Do P2X purinergic receptors regulate skeletal muscle blood flow during exercise?

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Buckwalter, John B., Jessica C. Taylor, Jason J. Hamann, and Philip S. Clifford. Do P2X purinergic receptors regulate skeletal muscle blood flow during exercise? Am J Physiol Heart Circ Physiol 286: H633–H639, 2004. First published October 9, 2003; 10.1152/ajpheart.00572.2003—Although there is evidence that sympathetic nerves release ATP as a neurotransmitter to produce vasoconstriction via P2X purinergic receptors, the role of these receptors in the regulation of blood flow to exercising skeletal muscle has yet to be determined. We hypothesized that there is tonic P2X receptor-mediated vasoconstriction in exercising skeletal muscle. To test this hypothesis, the effect of P2X receptor blockade on skeletal muscle blood flow was examined in six exercising mongrel dogs. P2X receptor antagonism was accomplished with pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonic acid (PPADs). Animals were instrumented chronically with flow probes on the external iliac arteries of both hindlimbs and a catheter in one femoral artery. PPADs (40 mg) was infused as a bolus into the femoral artery catheter during steady-state exercise at 6 miles/h. Intra-arterial infusion of PPADs increased iliac blood flow from 542 ± 55 to 677 ± 69 ml/min (P < 0.05) and iliac vascular conductance from 5.17 ± 0.62 to 6.53 ± 0.80 ml·min⁻¹·mmHg⁻¹. The PPADs infusion did not affect blood flow in the contralateral iliac artery. These data support the hypothesis that P2X purinergic receptors produce vasoconstriction in exercising skeletal muscle.

AT THE ONSET OF EXERCISE, there is a rapid increase in blood flow to exercising muscle as a result of extensive vasodilation of the skeletal muscle vasculature (5, 8, 19, 34). Although there are large increases in vascular conductance in exercising skeletal muscle, there is convincing evidence that sympathetic vasoconstrictor tone in the exercising muscle remains. Thus at one point the ability of the sympathetic nervous system to restrain blood flow in active skeletal muscle was questioned (15, 20, 27, 30), it is generally accepted that there is sympathetic restraint of skeletal muscle blood flow during exercise (4, 9, 19, 24, 37, 38, 45). Recent studies from our laboratory (4, 9, 19) and others (37) have clearly shown that α-adrenergic receptors mediate vasoconstriction in exercising skeletal muscle even during intense exercise. Traditionally sympathetic vasoconstriction has been characterized by the release of norepinephrine from sympathetic nerve terminals, which stimulates α-adrenergic receptors to produce contraction of vascular smooth muscle. However, in recent years, strong evidence has emerged indicating other neurotransmitters are released from the sympathetic nerve terminal and mediate vascular tone. One such neurotransmitter thought to be coreleased with norepinephrine is ATP (14, 22, 25, 31). The release of ATP is thought to stimulate a purinergic receptor (P2). Two types of P2 purinergic receptors have been shown to exist: 1) P2X, which mediates vasoconstriction; and 2) P2Y, which mediates vasodilation (12). P2X receptors are localized on vascular smooth muscle cells and are preferentially stimulated by ATP from sympathetic nerve endings (21). Indeed, P2X receptor stimulation has been shown to produce vasoconstriction in the hindlimb of the anesthetized cat and rat (1, 23). Furthermore, levels of circulating ATP have been shown to increase in an exercise intensity-dependent manner (18). A recent study from our laboratory provided credible evidence that P2X purinergic receptor stimulation can produce vasoconstriction in both resting and exercising canine skeletal muscle (7). However, it remains unknown whether P2X purinergic receptors tonically regulate skeletal muscle blood flow during exercise.

The purpose of this study was to examine the effect of P2X receptor blockade on the skeletal muscle vasculature during exercise. We hypothesized that tonic P2X purinergic receptor-mediated vasoconstriction exists in exercising skeletal muscle. Thus antagonism of these receptors would result in an increase in blood flow and vascular conductance in exercising skeletal muscle.

