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Adenosine Release into Venous Plasma during Free Flow Exercise (42266)

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Abstract. We 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 10 min at 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/100 g) went from -0.1 ± 0.1 at rest to 6.6 ± 4.6 during 6-Hz exercise. Isoproterenol infusion, which caused an increase in blood flow equivalent to 6-Hz exercise, did not result in increased R_{ado}. Infusion of the 5'-nucleotidase inhibitor, α, β, methylene adenosine 5'-diphosphate (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 twitch exercise and that this results in increased release of adenosine into venous plasma.

Adenosine has been proposed as a mediator of exercise vasodilation in skeletal muscle. One of the criteria of acceptance for such a mediator is that its concentration must reach vasodilator levels in the vicinity of resistance vessels during exercise. Several studies have addressed this question by measuring the tissue content of adenosine. Tissue adenosine content increases during ischemic contractions and during exercise with restricted blood flow (1–4). This is taken as evidence that adenosine reaching vascular smooth muscle is increased. The results for tissue adenosine during free flow exercise are not so clear. Steffen *et al.* reported that tissue adenosine increases in canine gracilis muscle during 1- and 2-Hz contractions (5). In a previous study, we exercised canine calf muscles at 2 and 6 Hz for 10 min under free flow conditions and were unable to detect a significant change in the tissue content of adenosine (3).

The measurement of tissue adenosine content assumes that most of the adenosine measured in a tissue sample is distributed in the extracellular space [see Ref. (3) for the argument in favor of this assumption]. It has since become apparent, however, that much of the adenosine measured in tissue may not be free in the interstitium (6–8). If this is the case there may be a high "background" concentration of adenosine which does not have access to vascular smooth muscle. This means that it is possible that interstitial fluid adenosine concentration did rise in our experiments, and that we were unable to detect the increase against a high background concentration of nonvasoactive adenosine. An alternative possibility is that vasoactive adenosine could be confined to a small perivascular region (9). Again, it might not be possible to detect this small amount of adenosine in a whole tissue content measurement.

The present study is a further test of the possibility that increased adenosine is present in a perivascular compartment during exercise. We reasoned that if adenosine concentration increases in a region immediately around capillaries, some of it should appear in venous plasma draining the muscle. For this reason, we measured adenosine release into venous plasma during sustained twitch exercise.

Methods. We used 11 male mongrel dogs (15–40 kg), anesthetized with intravenous sodium pentobarbital (30 mg/kg), supplemented as required. 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

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and were maintained within the range of normal values supplied by Feigl and D'Alecy (10). Average arterial blood gases just before the exercise bout were PO_2, 103 ± 2 mm Hg and PCO_2, 36 ± 1 mm Hg; and pH was 7.42 ± 0.01. Esophageal temperature was maintained between 37 and 39°C with thermostatically controlled heating pads.

We used an isolated hindlimb muscle preparation which we have previously described in detail (11). 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 2–5 V, 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 skeletal 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 the cannulated popliteal artery. A constant pressure pump (12) 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 a saline-soaked gauze and covered with Saran wrap to prevent evaporation. The preparation was maintained at 35–37°C using an incandescent lamp. A brachial artery was cannulated for the measurement of arterial blood pressure and heart rate. After completion of the surgery, the preparation was allowed approximately 30 min for equilibration. Mean and pulsatile systemic pressure, muscle perfusion pressure, gastrocnemius tension, and blood flow were continuously recorded on a Grass Model 7 polygraph.

Protocols. 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. The handling and processing of these samples are 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 the muscle was stimulated at a rate of 6 Hz for a 10-min period during which the perfusion pressure was maintained constant at 100 mm Hg. After 10 min, blood samples were drawn as described above. For a period of at least 5 min prior to sample collection, as well as during the sampling period, muscle blood flow was steady. The exercise was then terminated and control samples were taken after flow had returned to the resting level. Isoproterenol was then infused proximal to the pump at a rate which elicited flow increases slightly greater than during 6-Hz exercise. The doses infused ranged from 3 to 28 μg/min (calculated plasma concentration from

0.08 to 1.8 μM). As soon as a steady-state blood flow was reached, blood samples were collected as previously described. At the conclusion of each experimental intervention, flow was allowed to return to resting levels. We then waited at least 10 additional min, and collected post control blood samples.

