers Locke solution, only negligible quantities of ATP can be recovered in the venous effluent. Thus extracellular enzymes are capable of rapidly destroying extracellular ATP. Thus, it seems very unlikely that the ATP release seen by Forrester and Chen originated from skeletal muscle. It seems more likely that venous ATP came from some cell type (formed element) of the blood. Until the origin of the increased venous ATP seen during exercise can be determined, a role for ATP can not be substantiated.

10. Adenosine

The ADO hypothesis may be stated as follows: The increases in vascular conductance which occur during skeletal muscle exercise are caused by increases in the ADO concentration in the immediate vicinity of the arteriolar vascular smooth muscle. An ideal test of the ADO hypothesis would be to determine whether the changes in vascular conductance during exercise are correlated with changes in ISF [ADO]. Since direct measurements of ISF ADO are currently impossible, researchers have resorted to measuring variables which are thought to reflect changes in ISF [ADO]. One variable which has been extensively used is the total tissue ADO content. The rationale for using tissue

ADO as an index of ISF ADO was that the kinetics for intracellular adenosine deaminase and adenosine kinase were such that it was unlikely that any appreciable free ADO could exist within the cytoplasm. Evidence supporting this idea was obtained with the demonstration that red blood cell ghosts, incubated with a 5 uM concentration of radiolabelled ADO, did not demonstrate any significant free, cellular ADO (Schrader et al., 1972).

In 1964 the ADO hypothesis was formally tested by Berne and associates for skeletal muscle blood flow regulation. In this initial study the authors wished to determine whether skeletal muscle was capable of producing ADO under severly ischemic conditions, as had been demonstrated in heart muscle just one year earlier (Imai et al., 1964). These investigators were unable to detect an increase in ADO levels in anoxic skeletal muscle, and thus concluded that it was unlikely that ADO played a role in skeletal muscle blood flow regulation. The explanation given for the disparate results between heart and skeletal muscle was that in skeletal muscle the predominant pathway of AMP breakdown is to IMP, whereas in heart there is significant degradation of AMP to ADO.

Using a bioassay technique, Scott and co-workers (1965) were the first to find evidence in favor of ADO release from skeletal muscle during ischemic perfusion at rest and exercise. They found that the venous blood from the contracting muscle caused vasodilation in a forelimb but vasoconstriction in a kidney. The only two endogenous substances known to elicit these responses are AMP and ADO. Because of these results Berne and co-workers reopened their investigation of the ADO hypothesis in skeletal muscle. Dobson et al. (1971), were the first to quantitate the changes in muscle ADO levels associated with the

vasodilation caused by a severe ischemic challenge. They found that stimulation of a dog hindlimb preparation at 20-30 Hz, for a period of 5 min without any blood flow resulted in a twofold increase in tissue ADO. After restoring blood flow, at the end of the exercise period, they measured a fivefold increase in the ADO concentration of the venous blood. In a similar study Bockman et al. (1975), reported that crystaloid perfused rat hindlimb preparations were able to release ADO into the venous effluent after two minutes of muscle contraction. The experimental conditions of these early studies were obviously unphysiological. They were important, however, in demonstrating that skeletal muscle indeed had the metabolic machinery necessary for ADO production.

It had been previously determined that in skeletal muscle AMP is degraded primarily to IMP (Imai et al., 1964). This raised some questions about the ADO hypothesis. Recognizing this dillema, Rubio and Berne (1973) performed histochemical studies aimed at localizing 5'Nucleotidase, the enzyme responsible for the formation of ADO from AMP. They determined that the enzyme is localized in the vascular endothelium, and in discrete regions of skeletal muscle tissue near blood vessels. Based on these data the authors suggest that in skeletal muscle ADO can be produced in localized regions near blood vessels where it is needed to cause vasodilation, while in the bulk of the tissue adenine nucleotide degradation may still proceed to IMP.

Bockman et al. (1976) attempted to test the ADO hypothesis under more physiological conditions than had been performed previously. They found that when they exercised dog calf muscle isotonically at 2-4 Hz while perfusing the muscle at constant resting flow, tissue ADO content

was increased at 10 min but not at 5 min. The authors concluded that the data suggest a role for ADO in sustained active hyperemia. It must be noted that although the conditions of this experiment were indeed more physiological than had been previously used, the muscles were perfused at constant low flows and thus these data can not be used as evidence for ADO in normal exercise hyperemia.

Tabaie et al. (1977) tested the ADO hypothesis pharmacologically with the use of theophylline, a competitive antagonist of the ADO receptor. They found that exercise vasodilation was significantly reduced in the presence of theophylline. Since in these experiments flow was also held constant at the resting level, these data offer pharmacological evidence corroborating Bockman's study. Honig and Frierson (1980) repeated the experiments of Tabaie. Instead of infusing a vasoconstrictor to correct for the change in initial resistance due to theophylline, they made an analysis of initial resistance vs. the change in resistance, to predict the resistance change expected with exercise at a lower initial resistance. They concluded that theophylline had no effect on exercise vasodilation, and that the observations of Tabaie could be explained as exercise vasodilation measured in the presence of a vasoconstrictor. In Tabaie's defense, it should be noted that after he administered NE or ADH to correct for the change in resistance due to theophylline, vascular sensitivity was tested by bolus injections of two different vasodilators. He determined that the dilations were no different before or after administration of theophylline and NE or ADH. In light of this controversy it is difficult to conclude what the effect of theophylline is on exercise vasodilation.