MATERIALS AND METHODS

Experimental procedures described below were approved by the Institutional Animal Care and Use Committees of the Medical College of Wisconsin and Veterans Affairs Medical Center and conducted in accordance with the American Physiological Society’s “Guiding Principles in the Care and Use of Animals.” Mongrel dogs were examined in two different series of experiments. Anesthetized dogs were used to demonstrate the effectiveness and selectivity of the P2X receptor antagonist, whereas conscious dogs were used to determine the effect of P2X receptor blockade on skeletal muscle blood flow during exercise.

Selectivity of blockade. To establish the effectiveness and selectivity of pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonic acid (PPADs; Sigma, St. Louis, MO) in the canine skeletal muscle vasculature, four dogs (9.5–10.3 kg) were studied under anesthesia. Anesthesia was induced with a mixture of 100 mg/kg of α-chloralose and 500 mg/kg of urethane and maintained by infusions of ~20 and 100 mg·kg⁻¹·h⁻¹, respectively. The dogs were ventilated mechanically. At the end of the experiments, dogs were given an overdose of anesthesia and euthanized with a bolus infusion of saturated potassium chloride solution sufficient to eliminate the heartbeat. Arterial blood pressure was measured by a pressure transducer attached to a cannula in a carotid artery. These dogs were acutely instrumented with

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ultrasonic transit time flow probes (Transonic Systems; Ithaca, NY) on the external iliac arteries of both hindlimbs and a catheter in one femoral artery for drug infusion. Dose-response curves were created by intra-arterial infusion of the selective P2X agonist α,β-methylene ATP (Sigma) and the selective α-adrenergic receptor agonist phenylephrine (Baxter; Deerfield, IL) before and after a single bolus infusion of 40 mg of PPADs. Because hindlimb blood flow varied over the duration of the experiment, doses of the agonists were adjusted to compensate for a change in baseline blood flow the same as in previous experiments from our laboratory (10, 11). This adjustment ensured that effective concentration of the bolus remained the same despite an increase or decrease in hindlimb blood flow. Doses of α,β-methylene ATP were 0.1, 1, and 10 μg/ml of external iliac blood flow. Doses of phenylephrine were 0.01, 0.1, and 1 μg/ml of external iliac blood flow. Infusions of α,β-methylene ATP and phenylephrine were alternated every 5 min with at least 10 min passing between agonist infusions of the same drug. In pilot studies, this was shown to be sufficient to prevent any tachyphylaxis. Dose-response curves were generated over roughly a 30-min period before and 30-min period after P2X receptor blockade with PPADs. Because vascular responsiveness is best described in vivo by using the percent change in vascular conductance from baseline (6), dose-response curves are expressed as a dose (x-axis) by percent change in conductance (y-axis).

**P2X receptor blockade during exercise.** Six mongrel dogs (20–25 kg) were selected for their willingness to run on a motorized treadmill. The animals were chronically instrumented in a series of sterile surgical procedures, which have been previously described (4, 9). Briefly, the first surgical procedure placed the carotid arteries in skin tubes on the neck so that they could be cannulated percutaneously to measure arterial blood pressure (32, 36). During the second surgery, all dogs were instrumented with ultrasonic transit time flow probes (4 mm; Transonic Systems) around the external iliac arteries to measure skeletal muscle blood flow in each hindlimb. Because there are no changes in blood flow to skin and bone during exercise, the increase in iliac blood flow during exercise represents skeletal muscle blood flow (29). In the final surgery, a nonocclusive, heparinized catheter (0.045-in. outer diameter, 0.015-in. inner diameter, 60-cm length; Data Science, St. Paul MN) was chronically implanted into the femoral artery to permit drug infusions distal to the flow probes. For these surgical procedures, anesthesia was induced with thiopental sodium (15–30 mg/kg; Gensia Pharmaceuticals; Irvine, CA) and maintained with 1.5% halothane (Halocarbon Laboratories; River Edge, NJ). Antibiotics (cefazolin sodium; Apothecon, Princeton, NJ) and analgesic drugs (0.3 mg buprenorphine hydrochloride; Reckitt and Coleman, Kingston-upon-Hull, UK) were given postoperatively. To maintain patency, the femoral catheter was flushed daily with saline and filled with a heparin solution (100 IU heparin/ml in 50% dextrose solution). The dogs were given at least 2 days to recover from the final surgery before any experiments were performed.