In four experiments, α, β, methylene adenosine 5'-diphosphate (AOPCP), an inhibitor of 5'-nucleotidase, was infused during 6-Hz exercise. Beginning 5 min after initiating exercise, AOPCP was infused intraarterially at a rate which established a plasma concentration between 20 and 87 μM. Blood samples were collected after 10 min of exercise (5 min of AOPCP infusion). The resting blood samples from these muscles were collected in tubes containing sufficient AOPCP to produce a plasma concentration of 50 μM.

After completion of the experiment 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. These weights did not differ from the weights of the contralateral muscles.

Sample preparation and adenosine assay. Adenosine was assayed as previously reported (13); a brief description follows. Blood samples (3 ml) were dispensed into tubes containing 250 μl of solution containing dipyridamole (26 μM), erythrononylhydroxyadenosine (EHNA; 3 μM), and methanol (5%) in isotonic saline. In six experiments AOPCP (50 μM) was also included in the collection fluid. Blood samples were centrifuged and 1 ml of supernatant was acid precipitated and neutralized.

Adenosine in the neutralized extract was assayed by high-pressure liquid chromatography (HPLC). One hundred microliter samples were injected onto either a Bondapak C18 (Waters Associates) or a Partisil-5 ODS (Whatman, Inc.) column. A linear gradient of 70/30 methanol/water (v/v) against 4 mM KH_2PO_4 began with 0% and ended 20 min later with 40% 70/30 methanol/water. 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 adenosine deaminase-treated samples taken during the same period, and (3) an appropriately larger corresponding peak in spiked recovery samples collected at the same time. This adenosine assay can detect plasma concentrations as low as 0.02 μM and has a recovery of 101 ± 7% (13).

Data analysis and statistics. 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 (14). Oxygen consumption ($\dot{V}O_2$) was calculated from arterial and venous O_2 content and blood flow. There were no differences in pre- and postintervention control samples bracketing an intervention, so they were averaged for each dog to give one control value. Data were grouped according to intervention and analyzed for statistical differences using Student's t test. Values stated in the text are means ± SD. All chemicals except dipryidamole were supplied by Sigma. Dipyridamole was a gift from Boehringer Ingleheim.

Results. *Adenosine release during free flow at six twitches/sec.* Adenosine release during rest and steady-state exercise is shown in Table I. Exercise caused blood flow to increase thir-

TABLE I. ADENOSINE RELEASE DURING EXERCISE AND ISOPROTERENOL INFUSION

	Flow (ml/100 g·min)	Oxygen consumption (ml/100 g·min)	Venous PO_2	Plasma [ADO] (μM)		Adenosine release (nmole/100 g·min)
				A	V	
Rest (5)	10.0 ± 0.3	0.3 ± 0.1	49 ± 5	0.08 ± 0.03	0.06 ± 0.02	−0.1 ± 0.1
Exercise (5)	129 ± 25*	15.0 ± 0.9*	26 ± 4*	0.05 ± 0.02	0.14 ± 0.07*	6.6 ± 4.6*
Isoproterenol (4)	168 ± 54*	0.5 ± 0.5	89 ± 7*	0.06 ± 0.03	0.05 ± 0.02	−1.3 ± 2.3

Note. A = arterial, V = venous. Numbers in parentheses are number of experiments for which adenosine release data were available. Values are means ± SD.

* $P < 0.05$ compared to value at rest.

