Interstitial ATP and Norepinephrine Concentrations in Active Muscle

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Background—Sympathetic nervous system activity increases with exercise in normal subjects. Heightened peripheral sympathetic nervous activity and the resultant increased neurovascular levels of norepinephrine (NE) evoke vasoconstriction and serve to maintain blood pressure and perfusion to vital organs. Previous work demonstrated that the interstitial ATP concentrations ([ATP]i) rise in contracting skeletal muscle, and it is known that sympathetic nerves have purinergic P2X receptors. Thus, in this report we tested the hypothesis that elevated ATP would stimulate these receptors and increase interstitial NE concentrations ([NE]i).

Methods and Results—Muscle interstitial samples were collected from microdialysis probes inserted in the skeletal muscle of rats, and dialysate concentrations of ATP and NE were determined by the high-performance liquid chromatography method. Stretch (0.5 kg of tension) increased [ATP]i by 68% (P<0.05) and [NE]i by 45% (P<0.05) in active muscle. The rise in [NE]i was linearly linked to the elevated [ATP]i (r=0.878, P<0.001). [NE]i was also elevated by 76% (P<0.05) after ATP (3 μmol/L) was injected into the arterial blood supply of the hindlimb muscles. The [NE]i response to muscle stretch was blunted after the P2X receptor antagonist pyridoxal phosphate-6-azophenyl-2',4'-disulfonic acid (PPADS) was given. Finally, this response was potentiated by the nucleotidase inhibitor 6-N,N-diethyl-β,γ-dibromomethylene-D-adenosine-5′-triphosphate (ARL67156).

Conclusions—ATP released by skeletal muscle during stretch stimulates P2X receptors on the sympathetic nerves and increases the concentration of NEi in the muscle interstitium. (Circulation, 2005;111:2748-2751.)

Key Words: nervous system, sympathetic • exercise • blood flow

The sympathetic nervous system is activated during exercise. This contributes to increases in arterial blood pressure, heart rate, myocardial contractility, and peripheral vasoconstriction.1-3 It is thought that afferent input arising from contracting skeletal muscle causes increases in sympathetic nervous system activity.1,4 This muscle reflex responds to metabolic stimulation as well as to mechanical deformation of the muscle afferent receptive field.5,6 When this system is activated, sympathetic tone increases, and hemodynamic adjustments occur.7-9 For example, when the sympathetic nervous system is engaged, norepinephrine (NE) is released from the neurovascular junction, evoking vasoconstriction within a given vascular bed.1-3,10,11 Recent work suggests that ATP released from skeletal muscle stimulates and sensitizes thin fiber muscle afferents and contributes to this exercise pressor reflex.12-14 Recent studies from this laboratory have shown that muscle interstitial ATP concentrations ([ATP]i) increase with contraction.15 Of note, mechanical stimulation of muscle per se is a sufficient stimulus to raise interstitial ATP because muscle stretch in the absence of contraction raises [ATP]i. In addition to its sensory effects,12,13 ATP can affect adrenergic transmission by acting on purinergic receptors on sympathetic nerve endings.16 In the cultured cervical ganglion neurons and cardiac synaptosomes, ATP-sensitive P2X purinoceptors have been shown to enhance NE exocytosis.17,18 Thus, we postulated that increasing muscle ATPi would increase interstitial NE concentrations ([NE]i) by activation of purinergic P2X receptors. In this report, we examined ([NE]i) during stimulation of muscle mechanoreceptors by stretch before and after arterial injection of P2X receptor blocker into the blood supply of skeletal muscle.

Methods

Surgical Preparation

All procedures outlined in this study were approved by the Animal Care Committee of this institution. Sprague-Dawley male rats (weight, 250 to 350 g) were anesthetized by inhalation of isoflurane oxygen mixture. An endotracheal tube was inserted into the trachea and attached to a ventilator. Polyethylene catheters were inserted into the common carotid artery and external jugular vein for measurement of arterial blood pressure and for drug administration, respectively. A femoral artery catheter was placed for the injection of drugs into the arterial blood supply of the hindlimb muscles.19 The animals were ventilated, and respiratory parameters were monitored and main-
tained within normal ranges as previously described. Body temperature was maintained between 37.5°C and 38.5°C by a heating pad and external heat lamps, and fluid balance was stabilized by a continuous infusion of saline.