To determine whether or not ADO is released during normoxic exercise, Tominaga et al. (1980) infused tracer adenosine and observed the effect of exercise on the appearance of labelled ADO in venous plasma. An equation was derived to incorporate the experimental results and compute an ADO release rate. It is difficult, however, to evaluate the meaning of this equation since close examination of the units reveals that the equation computes a concentration, not a release rate.

The most direct test of the ADO hypothesis for normoxic exercise hyperemia was performed in my laboratory by Phair and Sparks in 1979. In this study tissue ADO content was measured in dog calf muscle exercised at 2 and 6 Hz under free flow conditions. Even with a very sensitive ADO assay they were unable to detect any significant changes in tissue ADO content. Similar results were subsequently found in exercising red and white cat muscle, under free flow conditions (Bockman and Mckenzie, 1979). Recently however, Bockman et al. (1982) observed increases in gracilis muscle ADO content under the same conditions. The studies demonstrating no increase in tissue ADO content during exercise do not support a role for ADO; however, there are problems associated with the use of tissue measurements which preclude rejection of the ADO hypothesis. Measurement of tissue ADO content would be an accurate index of ISF [ADO] providing all the measured ADO were in the interstitial space. Recently it has become evident that a large portion of the ADO measured in tissue is probably not free in the interstitial space; it is possible that as much as 90% of measured tissue ADO is intracellular (Sparks and Fuchs, in press). Evidence suggesting compartmentalization of tissue ADO was found by Schrader and Gerlach (1976). After prelabeling the adenine nucleotide pool of hearts with

labelled precursors, it was found that the specific activity of released ADO was very high in comparison with the specific activity of its tissue precursor pool; suggesting the existence of at least 2 pools of ADO. Schrader also argues that if all the ADO measured in tissue existed in the ISF, based on the vasoactive potency of ADO, blood flow ought to be much greater than has actually been observed. Additional evidence for the compartmentation hypothesis was provided by the observation that the intracellular enzyme S-adenosylhomocysteine hydrolase had ADO binding properties, and thus could protect ADO from degradation by adenosine kinase and adenosine deaminase (Ueland and Saebo, 1979).

Thus changes in tissue ADO content may not reflect changes in ISF [ADO]. In the study by Phair and Sparks (1979), changes in ISF [ADO] may have occurred during free flow exercise, but against a high backround level of compartmentalized ADO these changes might have been undetectable. Another possibility is that changes in ISF [ADO] may have occurred during exercise in a small perivascular space, but against the high backround ADO this might also be undetectable. Evidence to substantiate this latter possibility is provided by the histochemical localization of 5'nucleotidase near skeletal muscle blood vessels (Rubio et al., 1973). Phair and Sparks considered these two possibilities and therefore performed additional experiments to test them. They determined the vasoactive potency of infused ADO and calculated that if a dilatory concentration of ADO had been established during exercise they would have detected it, providing that it existed in a compartment >10% of the ISF. In order to determine the potency of ADO, Phair and Sparks assumed that arterial plasma ADO equilibrated with ISF ADO. Since we are now aware that the endothelium has an extraordinary

capacity to accumulate ADO (Pearson et al., 1978), this assumption may not be valid. The ramifications of this are the following: if the vascular endothelium represents a metabolic sink for ADO, then a determination of the vasoactive potency of ADO based on exogenous ADO infusions may be a gross underestimation. This would make the detection of vasoactive ADO concentrations impossible given the current sensitivity of tissue ADO measurements.

To summarize: Given the result that tissue ADO content did not increase during 10 min of free flow exercise, there are three alternative explanations which preclude rejection of the ADO hypothesis. First, the concentration of ADO in the interstitium necessary to cause exercise vasodilation might be too low to cause a detectable increase in tissue content, given the possibility of a relatively high backround intracellular content. Second, vasoactive ADO could be confined to a small perivascular region surrounding blood vessels, leaving most of the interstium free of ADO. Again, it might be impossible to detect this small compartment of ADO in a whole tissue measurement. Third, ADO may be released during exercise from preformed intracellular stores. One might expect to find a decrease in tissue ADO, due to the subsequent metabolism of the released ADO.