All experiments were performed in a laboratory in which the temperature was maintained below 20°C to minimize heat accumulation during exercise. On the day of the experiment, the dog was brought to the lab and a 20-gauge intravascular catheter (Innyte; Becton Dickinson, Sandy, UT) was inserted retrogradely into the lumen of the carotid artery. The catheter was attached to a solid-state pressure transducer (Ohmeda; Madison, WI), and the flow probes were connected to a transit time flowmeter (Transonic Systems).

Tonic P2X receptor-mediated vasoconstriction in skeletal muscle was examined with intrafemoral infusions of PPADs, a P2X receptor antagonist. This antagonist was chosen for its ability to be dissolved easily in an aqueous solution in concentrations sufficient to antagonize P2X receptors in vivo and to be infused in a conscious, chronically instrumented dog without detrimental side effects. Intra-arterial bolus infusions of 40 mg of PPADs were given at rest and during steady-state exercise while the dogs ran on the treadmill at a moderate exercise intensity of 6 miles/h (9.7 km/h).

**Data analysis.** For all experiments, a computer (Macintosh G3) using a PowerLab system (ADInstruments; Castle Hill, Australia) was used to record (at 100 Hz) arterial blood pressure and right and left external iliac blood flow during all experiments. Data were analyzed off-line by using PowerLab software to calculate mean arterial pressure, heart rate, iliac blood flow, and iliac vascular conductance (blood flow/mean arterial pressure). Vascular conductance was calculated rather than vascular resistance, because conductance better reflects vascular tone when the experimental manipulation causes a change primarily in flow and not pressure (28). For the anesthetized preparation, control measurements were averaged over 20 s before the agonist infusion and the nadir 1-s average for vascular conductance was chosen as the peak response. In the exercising dogs, control measurements were averaged over 30 s before PPADs infusion. After the PPADs infusion, all variables were averaged over 5-s intervals (500 consecutive data points). Data in the conscious animals were taken when blood flow stabilized after the PPADs infusion. The effect of infusing the solvent vehicle for PPADs was examined in three exercising animals.

A level of *P* < 0.05 was used to establish statistical significance during all analysis. In the exercising dogs, statistical analyses of the data were performed with a one-way repeated-measures analysis of variance for all hemodynamic variables to examine the effect of the infusion of PPADs. For the anesthetized preparations, statistical analyses of the data were performed with a three-way (PPADs × agonist × time) repeated-measures analysis of variance. The percent changes in vascular conductance from baseline after the infusion of the agonists were calculated for each individual dog and analyzed with a two-way (PPADs × agonist) repeated-measures analysis of variance. Where significant *F*-ratios were found, a Tukey’s post hoc test was performed. All data are expressed as means ± SE.

**RESULTS**

In the anesthetized dogs, intra-arterial infusion of α,β-methylene ATP and phenylephrine produced localized vasoconstriction in the experimental limb ( *P* < 0.05) without corresponding changes in blood flow or conductance in the contralateral limb ( *P* > 0.05). The dose-response curves to intra-arterial infusions of α,β-methylene ATP and phenylephrine before and after administration of PPADs are depicted in Figs. 1 and 2. Intra-arterial infusions of α,β-methylene ATP produced significantly less ( *P* < 0.01) vasoconstriction after antagonism of P2X purinergic receptors (Fig. 1). In contrast, vascular responsiveness to intra-arterial infusions of phenylephrine was unaltered by the presence of PPADs ( *P* > 0.05) (Fig. 2).