Decerebration was performed as previously described. Once this procedure was completed, anesthesia was removed from the inhaled mixture. The triceps surae muscle was isolated, and the calcaneal bone of the hindlimb was cut. The Achilles tendon was connected to a force transducer for the measurement of muscle tension during passive muscle stretch. The pelvis was stabilized in a spinal unit, and the knee joints were secured by clamping the patellar tendon to a spinal unit.

**Microdialysis**
The skin directly over the triceps surae muscles of both legs was dissected away, and 4 microdialysis probes were inserted into the muscle of each leg. Briefly, the probes were inserted into the muscle via a cannula in the direction parallel to muscle fiber orientation. After insertion, the microdialysis probes were attached to a perfusion pump and perfused at a rate of 2.5 μL/min with Ringer’s solution.

The semipermeable fibers with a molecular weight cutoff of 30 000 (0.20 mm ID, 0.22 mm OD; Spectrum Laboratories) were used to construct the microdialysis probes. Each end of a single fiber was inserted ∼1 cm into a hollow polyamide tube (0.25 mm ID, 0.36 mm OD) and glued. The length of the probe fibers was 2.0 cm. The dialysate was collected in 250-μL microcentrifuge tubes and immediately sealed (to prevent evaporation) and stored at −80°C until being analyzed.

**ATP and NE Measurements**
Because the dialysate samples were ultraclean, purification was not required before analysis. Dialysate ATP as well as NE was measured by high-performance liquid chromatography methods. All metabolites were determined in each of the dialysis probes.

**Experimental Protocols**
After the microdialysis probes were inserted, a 60-minute equilibration period was allowed. Muscle stretch was then performed with the use of a rack and pinion attached to the Achilles tendon of the rats. Each bout of stretch was maintained for 10 minutes. There was a 60-minute rest period between each period of muscle stretch. Dialysate was collected before, during, and after each workload.

**Study 1: Muscle Stretch (n=4)** Baseline tension was adjusted to 0.1 kg. Muscle stretch was performed and maintained for 10 minutes at 0.25, 0.5, and 1.0 kg.

**Study 2: Arterial Injection of ATP (n=6)** After a control saline injection, ATP was injected into the blood supply of the hindlimb muscles at 3 different concentrations: 0.3, 3, and 30 μmol/L, respectively. The volume of the injectate was 0.1 mL, and the duration of each injection was ∼2 minutes. Control data were obtained 10 minutes before each injection. Experimental data were collected for 10 minutes after the injection of ATP. There was a 60-minute rest period between each injection.

**Study 3: Muscle Stretch After PPADS and ARL67156 (n=6)** After control data (saline injection) were obtained, 5 mmol/L of pyridoxal phosphate-6-azophenyl-2',4'-disulfonic acid (PPADS) (a P2 receptor blocker)12 was injected via the femoral artery. A 10-minute stretch (0.5 kg of muscle tension) was then performed, and dialysate was collected during stretch. Recovery data were collected for 10 minutes after stretch. Next, 30 μmol/L of 6-N,N-diethyl-β-γ-dibromomethylene-β-adenosine-5'-triphosphate (ARL67156) (a nucleotidase inhibitor) was injected before and after stretch. Stretch was then repeated in the presence of PPADS. This dose of ARL67156 has been reported to effectively inhibit nucleotidase.18

The volume of injectate was 0.1 mL.

**Results**
Figure 1 shows the values for muscle [NE]i at rest throughout the experiment. NEi stabilized 40 minutes after probe insertion, and there were no differences in the resting muscle [NE]i of both legs (n=12).

**ATP and NE Responses by Muscle Stretch**
Basal values for dialysate ATP and NE were 0.16±0.04 μmol/L and 2.12±0.06 mmol/L, respectively. Stretch raised muscle [ATP]i and [NE]i in the experimental leg (n=4; Figure 2). The rises in ATP and NE were tension related. Furthermore, the rise in NEi was linearly linked to the rise in ATPi. Stretch did not significantly increase NEi (6%) and ATPi (2%) in the control leg. Baseline value for mean arterial pressure before muscle stretch was 95±8 mm Hg. Mean arterial pressure increased by 12.3±2.6 mm Hg during stretch. Furthermore, stretch (0.5 kg tension) increased muscle NEi concentration 45±6% in the stretched leg (P<0.05 versus control) and 7±4% in the control leg. This suggests that the elevated NEi was unlikely to be a reflex effect.