Although these hypotheses represent three different explanations for which a role for ADO can be reconciled, given the negative results obtained from tissue measurements, they all have one feature in common i.e. they all specify that a perivascular, vasoactive [ADO] is established during exercise. I reasoned that if in fact this occurs, some of the perivascular ADO should diffuse through the capillary wall

and enter the capillary plasma. Further, this released ADO ought to appear in the venous blood, providing the blood doesn't completely degrade the ADO. It was recently shown in our laboratory, that when a good "stop" solution is used, blood degradation of ADO is slow enough to allow its quantitation in plasma by HPLC (Manfredi and Sparks, in press). With this in mind I felt a more rigorous test of the ADO hypothesis could be performed. It was the purpose of this thesis to test the following hypothesis. The increases in vascular conductance during 6 Hz free flow exercise are caused by increased ISF [ADO]. I have tested the hypothesis by measuring arterial and venous plasma [ADO] and calculating ADO release. This test is based on the assumption that increased ISF [ADO] results in increased release of ADO into venous plasma which can be detected by increases in adenosine release. (ADO release measurements can be interpreted independently of the nature of the cellular ADO compartment of muscle).

II. MATERIALS AND METHODS

A. Canine Hindlimb Preparation

Male mongrel dogs weighing between 15 and 40 kg were used. Anesthesia was produced by intravenous sodium pentobarbital (30 mg/kg) supplemented throughout the experiment. To prevent clotting, an initial dose of heparin (750 units/kg) was administered just prior to cannulation of the muscle preparation, with hourly supplements of 100 units/kg. The dogs were ventilated so as to maintain arterial PCO_2 within the normal range. If necessary, the inspired room air was supplemented with 100% O_2 to bring the arterial PO_2 into the normal range. When metabolic acidosis (pH < 7.35) occurred, it was corrected by an intravenous drip of 1.5% $NaHCO_3$. Blood gases were monitored throughout the experiment and were maintained within the range of normal values supplied by Feigl and D'Alecy (1972). Esophageal temperature was maintained between 37-39°C with thermostatically controlled heating pads.

I used an isolated calf muscle preparation which we have previously described in detail (Mohrman and Sparks, 1973). The hindlimb was skinned by electrocautery, and the paw was vascularly isolated by ligating the anterior tibial artery and by securely tightening a hose clamp around the tibia and overlying tendons just proximal to the paw. The thigh muscles surrounding the femur were transected just proximal to the knee, as were all other structures in that region except for the major artery and vein. All small branch vessels not entering or exiting the muscles were ligated and cut. Two holes were drilled in the femur; the first was used for plugging the marrow with petroleum jelly-soaked

cotton balls, and the second for mechanically anchoring the limb. The sciatic nerve was ligated and cut, and the peripheral end was placed on bipolar silver electrodes. The portion of the calcaneus to which the gastrocnemius tendon inserts was transected and firmly attached to a specially adapted Grass force transducer for the measurement of gastrocnemius tension. Muscle length and stimulus voltage were adjusted to give maximum tension. The stimulation parameters were 2-5V, 0.2 msec and 6 Hz. These stimulus parameters excite skeletal muscle motor fibers but not sympathetic fibers, as confirmed by the absence of a vascular response after somatic neuromuscular blockade. The popliteal vein was cannulated just proximal to its bifurcation. The open end of the venous cannula, which was no more than 5 cm above the popliteal vein, emptied into a reservoir funnel from which a roller pump returned the blood to the contralateral femoral vein. The contralateral femoral artery supplied blood for perfusion of the calf through tne cannulated popliteal artery. A constant pressure pump (Mohrman, 1980) and a Zepeda electromagnetic flow probe were interposed in the arterial perfusion line. The flowmeter was calibrated by linear regression of multiple timed collections of venous outflow on the corresponding oscillograph pen deflections. Perfusion pressure was monitored at the tip of the perfusion cannula via a Statham pressure transducer. Side taps in both the arterial and venous lines were used to sample blood for the analysis of oxygen content and adenosine concentration. The calf, as well as other skinned tissue exposed to the air, was wrapped in saline-soaked gauze and covered with Saran wrap to prevent evaporation. The ipsilateral brachial artery was cannulated for the measurement of arterial blood pressure and heart rate. After completion of the

surgery, the preparation was allowed approximately 15 minutes for equilibration. Mean and pulsatile blood pressure, perfusion pressure, gastrocnemius tension and blood flow were continuously recorded on a Grass Model 7 Polygraph.

B. Protocol

Before beginning the experiment, arterial blood gases, pH and hematocrit were measured and adjusted to the normal values if necessary. Two 3 ml blood samples were simultaneously drawn from the arterial and venous lines for the measurement of plasma adenosine concentration. In addition, a sample was drawn to determine the recovery of adenosine added to the sample, and another sample was treated with adenosine deaminase to check the specificity of the adenosine assay. Handling and processing of these samples is described below. Arterial and venous blood samples were drawn anaerobically for the determination of PO_2, PCO_2, pH, hematocrit, and hemoglobin content. Venous outflow was then measured by timed collection, and the occlusive zero of the electromagnetic flowmeter was checked by turning off the pump.

In five dogs, two experimental maneuvers were performed in randomized order:

1. 6 Hz free flow exercise

The muscle was stimulated at a rate of 6 Hz for a ten minute period during which the perfusion pressure was maintained constant at 100 mmHg. After ten minutes, blood samples were drawn as described above. A timed collection of the venous outflow from the muscle was performed. For a period of at least five minutes prior to sample collection, as well as during the sampling period, muscle blood flow was steady. The exercise

was then terminated and I waited until flow returned to control and took blood samples for blood gas and alveolar determinations.