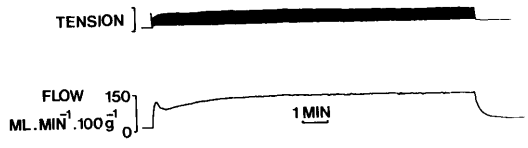

FIG. 1. Blood flow and gastrocnemius muscle tension during 6-Hz stimulation of hindlimb muscle preparation. Perfusion pressure was constant at 100 mm Hg. Blood samples for adenosine measurement were drawn after 10 min of exercise.

teenfold and $\dot{V}O_2$ to increase fiftyfold (Fig. 1). Venous plasma [ADO] increased more than twofold and R_{ado} increased from -0.1 ± 0.1 to 6.6 ± 4.6 nmole/min/100 g.

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 at least as much as did 6-Hz exercise. The results are presented in Table I. Isoproterenol caused at least as large a change in blood flow, but neither increased $\dot{V}O_2$ nor release of adenosine was observed.

Effect of AOPCP on release of adenosine during exercise. A possible explanation for the increased venous plasma [ADO] is that adenine nucleotides were released from formed elements in blood (e.g., platelets) and were subsequently degraded to adenosine via ecto-5'-nucleotidase (25). We tested this hypothesis in four experiments by repeating the exercise protocol with AOPCP in the collecting tubes in a concentration ($50\mu M$) which greatly inhibits formation of adenosine from AMP over the time period used in this experiment (8, 15). In addition, AOPCP was infused intraarterially during the final 5 min of exercise. Results from these experiments are presented in Table II. The increases in blood flow and $\dot{V}O_2$ were similar to those without AOPCP (Table I). Exercise still doubled venous plasma adenosine concentration.

Discussion. Our experimental results demonstrate the following: (i) There is a significant increase in venous adenosine concentration and in adenosine release during sustained normoxic exercise hyperemia. (ii) Increased blood flow caused by isoproterenol is not associated with increased venous adenosine. (iii) Exercise still results in increased venous adenosine in the presence of an ecto-5'-nucleotidase inhibitor, AOPCP. These results support the hypothesis that increased interstitial adenosine concentration occurs during free flow exercise vasodilation.

Previous studies have demonstrated that adenosine release increases during exercise when flow is restricted. Using a bioassay technique, Scott *et al.* (16) provided evidence that adenosine (or AMP) is released from dog skeletal muscle during ischemic exercise. Release of adenosine from severely ischemic muscle was then confirmed using chemical methods (1). Release of adenosine was again demonstrated by Tominaga *et al.* (17) under constant flow (but less severe) exercise conditions. Tissue content of adenosine also increases during exercise when flow is held constant (2), and during ischemia in resting muscles (18).

There has been scant evidence favoring increased interstitial adenosine during twitch exercise with free flow. We measured tissue adenosine content of dog anterior calf muscle and did not find a significant increase (3). Bockman and McKenzie (19) reported no increase in tissue adenosine of either red or white

TABLE II. ADENOSINE RELEASE DURING EXERCISE IN THE PRESENCE OF AOPCP

	Flow (ml/100 g·min)	Oxygen consumption (ml/100 g·min)	Venous PO_2	Plasma [ADO] (μM)		Adenosine release (nmole/100 g·min)
				A	V	
Rest (6)	16 ± 10	0.24 ± 0.1	57 ± 6	0.03 ± 0.02	0.05 ± 0.03	0.2 ± 0.2
Exercise (4)	122 ± 16*	17.3 ± 1.0*	25 ± 4*	0.07 ± 0.01	0.13 ± 0.01*	4.3 ± 2.7*

Note. AOPCP was present in the collection tube for all samples and was infused for the last 5 min of a 10-min exercise bout.

* $P < 0.05$ compared to value at rest.

feline muscle during free flow exercise, but Steffen *et al.* reported an increase in tissue content during free flow exercise of dog gracilis muscle (5). During 1- to 10-sec tetanic contractions of dog gracilis muscle at free flow, Kille and Klabunde (20) have provided strong pharmacologic evidence that adenosine is responsible for a large part of the postcontraction hyperemia. These authors have subsequently shown that this result requires the cessation of flow during tetanic contraction (21). Using brief tetani or 1-min bouts of twitch contraction at 1 or 2 Hz, Honig and Frierson (22) concluded that the adenosine antagonist theophylline had no effect on exercise vasodilation. Thus, the mechanism of exercise vasodilation may be different for twitch versus prolonged tetanic contractions.