**Effect of ATP on [NE]i**
ATP (3 μmol/L) injected into blood supply of the hindlimb muscles raised [NE]i (n=6; Figure 3A). Of note, an increase in NE was not noted at the 30-μmol/L dose. ATP did not affect [NE]i in the control leg (Figure 3A). Blood pressure was unchanged by the ATP injections (94±6 mm Hg before and 95±4 mm Hg after injection).
Effects of PPADS and ARL67156 on [NE]i Response

Arterial injections of PPADS (5 mmol/L) (n = 6) significantly attenuated the [NE]i response by muscle stretch in the stimulated muscle (Figure 3B). ARL67156 (30 μmol/L) was injected to attenuate endogenous ATP metabolism by the nucleotidase (n = 6). The [NE]i response due to muscle stretch in the stimulated muscle was augmented by the nucleotidase inhibitor (Figure 3B). The effect of ARL67156 on NEi was blunted by the prior injection of PPADS (Figure 3B). There was no change in the NEi response in the control muscle. PPADS and ARL67156 injection did not alter blood pressure.

Discussion

The results of these studies suggest that muscle stretch is a sufficient stimulus to raise NEi. This effect is likely to occur via a local mechanism because muscle NEi was not significantly increased in the control leg. Moreover, the rise in NE is linearly linked to [ATP]i, and the response can be attenuated by purinergic receptor blockers and accentuated by ectonucleotidase blockers. Thus, this study suggests a novel pathway for the regulation of NE release from muscle sympathetic nerve terminals. ATP, largely released from exercising muscle,15,23 stimulates presynaptic P2X receptors and increases NE exocytosis. In this report we found that the highest dose of infused ATP did not raise NE. The reason for this finding is not clear, although it is possible that the highest infused dose stimulated NE uptake 1 mechanism.24 This local response is counterbalanced by endogenous ectonucleotidase activation. When the nucleotidase activity is accentuated, ATP and NE levels would fall.

In the present study it is possible that the injected ATP could have stimulated muscle afferent fibers and reflexly increased NE release from the sympathetic nerves. However, our data (Figure 3A) show that ATP did not elevate NEi in the opposite control hindlimb. This suggests that the rise in NEi was not due to a reflex effect.

It should also be noted that the concentration of ATP used in this report was lower than those used in a prior report in which ATP stimulated sensory afferents.12 Finally, if ATP had stimulated sensory afferents, we would have expected to see a rise in blood pressure with the ATP infusions, and we did not.

In addition, it is possible that muscle stretch could have stimulated muscle mechanoreceptors. This in turn could have resulted in an increase in muscle sympathetic nerve activity and NEi. Our data show that muscle stretch increased blood pressure. However, although muscle stretch raised blood pressure, it did not lead to an increase in NEi in the control leg. Thus, the rise in NEi in the stretched leg was not due to engagement of a muscle mechanoreflex.

The results of these studies raise several important questions. First, what role does this local pathway play during exercise? It is known that sympathetic tone rises with exercise, leading to the release of NE within the exercising muscle. The mechanism described here may play an important role in balancing muscle contractile activity with NE release. Second, what role does ectonucleotidase activity play in determining regional blood flow during exercise? The results of these studies suggest that ectonucleotidase activity
may not only increase the concentrations of adenosine and other dilators but also lead to reduced NE release. Thus, ectonucleotidase activity may be a crucial factor in determining exercise capacity, response to exercise in disease, and regional blood flow responses. Finally, what role does interstitial ATP metabolism play in disease processes associated with high catecholamine levels, such as heart failure? For example, do subjects with heart failure have high ATP levels and low ectonucleotidase levels? This would lead to sympathetic constriction coupled with reduced adenosine-mediated dilation. Clearly, additional experiments are necessary to address these issues.

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