2. Isoproterenol infusion

Isoproterenol was infused intraarterially proximal to the pump at a rate which elicited flow increases similar to 6 Hz exercise hyperemia. The doses infused ranged from 3 to 28 μg/min. As soon as a steady state was reached with respect to blood flow, blood samples were collected as previously described. At the conclusion of each experimental intervention, flow was allowed to return to resting levels. I then waited at least ten additional minutes, and collected post-control blood samples.

In six additional experiments, the effect of α,β-methylene adenosine 5'-diphosphate (AOPCP) was studied. In all dogs AOPCP (50 μM) was added to the collecting tubes for control blood samples. In four dogs AOPCP was also added to the collecting tube for the blood samples obtained during exercise. In two of these dogs and in two others, AOPCP was infused so as to establish a plasma concentration of AOPCP ranging from 20 μM to 87 μM 5 minutes after starting the exercise period. After ten minutes of exercise, and thus five minutes of AOPCP infusion blood samples were collected.

After completion of the post-control period, the animals were euthanized with an overdose of pentobarbital. The calf muscles were removed and weighed so that blood flow could be normalized to the mass of tissue perfused. The tissue weights did not differ from the weights of the contralateral muscles.

C. Sample Preparation and Adenosine Assay

Blood samples for adenosine analysis were gently drawn into 3 ml syringes and were dispensed into tared collecting tubes on ice. Each of these tubes contained 250 µl of a collection solution containing dipyridamole (26 µM), erythrononylhydroxyadenosine (EHNA) 3 µM, and methanol (5%) in isotonic saline. In seven experiments of series I, AOPCP (50 µM) was also included in some tubes. For the recovery sample in each experimental period, the collection solution also contained approximately 0.3 nmole adenosine, which was sufficient to raise plasma adenosine concentration in collected samples to about 0.30 µM. Less than thirty seconds elapsed from the beginning of sample collection until the sample was mixed in the collection solution. Blood samples were centrifuged and one ml of supernatant was added to 250 µl 35% $HCLO_4$, vortexed, and placed on ice. This procedure was followed for all samples except for those treated with adenosine deaminase (ADA). The collection solution for these samples contained 0.05 mg ADA (Sigma, Type I) in place of EHNA. These samples were incubated ten minutes at room temperature and then treated like the other samples. After treatment with $HCLO_4$, samples were centrifuged at 32,000 x g for fifteen minutes. The supernatant was decanted and neutralized with approximately 110 µl of 7 M K_2CO_3. Samples were again centrifuged to remove the resulting precipitate and 700 µl of supernatant was stored at -20°C until analyzed.

Adenosine was assayed by high pressure liquid chromatography (HPLC). One hundred l samples were injected onto either a uBondapak C18 (Waters Associates) or a partisil-5 ODS (Whatman, Inc.) column. A linear gradient of 70/30 methanol/water (v/v) against 4mM KH_2PO_4 began

with 0% and ended twenty minutes later with 40% 70/30 methanol/water. Column flow was usually 1.5 ml/min. For the analysis of certain experiments, slightly different flows (1.2 to 1.8 ml/min) gave better resolution of the adenosine peak. This adenosine assay has adequate sensitivity and precision (Manfredi and Sparks, in press). The limit of the sensitivity of the assay is approximately 4 pmoles of ADO in a water solution.

The adenosine absorbance peak (at 254 nm) was identified by (1) correspondence of its retention time with that of standards, (2) absence of a corresponding peak in ADA-treated samples taken during the same period, and (3) an appropriately larger corresponding peak in recovery samples collected at the same time. Any period in which the recovery sample concentration was less than 80% of the predicted value was rejected. All periods in which ADA samples gave a peak greater than 30% of the corresponding adenosine peak in paired, untreated samples were rejected. When an ADA sample exhibited a measurable peak less than 30% of its paired sample, the residual peak was subtracted from the paired peak. Frequently an unknown substance which absorbs at 254 nm co-eluted with adenosine, precluding adequate resolution of the adenosine peak. Approximately one-third of the experiments analyzed could not be accepted for this reason. Occasionally, such interference was present in one period but not others in the same animal.

D. Data Analysis and Statistic

Adenosine release (R_{ADO}) was calculated as the venous minus arterial difference in plasma adenosine concentration multiplied by plasma flow. Blood O_2 content was calculated from PO_2, pH and

hemoglobin concentration using a nomogram for dog blood. Oxygen consumption (VO_2) was calculated from arterial and venous O_2 content and blood flow. In each animal, the data from an experimental intervention were accepted only when acceptable peaks were obtained from the intervention samples and either the pre- or post-intervention control samples. In cases where both the pre- and post-intervention control samples bracketing an intervention were successfully analyzed, the results were averaged to give one control value. Each preparation was used for either one or two interventions (exercise, isoproternol, exercise plus AOPCP). Because not all experimental periods yielded acceptable adenosine measurements, data were grouped according to intervention rather than paired for each preparation. Grouped data were analyzed for statistical significance using Student's t-test. Arterial and venous plasma adenosine concentrations were compared using the paired t-test. Values stated in the text are means $\pm$ standard deviation.