This paper reports the first data showing that venous adenosine concentration increases during free flow twitch exercise. A possible explanation for increased venous adenosine is an increased gradient for diffusion of adenosine into capillary plasma as a result of increased interstitial adenosine concentration. Before accepting that possibility, we must consider the other factors which could influence adenosine release independently of changes in interstitial adenosine. These include (i) transport of adenosine across the capillary wall, (ii) plasma flow, (iii) uptake and metabolism of adenosine by red blood cells and/or endothelium, and (iv) the release of adenosine or its precursor, ATP, directly into plasma from endothelium or formed elements of blood.

We believe that our experiments with isoproterenol rule out the possibility that the increased venous adenosine observed during exercise was the direct result of increased plasma flow or capillary surface area. The flow increase caused by isoproterenol was greater than that caused by exercise and yet no increase in venous plasma adenosine concentration occurred. Although we have no direct measure of the increase in capillary surface area caused by isoproterenol in our preparation, an increase during β-adrenergic stimulation has been demonstrated by other investigators (23).

Red blood cells take up and metabolize adenosine. In the case of dog blood, however, added adenosine disappears at only 20% per minute. This relatively slow rate may be due to the lack of a membrane ADO carrier on dog RBCs (24). Because the transit time from capillary to collection tube is less than 1 min in our experiments, this cannot be a major factor determining the measured release of adenosine.

If adenosine is deposited in plasma from a source other than the interstitium, venous adenosine concentraton or adenosine release cannot be used as an indicator of interstitial adenosine concentration. We know of no evidence that adenosine itself is released from endothelial cells or the formed elements of blood during exercise, but this possibility cannot be ruled out. In favor of this possibility is the observation that cultured coronary endothelial cells release adenosine when exposed to high CO_2 (26).

ATP may be released during exercise (27), and if this happens, its degradation to adenosine in blood could result in increased venous plasma adenosine. In fact, if transit through an exercising muscle increases the tendency of formed elements to release ATP, collection of blood itself could serve as the major stimulus for release of ATP in venous samples. Since our standard collection solution stops adenosine removal from plasma but not its formation, increased adenosine concentration could be caused by this artifact. We tested this possibility by adding an inhibitor of ecto-5′-nucleotidase, AOPCP, to our collection solution and by infusing it during exercise. AOPCP (50 μM) inhibits adenosine formation from AMP by at least 85% (8, 15). Its presence caused no change in arterial or venous plasma adenosine concentrations during exercise. Thus, we 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.

The above considerations lead to the conclusion that it is likely that the observed increase in adenosine release is the result of either a release of adenosine from endothelial cells directly into capillary plasma or an increase in interstitial adenosine concentration. If it represents an increase in interstitial adenosine concentration, this would appear to contradict our earlier study in which we did

not observe an increase in tissue content. An explanation of this paradox could be that the increase in interstitial adenosine during exercise is too small to be detected by measurements of total tissue adenosine content. This possibility is supported by recent studies indicating that most of the adenosine measured in tissue samples is intracellular (6–8). Against a large background concentration of intracellular adenosine, it becomes difficult to detect changes in interstitial adenosine concentration.

Demonstration of adenosine release during exercise is not by itself sufficient to demonstrate that adenosine causes exercise vasodilation. We do not know whether the interstitial concentration associated with this release is sufficient to cause the observed dilation. If it is, we might expect a positive correlation between adenosine release and exercise blood flow among the different animals in this study. We did not observe such a correlation ($r = -0.05$). The question of causality between adenosine release and exercise vasodilation remains open.

In conclusion, venous adenosine concentration increases during vigorous sustained twitch exercise at free flow. This could be the result of an increase in interstitial adenosine concentration or the release of adenosine from endothelial cells. Until more is known about the nature of capillary transport of adenosine, it will not be possible to determine whether the magnitude of the adenosine release is sufficient to account for free flow exercise vasodilation.

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