In the conscious dogs, baseline hemodynamic variables during a bout of moderate exercise were as follows: heart rate, 182 ± 11 beats/min; mean arterial pressure, 107 ± 4 mmHg; experimental limb blood flow, 542 ± 55 ml/min, and conductance, 5.17 ± 0.62 ml·min⁻¹·mmHg⁻¹; and control limb blood flow, 618 ± 42 ml/min, and conductance, 5.82 ± 0.42 ml·min⁻¹·mmHg⁻¹. Infusion of the solvent vehicle during exercise did not alter vascular conductance in the experimental limb (4.11 ± 0.29 to 4.04 ± 0.46 ml·min⁻¹·mmHg⁻¹). After PPADs infusion, control limb blood flow and conductance were unchanged ( *P* > 0.05). In contrast, intra-arterial infusion of PPADs produced substantial changes ( *P* < 0.05) in vascular tone in the experimental limb, as can be seen in the original tracing from one experiment shown in Fig. 3. In response to infusion of PPADs, there were initial decreases in blood flow in the experimental limb followed by a sustained increase in blood flow. There were no corresponding alterations in blood
flow to the contralateral limb. Figures 4 and 5 give blood flow and vascular conductance values for each dog before and after the infusion of PPADS. P2X receptor blockade resulted in a significant increase in experimental limb blood flow and conductance in every dog. The average increase in vascular conductance during exercise was 26 ± 4.5%. Additional experiments showed that the infusion of PPADS at rest also produced an increase in experimental limb blood flow (120 ± 84 to 280 ± 40 ml/min) and experimental limb conductance (1.42 ± 0.27 to 2.70 ± 0.52 ml-min⁻¹-mmHg⁻¹).

**DISCUSSION**

The salient new finding in this study is that P2X purinergic receptors in the arterial vasculature mediate tonic vasoconstriction in active skeletal muscle. This was clearly demonstrated by the increase in vascular conductance to exercising muscle that occurred in every dog after P2X receptor blockade. These results are the first demonstration of restraint of blood flow during exercise by a nonadrenergic postsynaptic receptor on vascular smooth muscle. The interruption of ongoing purinergic vasoconstriction by intra-arterial infusions of PPADS establishes a role for P2X receptors in the regulation of skeletal muscle blood flow during exercise and has important implications for the regulation of blood pressure during exercise.

Regulation of vascular tone by the sympathetic nervous system is traditionally thought to involve the release of norepinephrine from sympathetic nerve terminals resulting in the stimulation of α-adrenergic receptors to produce vasoconstriction. However, there is strong evidence that ATP acts as a neurotransmitter in vascular smooth muscle and is coreleased with norepinephrine from sympathetic nerves (14, 22, 25, 31). ATP can stimulate two different subtypes of P2 purinergic receptors. The generally accepted subdivision of P2 purinergic receptors include vasoconstrictive P2X receptors, which are ligand-gated ion channel receptors, and P2Y receptors, which mediate vasodilation via G protein coupling (2, 35). P2X receptors have been shown to be present on vascular smooth muscle and to be preferentially stimulated by ATP released from sympathetic nerve endings (21). P2Y receptors are found predominantly on endothelial cells, but there is limited evidence for this receptor subtype on vascular smooth muscle (14). A role for P2X purinergic receptors in the regulation of vascular tone is consistent with several studies that have shown that stimulation of neurotransmitter release produces vascular smooth muscle contractions independent of α-adrenergic receptors (12, 24, 32, 43). These nonadrenergic contractions can be abolished with desensitization of P2X purinergic receptors (13), suggesting that the vasoconstriction is mediated by ATP or a related purine nucleotide. Sympathetic vasoconstriction independent of α-adrenergic receptors is consistent with data provided in the present study.

Although our lab (4, 9, 19) and others (24, 37) have demonstrated sympathetic restraint of blood flow to exercising skeletal muscle mediated by α-adrenergic receptors, it was unknown whether P2X receptors mediate vasoconstriction in exercising skeletal muscle. Bivalacqua et al. (1) and Johnson et al. (23) showed P2X receptor stimulation will produce vasoconstriction in the hindlimb of anesthetized animals. We (7) recently extended those findings in a conscious animal model by demonstrating that P2X receptor stimulation will produce vasoconstriction in resting and exercising skeletal muscle. These data coupled with the findings of Bivalacqua et al. (1) and Johnson et al. (23) led us to hypothesize the existence of tonic sympathetic restraint of blood flow to exercising skeletal muscle by P2X receptors. The present study is the first to demonstrate that P2X purinergic receptors mediate tonic vasoconstriction in active skeletal muscle. These findings suggest a role for P2X purinergic receptors in the regulation of blood pressure during exercise.