III. RESULTS

A. Adenosine release during free flow at 6 twitches/sec.

Adenosine release during rest and steady state exercise is shown in Table I. Exercise caused vascular conductance to increase 14-fold and VO_2 to increase 55-fold. R_{ADO} increased from -0.1±0.1 to 6.6±4.6 nmoles/min/100g.

B. Adenosine Release during infusion of Isoproterenol.

We wished to test whether the increase in R_{ADO} seen with exercise would occur if blood flow were increased in the absence of exercise. We evaluated this possibility by infusing enough isoproterenol to raise blood flow as much as did 6 Hz exercise. The results of this experiment performed on four animals are presented in Table I. Isoproterenol caused larger changes in blood flow than were observed with exercise, but neither increased VO_2 nor release of adenosine was observed.

C. Effect of AOPCP on release of adenosine during exercise.

Another possibility for the observed exercise-induced R_{ADO} is that the increased venous plasma [ADO] resulted from the release of adenine nucleotides from formed elements in blood (e.g., platelets) and their subsequent degradation to adenosine via ecto-5'-nucleotidase. This is a possibility because our sample collecting solution prevents adenosine removal from plasma but not its formation. We reasoned that if this possibility is correct, addition of an inhibitor of ecto-5'-nucleotidase, AOPCP, to the collecting solution should prevent the increases in venous adenosine. We tested this hypothesis in four

experiments by repeating the exercise protocol with AOPCP in all the collecting tubes in a concentration (50 μM) which greatly inhibits formation of adenosine from AMP over the time period used in this experiment (Burger and Lowenstein, 1970; Shutz et al., 1981). The values obtained for R_{ADO} in these experiments would then represent only that adenosine which has been formed prior to its mixture with the collection solution. Results from these experiments are presented in Table II. The increases in blood flow and VO_2 were not significantly different from the remainder of the preparations (Table I). When AOPCP was present in the collection tubes, R_{ADO} increased from 0.2±0.2 to 3.8±2.4 nmole/min/100g during exercise ($p < .05$). This R_{ADO} was not significantly different from the value without AOPCP (6.6 ± 4.6 nmol/min/100g).

D. R_{ADO} in the presence of AOPCP infusion.

Given the results of the previous experiment, we reasoned that the remaining ADO released during exercise may be the result of the extracellular degradation of released adenosine nucleotides by ecto-5'-nucleotidase occurring during intravascular transit through the muscle. We tested this possiblility by establishing 20-87 μM AOPCP in arterial plasma during exercise. Arterial samples were collected distal to the infusion site, so that AOPCP was present in both arterial and venous samples. Exercise produced statistically significant increases in flow and VO_2 in the presence of an AOPCP infusion (Table II). These increases were not significantly different from the values obtained in

the absence of the AOPCP infusion. R_{ADO}, 4.3±2.7 nmole/min/100g, was significantly higher than rest, but also was not different from R_{ADO} in the absence of AOPCP.

Table I. Effect of 6 Hz twitch exercise and isoproterenol on blood flow VO_2 and adenosine release of the canine hindlimb perfused at free flow.

Date	Period	Flow ml/100g·min	VO_2 ml/100g·min	Venous PO_2 mm Hg	Plasma [ADO] (M) A	V	RADO nmoles/100g·min
4/9	CNL	8.5	.193	56	.075	.076	-.005
	EX	100.0	13.9	20	.060	.153	5.330
	ISO	122.0	.18	80	.045	.062	1.21
4/22	CNL	7.7	.28	50	.055	.046	-.011
	EX	112.1	15.9	22	.026	.179	10.04
	ISO	130.9	1.3	89	.025	.028	.178
5/20	CNL	8.2	.264	44	.100	.074	-.138
	EX	122.5	14.4	27	.048	.092	3.300
	ISO	--	--	--	--	--	--
5/22	CNL	14.7	.40	47	.042	.036	-.048
	EX	153.5	13.7	29	.020	.040	1.81
	ISO	239.6	.45	96	.103	.076	-3.494
5/27	CNL	7.4	.18	47	.111	.049	-.268
	EX	156.3	14.8	30	.072	.215	12.62
	ISO	179.5	.18	90	.079	.041	-3.757
AVG	CNL	9.3 ± 3.0	$.263\pm.09$	49 ± 5	$.077\pm03$	$.056\pm.02$	$-.094+.11$
AVG	EX	129.0 ± 25*	$14.5\pm.90$*	26 ± 4*	$.045\pm.02$	$.136\pm.07$*	6.62 ± 4.6*
AVG	ISO	168.0 ± 54*	$.53\pm.53$ +	89.0 ± 7*+	$.063\pm.04$	$.052\pm.02$	-1.47 ± 2.5+

All values expressed as mean $\pm$ SD. * designates significant change from control ($P<0.05$).
+ designates significant difference between EX and ISO ($P<0.05$).

Table II. The effect of AOPCP on the changes of blood flow VO_2 and RADO associated with 6 Hz twitch exercise.

Date	Period	Flow ml/100g·min	VO_2 ml/100g·min	Venous PO_2 mm Hg	Plasma [ADO] (M) A	V	RADO nmoles/100g·min
6/17	CNL	11.9	.213	49	.030	.027	-.020
	EX	124.1	13.12	28	.008	.041	2.211
	EX/INF	--	--	--	--	--	--
6/30	CNL	27.6	.336	59	.035	.061	.416
	EX	167.6	17.28	26	.037	.067	2.967
	EX/INF	131.5	18.17	22	.109	.136	2.025
7/15	CNL	10.8	.181	57	.073	.068	-.037
	EX	122.4	19.32	19	.026	.136	7.370
	EX/INF	106.2	17.26	23	.081	.121	2.104
7/16	CNL	27.8	.252	66	.026	.050	.358
	EX	143.8	12.58	30	.018	.049	2.544
	EX/INF	--	--	--	--	--	--
7/23	CNL	10.9	.212	55	.029	.098	.317
	EX	--	--	--	--	--	--
	EX/INF	138.5	16.35	30	.045	.128	5.670
8/11	CNL	5.8	--	--	0.00	0.00	0.00
	EX	--	--	--	--	--	--
	EX/INF	109.9	--	--	.033	.151	7.458
AVG	CNL	15.8 ± 10	$.239 \pm .06$	57 ± 6	$.032 \pm .02$	$.05 \pm .03$	$.172 \pm .21$
AVG	EX	139.5 ± 21*	15.57 ± 3.3*	26 ± 5*	$.022 \pm .01$	$.073 \pm .04$	3.773 ± 2.4*
AVG	EX/INF	121.5 ± 16*+	$17.26 \pm .9$*+	25 ± 4*+	$.07 \pm .03$	$.130 \pm .01$*+	4.314 ± 2.7*

All ADO collection tubes contained ~ 50 M AOPCP. All values expressed as mean $\pm$ SD.
* designates significant change from control ($P<0.05$).
+ designates significant difference between EX with and without AOPCP ($P<0.05$).

IV. DISCUSSION

My experimental results demonstrate the following: (1) There is a significant increase in R_{ADO} during sustained normoxic exercise hyperemia. (2) Increased blood flow caused by isoproterenol is not associated with increased R_{ADO}. (3) Exercise results in increased R_{ADO} even in the presence of an ecto-5'-nucleotidase inhibitor, AOPCP.

A. Stimulus For ADO Production

Previous studies have demonstrated that adenosine release increases during exercise when flow is restricted. Using a bioassay technique, Scott et al. (1965) provided evidence that adenosine (or AMP) is released from dog skeletal muscle tissue during ischemic exercise. Release of adenosine from severely ischemic muscle was then confirmed using chemical methods (Rubio et al., 1973). Release of adenosine was again demonstrated by Tominaga et al. (1980) under constant flow (but less severe) conditions. Tissue content of adenosine also increases during exercise when flow is held constant (Bockman et al., 1976). Although it is well established that hypoxia represents a sufficient stimulus for cellular increases in ADO production, these data can not be used to support a role for ADO during free flow, exercise hyperemia. It is quite clear that under the conditions of severe cellular hypoxia, oxidative phosphorylation can not keep pace with the enhanced ATP hydrolysis associated with muscle contraction. This leads to the depletion of creatine phosphate stores, and quickly the [ADP] increases. By mass action through the myokinase reaction, an increase in [AMP] occurs (McGilvery and Murray, 1974). This supplies substrate for

5'Nucleotidase, for the subsequent formation of ADO. Further, 5'Nucleotidase activity is simultaneously enhanced by the decrease in [CrP] and [ATP] (which normally inhibit the enzyme), as well as the increase in free [Mg++] (Rubio et al., 1979).

The important question remains: Can we invoke a role for ADO in the vasodilation seen with physiological exercise. Or more specifically: If contracting muscle cells are allowed adequate amounts of O_2 to support ATP resynthesis, as occurs during free flow exercise, is there an adequate stimulus for enhanced ADO production. This question has been recently answered for heart muscle (Manfredi and Sparks, 1981). These investigators found that the increase in oxygen consumption associated with atrial pacing, was not accompanied by changes in ADO production. When a similar increase in VO_2, however, was induced by intravenous norepinephrine administration ADO production was increased. It was concluded that increases in oxygen consumption per se are not necessarily associated with increases in ADO production, and that a parallel activation of adenylate cyclase is a necessary prerequisite for enhanced ADO production.

Although highly oxidative skeletal muscle bears many similarities to heart, nicotinic receptor stimulation of skeletal muscle is not associated with adenylate cyclase activation (Posner et al., 1965). Thus if I assume that my data are indicative of enhanced muscle ADO production, the nature of the stimulus for this enhanced production remains to be answered. Whatever the pathway is, it is unlikely that it involves the cAMP cascade. I hold this view since when I infused

isoproterenol into my muscle preparation, which is known to stimulate cAMP production (Posner et al., 1965), we did not see increases in ADO release.