In addition to demonstrating that P2X purinergic receptors mediate tonic vasoconstriction in exercising skeletal muscle,
the data provide evidence of substantial P2X purinergic receptor-mediated vasoconstriction in resting skeletal muscle. This is consistent with other studies from our laboratory showing abundant sympathetic restraint of blood flow to resting skeletal muscle (4, 5, 9, 19). Indeed, total abolition of sympathetic vasoconstriction in skeletal muscle with hexamethonium produces greater percent changes in muscle vascular conductance at rest than during exercise (5).

Fig. 3. Original records from an individual dog during steady-state treadmill exercise at 6 miles/h with P2X receptor antagonism. The bar indicates an intra-arterial infusion of the P2X antagonist PPADS. Initially, there was a transient vasoconstriction followed by a sustained elevation in blood flow. Prolonged vasodilation represents interruption of tonic P2X receptor-mediated vasoconstriction. Note that there were no changes in blood flow or conductance in the control limb after the infusion of PPADS.

Fig. 4. Change in iliac blood flow with P2X receptor blockade in conscious, dynamically exercising dogs. The thick line represents means ± SE data for 6 dogs. In each dog, infusion of PPADS resulted in an increase in experimental limb blood flow.

Fig. 5. Change in iliac conductance with P2X receptor blockade in conscious, dynamically exercising dogs. The thick line represents means ± SE data for the 6 dogs. In each dog, infusion of PPADS resulted in an increase in experimental limb conductance.
Maintenance of a stable arterial pressure is of paramount importance during exercise to ensure adequate perfusion of exercising skeletal muscle. During the transition from rest to exercise, sympathetic vasoconstriction in skeletal muscle becomes progressively more important for the regulation of systemic blood pressure (40). Blood pressure at rest and during exercise is determined by two factors: cardiac output and peripheral vascular conductance. An increase in sympathetic vasoconstriction can reduce peripheral vascular conductance and produce an increase in mean arterial pressure. During moderate exercise, vasodilation in active skeletal muscle markedly increases skeletal muscle vascular conductance such that vascular conductance in active muscle represents the majority of total vascular conductance. During heavy exercise, the proportion of total vascular conductance contributed by exercising skeletal muscle is so great that even maximal vasoconstriction in nonexercising tissues will produce only modest changes in mean arterial pressure (6). Because sympathetic vasoconstriction of exercising skeletal muscle plays such a prominent role in the regulation of blood pressure during exercise, the demonstration in the present study of P2X receptor-mediated vasoconstriction of active skeletal muscle has important implications for the regulation of blood pressure during exercise. Further experimentation is needed to characterize the contribution of P2X purinergic receptor-mediated constriction to blood pressure regulation.

Although the present study clearly demonstrates tonic P2X receptor-mediated vasoconstriction in exercising skeletal muscle, the source of the ATP that stimulates these receptors is undetermined. Red blood cells, skeletal muscle, and sympathetic nerves all represent potential sources of ATP that are in close proximity to vascular smooth muscle. In response to mechanical deformation, human and animal red blood cells have been shown to release ATP that can regulate local vascular tone (43, 44). Whereas ATP from red blood cells may play a role in the regulation of vascular tone in many mammalian species, canine red blood cells do not release ATP in response to mechanical deformation (43, 44) and thus are not likely to be the source of P2X stimulation in the present study. Another possible source of ATP is skeletal muscle. Large quantities of ATP are synthesized by skeletal muscle during exercise to fuel muscle contractions. Indeed, circulating levels of ATP have been shown to increase in an exercise intensity-dependent manner (18). Because P2X receptor blockade at rest (when ATP production was presumably low) produced substantial increases in vascular conductance, it is unlikely that skeletal muscle is the source. The final and most likely source of the ATP-stimulating P2X receptors in skeletal muscle is ATP released from sympathetic nerves innervating vascular smooth muscle. There is considerable evidence demonstrating that ATP acts as a neurotransmitter in vascular smooth muscle and is released from sympathetic nerves (14, 22, 25, 31). In addition, P2X receptors on vascular smooth muscle have been shown to be preferentially stimulated by ATP released from sympathetic nerve endings (21). Therefore, we believe that PPADs interrupted tonic P2X receptor-mediated vasoconstriction evoked by the release of ATP from sympathetic nerves innervating the skeletal muscle vasculature.