B. Interpretation of ADO Release Measurements

There has been scanty evidence favoring increased interstitial adenosine during exercise with free flow. Phair and Sparks (1979) measured tissue adenosine content of dog anterior calf muscle and did not find a significant increase. Bockman and McKenzie (1979) reported no increase in tissue adenosine of either red or white feline muscle during free flow exercise. However, this same group recently reported increased ADO content of dog gracilis muscle during free flow exercise (Bockman et al., 1982).

This thesis reports the first data showing that R_{ADO} increases during free flow exercise. Before reaching the conclusion that the observed increase in R_{ADO} is the result of increased interstitial ADO concentration, I must consider the other factors which may influence our measurement of R_{ADO}. These include (1) the capillary transport of adenosine, (2) plasma flow, (3) uptake and metabolism of adenosine by red blood cells and (4) the release of ADO or its precursor, ATP, directly into plasma from cells (formed elements) of the blood.

I believe that my experiments with isoproterenol rule out the possibility that the increased R_{ADO} observed during exercise was the direct result of increased plasma flow or capillary surface area. The flow increase caused by isoproterenol was as great as that caused by exercise and yet no R_{ADO} occurred. Although I have no direct measure of the increase in capillary surface area caused by isoproterenol in our

preparation, such an increase during beta-adrenergic stimulation has been demonstrated by other investigators (Lundvall and Janhult, 1976).

Red blood cells take up and metabolize adenosine. In the case of dog blood, however, they do so at a relatively slow rate (Manfredi and Sparks, in press). Therefore, they cannot be a major factor determining the measured release of adenosine in these experiments.

If adenosine is deposited in plasma from a source other than the interstitium, R_{ADO} cannot be used as an indicator of interstitial [ADO]. One possibility is that ATP is released into the venous plasma during exercise (Forrester and Lind, 1969). For many years Forrester has been reporting that during muscle exercise, there is an increase in venous [ATP]. If this also occurs in my experiments, ATP degradation to adenosine in blood could result in increased R_{ADO}. For reasons previously discussed (see introduction), I feel that the increased ATP that is measured in the venous blood during exercise probably originates from the formed elements in the blood. If passage through an exercising muscle increases the tendency of a formed element to release ATP, collection of blood itself could serve as the major stimulus for release of ATP in venous samples. Regardless of the origin, however, venous ATP has the potential to break down to ADO in my experiments since my collection solution stops adenosine removal from plasma but not its formation.

I tested this possibility by adding an inhibitor of ecto-5'-nucleotidase, AOPCP, to the collection solution. AOPCP (50 μM) inhibits adenosine formation from AMP by least 85% (Burger and Lowenstien, 1970; Shutz et al., 1981). When I added 50 μM AOPCP to my collection solution, R_{ADO} still increased significantly during 6 Hz

exercise. However, there was a statistically insignificant ($p = .30$) decrease in R_{ADO}. This could have meant that most of the nucleotide degradation occurred during intravascular transit before reaching the collection tubes. I tested this hypothesis by infusing AOPCP (plasma concentration, 20-87 μM) during exercise. Even in the presence of an AOPCP infusion, exercise was still associated with significant increases in R_{ADO} (Table II). In addition, there was no further reduction in R_{ADO} during exercise than when AOPCP was present only in the collection tubes. Even when all the AOPCP data are combined there is not significantly less R_{ADO} during exercise as compared to experiments in which no AOPCP was used. Thus, I conclude that the increased adenosine found in venous plasma during exercise is not primarily the result of blood degradation of adenine nucleotides via ecto-5'-nucleotidase.

Another possibility is that ADO itself is released from the formed elements of the blood, or from the vascular endothelium during exercise. Formed elements do not contain any significant free ADO, however, under conditions of hypoxia they can produce it (Edwards et al., in press; Matsumoto et al., 1979). Since these cells probably never become hypoxic during exercise, it seems unlikely that they could be a direct source for ADO release during exercise. A more likely mechanism is that they release their stores of adenine nucleotides, due to some trauma associated with exercise, which are then degraded to ADO in blood. As discussed above, I have experimentally ruled out this possibility with my AOPCP experiment (Table I).

Endothelial cells, on the other hand, contain a rich store of ADO which is believed by some to include a free, unbound pool. In fact cultured endothelium has been shown to release ADO under basal

conditions (Nees and Gerlach, in press). One can argue then that if in vivo endothelium behaves like its cultured counterpart and releases ADO at a rate proportional to a concentration gradient across its membrane, then the increased plasma flow during exercise could be responsible for maintaining this gradient and therefore could explain the increased release of ADO during exercise. I can refute this argument, however, on the basis that my isoproterenol induced increases in flow were not associated with any significant ADO release (Table II). Cultured endothelium has been shown to release ADO at a faster rate when exposed to hypoxia (Nees et al., 1979), or under catecholamine stimulation (Nees and Gerlach, in press). Under the conditions of free flow exercise however, the endothelium is never exposed to hypoxia (Duling and Pittman, 1975). Also my isoproterenol experiment suggests that the observed ADO release is not catecholamine mediated (Table III).