There are several strengths to the experimental protocol used in this study. Conscious, dynamically exercising dogs allowed natural patterns of muscle recruitment unachievable in anesthetized animal preparations. Effectiveness and selectivity of the pharmacological tools was demonstrated. Intra-arterial infusions of the selective P2X agonist α,β-methylene ATP produced vasoconstriction in the canine skeletal muscle vasculature that was significantly attenuated by P2X receptor antagonism with PPADs. Selectivity of PPADs was demonstrated by a lack of change in the magnitude of vasoconstriction produced with an α1-adrenergic receptor agonist. The ability to continuously measure blood flow also represents an important strength of the experimental design. Continuous hemodynamic measurements allow the examination of transient changes in skeletal muscle blood flow. Indeed, this permitted an interesting observation. Intra-arterial infusion of PPADs produced a transient decrease in hindlimb blood flow, followed by a prolonged hyperemia. We believe the prolonged hyperemia indicates an interruption of tonic P2X receptor-mediated vasoconstriction. However, the brief vasoconstriction associated with the infusion of PPADs may indicate interruption of P2Y receptor-mediated vasodilation. We considered the possibility that the vasoconstriction in the experimental limb might have been a myogenic response to the elevation in mean arterial pressure seen immediately after the infusion of PPADs. However, we rejected this idea because there was no corresponding vasoconstriction in the control limb. Whereas PPADs has been described as a selective P2X receptor antagonist (16, 17, 26), it appears likely that PPADs is a partial antagonist of P2Y receptors (3, 39). If endothelial P2Y receptors mediate skeletal muscle vasodilation during exercise, antagonism of these receptors with PPADs would likely result in vasoconstriction. In every dog, we saw a brief transient vasoconstriction followed by a prolonged increase in skeletal muscle vascular conductance. This may indicate a role for P2Y purinergic receptors in the regulation of skeletal muscle blood flow during exercise. However, the investigation of tonic P2Y receptor activation during exercise was beyond the scope of this study.

Lack of an available potent, selective P2X receptor antagonist is a limitation in the present study. In addition to blocking P2Y receptors, PPADs may have other undesired effects. Vigne et al. (46) reported that PPADs prevents intracellular calcium mobilization by a nonspecific mechanism that might involve the inhibition of inositol 1,4,5-trisphosphate channels. Another limitation in the present study is the inability of PPADs to distinguish P2X receptors on vascular smooth muscle from those on sympathetic nerve terminals. It has been shown previously that stimulation of presynaptic P2X receptors can enhance the release of norepinephrine from the sympathetic nerve terminal (41, 42). It is possible that in the present study, the increase in vascular conductance seen after the infusion of PPADs was due to the inhibition of norepinephrine release by blocking P2X receptors on the sympathetic nerve terminal. However, we do not believe this to be the case. In two dogs (data not shown), infusion of PPADs after α1 and α2-adrenergic receptor antagonism produced the typical biphasic response with a prolonged elevation in vascular conductance above baseline. These results suggest that PPADs-induced vasodilation is independent of α-adrenergic constrictor tone. On this basis, it is our contention that PPADs interrupts tonic P2X receptor-mediated vasoconstriction.

Findings in this study support the hypothesis that P2X purinergic receptors mediate tonic vasoconstriction in active skeletal muscle. To our knowledge, this is the first demonst-
tion of restraint of blood flow during exercise by purinergic receptors on vascular smooth muscle and has important implications regarding the regulation of blood flow and blood pressure during exercise.

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