The above considerations lead to the conclusion that it is very likely that the observed increase in R_{ADO} is the result of an increase in interstitial ADO concentration, even though an increase in tissue ADO content was not observed in an earlier study from my laboratory (Phair and Sparks, 1979). An explanation of this paradox would be that the increase in interstitial adenosine is too small to be detected in measurements of total tissue adenosine content. The following calculations are meant to gauge the likelihood that an undetectable (in total tissue) increase in interstitial adenosine could result in the observed release of adenosine during exercise.

Let me assume that the relationship between interstitial adenosine and arterial and venous plasma adenosine is determined by the capillary model of Sheehan and Renkin (20):

$$[ADO]_{ISF} = \frac{[ADO]_a e^{-PS/F} - [ADO]_v}{e^{-PS/F} - 1}$$

where:

$[ADO]_a$ = arterial plasma concentration of adenosine

$[ADO]_v$ = venous plasma concentration of adenosine

PS = capillary permeability surface area product for adenosine (I will use the value obtained for sucrose)

F = plasma flow

At rest, when $[ADO]_a$ is 0.08 ± 0.03 and $[ADO]_v$ is 0.06 ± 0.02 μM. If I assume PS = 5 ml min^{-1} $100g^{-1}$ and F = 5 ml min^{-1} $100g^{-1}$, $[ADO]_{ISF}$ is 0.05 μM. If this concentration is present in 0.15 ml of interstitial fluid per g of skeletal muscle, the tissue content would be 0.007 nmoles/g. A much higher amount, $1.1 \pm .22$ nmoles/g is observed (Phair and Sparks,1979). During exercise, using the same model with F = 70 ml min^{-1} $100g^{-1}$, PS = 20 ml min^{-1} $100g^{-1}$, $[ADO]_a = 0.05 \pm 0.02$ μM and $[ADO]_v = 0.14 \pm 0.07$ M, I find that $[ADO]_{ISF}$ = 0.41 μM. This concentration would account for 0.06 nmoles/g in tissue. Phair and Sparks measured a tissue ADO content of 1.5 ± 0.6 nmoles/g during exercise. Once again the portion of total tissue adenosine contributed by the interstitium could be lost in the noise of the tissue measurement.

In summary, because the concentration of adenosine measured in arterial and venous plasma is so low, my estimates of interstitial adenosine concentration are very low. My calculated increase in $[ADO]_{ISF}$ (0.05 to 0.41 μM) would not be detectable even if it were distributed in the entire interstitium. This calculation of tissue

content from measurements of arterial and venous adenosine concentration may not be correct because the model of adenosine transport may be vastly oversimplified. However, if our calculation is accurate, it suggests that most (>90%) of the adenosine measured in tissue must not be in the interstitial space. This fits well with the current concepts concerning compartmentation of adenosine in the heart (Olsson et al., 1979; Schrader and Gerlach, 1976; Shutz et al., 1981).

My data do not allow me to rule out the possibility that adenosine is limited to a relatively small perivascular space. This could be the result of localized release of adenosine from skeletal muscle cells (Rubio et al., 1973). Another possibility already discussed, is that adenosine is released from a cellular element of the vascular wall, e.g., endothelium (Nees et al., 1979). Either of these mechanisms could result in the release of adenosine observed in my experiments, but might not lead to a detectable rise in total tissue content.

C. Significance Of ADO Release Measurements

Assuming that I am correct in concluding that the ISF [ADO] increased during exercise, eventually I will want to know whether it is great enough to be responsible for exercise vasodilation. Dose response curves from intraarterial infusion would suggest that an arterial plasma concentration of greater than 10 μM adenosine is necessary to produce the vasodilation observed during exercise (Phair and Sparks, 1979). This is much higher than the ISF concentration of ADO which I have calculated above (0.41 μM). A few years ago this analysis would have probably led to a premature rejection of the ADO hypothesis. Currently, however, the capability of endothelial cell uptake of ADO is now greatly

appreciated (Pearson et al., 1978). Because of this, both my estimate of ISF [ADO] as well as the vasoactive potency of ADO are suspect, since both of these estimates were determined with the assumption that the endothelium represents only a passive barrier for ADO diffusion. Providing that ADO is not transported completely through the endothelial cell, the greater the capacity for endothelial cell uptake the closer those two estimates will be. If this was the case ADO might be quite important in exercise vasodilation. On the other hand, if transendothelial ADO transport exists, the two estimates may differ by an even greater amount and thus the importance of ADO might be negligible. Till more is learned about the role of the endothelium in the transport of ADO, it will be impossible to predict the relative importance of ADO in the vasodilation associated with free flow exercise.

V. SUMMARY AND CONCLUSIONS

In conclusion, increased R_{ADO} occurs during vigorous sustained exercise. This probably is the result of an increase in interstitial adenosine concentration. This increase in interstitial adenosine concentration could be confined to a small perivascular region, or distributed in the entire interstitium. Until more is known about the nature of capillary transport of adenosine, it will be difficult to state the quantitative importance of increased adenosine release during exercise vasodilation.

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