Adenosine Contributes to Blood Flow Regulation in the Exercising Human Leg by Increasing Prostaglandin and Nitric Oxide Formation

Stefan P. Mortensen, Michael Nyberg, Pia Thaning, Bengt Saltin, Ylva Hellsten

Abstract—Adenosine can induce vasodilation in skeletal muscle, but to what extent adenosine exerts its effect via formation of other vasodilators and whether there is redundancy between adenosine and other vasodilators remain unclear. We tested the hypothesis that adenosine, prostaglandins, and NO act in synergy to regulate skeletal muscle hyperemia by determining the following: (1) the effect of adenosine receptor blockade on skeletal muscle exercise hyperemia with and without simultaneous inhibition of prostaglandins (indomethacin; 0.8 to 1.8 mg/min) and NO (N\textsuperscript{G} -mono-methyl-L-arginine; 29 to 52 mg/min); (2) whether adenosine-induced vasodilation is mediated via formation of prostaglandins and/or NO; and (3) the femoral arterial and venous plasma adenosine concentrations during leg exercise with the microdialysis technique in a total of 24 healthy, male subjects. Inhibition of adenosine receptors (theophylline; 399±9 mg, mean ± SEM) or combined inhibition of prostaglandins and NO inhibited the exercise-induced increase in leg blood flow by 14±1% and 29±2% (P<0.05), respectively, but combined inhibition of prostaglandins, NO, and adenosine receptors did not result in an additive reduction of leg blood flow (31±5%). Femoral arterial infusion of adenosine increased leg blood flow from ~0.3 to ~2.5 L/min. Inhibition of prostaglandins or NO, or prostaglandins and NO combined, inhibited the adenosine-induced increase in leg blood flow by 51±3%, 39±8%, and 66±8%, respectively (P<0.05). Arterial and venous plasma adenosine concentrations were similar at rest and during exercise. These results suggest that adenosine contributes to the regulation of skeletal muscle blood flow by stimulating prostaglandin and NO synthesis. (Hypertension. 2009;53:993-999.)

Key Words: skeletal muscle ■ regional blood flow ■ theophylline ■ indomethacin ■ L-NMMA

Extracellular adenosine can function as a signaling molecule by matching blood flow with alterations in tissue oxygen supply or demand.\textsuperscript{1-3} In vivo evidence support a role for adenosine in skeletal muscle blood flow regulation by demonstrating the following: (1) femoral arterial adenosine infusion can induce vasodilation similar to that observed during intense exercise;\textsuperscript{6} (2) adenosine receptor blockade reduces limb blood flow during exercise;\textsuperscript{4,5} and (3) the interstitial adenosine concentration is tightly coupled to the increased in skeletal muscle blood flow during muscle contractions.\textsuperscript{6}

Adenosine sensitive P1 receptors (A\textsubscript{1}, A\textsubscript{2A}, and A\textsubscript{2B}) are located on vascular smooth muscle cells and in the endothelium of skeletal muscle.\textsuperscript{7} Reports regarding the mechanisms by which adenosine induces vasodilation are controversial. On one hand, adenosine has been suggested to induce vasodilation independent of the endothelium by direct action of P1 receptors on smooth muscle cells.\textsuperscript{8,9} On the other hand, in vitro and in vivo data also suggest a close interaction between endothelial P1 receptor stimulation and NO release.\textsuperscript{10-13} Furthermore, in vitro data have suggested that prostaglandins could be involved as a second messenger of adenosine by stimulating NO production.\textsuperscript{14} Single inhibition of NO\textsuperscript{15,16} or prostaglandin\textsuperscript{17-19} formation has little or no effect on exercising limb blood flow, but simultaneous inhibition of NO and prostaglandin formation lowers blood flow to exercising limbs by ~30%,\textsuperscript{19,20} suggesting that there is an interaction between the prostaglandin and NO systems. It remains unknown whether adenosine contributes to blood flow regulation via direct stimulation of smooth muscle cell P1 receptors and/or by stimulation of endothelial second messengers systems, eg, prostaglandins and NO. Furthermore, the possible interaction between prostaglandins and NO in response to adenosine stimulation has not been examined in humans.

The origin of intraluminal adenosine is thought to be the endothelium, degradation of intraluminal ATP, and/or the interstitial compartment. In vitro evidence from studies on laboratory animals suggests that adenosine is increased in the venous efflux of contracting muscle,\textsuperscript{21,22} as well as during...
hypoxia and ischemia.23 However, because of rapid uptake by red blood cells, as well as degradation in plasma, the half-life of adenosine is estimated to be <1 second.24 Therefore, despite the use of stop solutions and rapid centrifugation, adenosine determinations from blood samples are difficult to interpret, and the actual adenosine concentrations in the vascular beds supplying and draining resting and contracting muscles in humans remain unclear. Intravascular microdialysis probes allow in vivo determinations of actual adenosine levels in plasma without the problem of blood sampling and centrifugation. In the current study, this technique was applied to assess femoral arterial and femoral venous concentrations of adenosine.

Accordingly, the purpose of this investigation was to determine the following: (1) whether there is interaction or redundancy of adenosine with prostaglandins and NO during exercise in the control of blood flow; (2) whether adenosine-induced vasodilation is mediated by the action of prostaglandins and/or NO; and (3) arterial and venous plasma adenosine concentrations at rest and during exercise with the use of intravascular microdialysis probes. We hypothesized that adenosine, prostaglandins, and NO act in synergy to contribute to skeletal muscle hyperemia.

**Materials and Methods**

Twenty-four physically active male subjects with a mean (±SD) age of 24±1 years, body weight of 80±2 kg, height of 185±1 cm, and VO$_2$max of 48±1 mL/min per kilogram participated in 3 studies, whereas 6 of the subjects participated in 2 of the studies separated by 2 to 4 weeks. The subjects were informed of any risks and discomforts associated with the experiments before giving their informed, written consent to participate. The study was approved by the Ethics Committee of Copenhagen and Frederiksberg (KF 01-013/96 and KF 11289201), and all of the procedures followed were in accordance with institutional guidelines.

The subjects refrained from caffeine, alcohol, and exercise for 24 hours before the study. On the day of the experiment, they arrived at the laboratory 1 hour before the experiment after a light breakfast. Catheters were placed into the femoral artery and vein of the experimental leg under local anesthesia, and a thermostat was advanced through the femoral venous catheter for determination of femoral venous temperature. In studies 1 and 2, an additional catheter was placed in the femoral artery of the nonexperimental leg. In study 3, 2 microdialysis probes (IView, CMA Microdialysis AB) were inserted in the distal direction of the femoral artery and of the femoral vein of the experimental leg.

**Study 1: The Effect of Adenosine Receptor Blockade and/or NO and Prostaglandin Inhibition on Skeletal Muscle Hyperemia**

In study 1, the subjects completed 1 minute of passive one-leg knee-extensor exercise followed by 4 minutes of active exercise (24±1 watts [W]; ie, 30% of peak workload) under the following conditions: saline (control) and theophylline (P1 receptor blocker; Sygehus Apotekerne; n=7) or control, indomethacin (INDO; cyclooxygenase inhibitor)+N$^6$-mono-methyl-L-arginine (l-NMMMA; NO synthase [NOS] inhibitor), and INDO+l-NMMMA+theophylline (n=7; Figure 1). Saline, INDO (120 μg/min per kilogram of leg mass; Confortid, Alphapharma), and/or l-NMMMA (4 mg/min per kilogram of leg mass; Clinalfa, Bachem) were infused into the femoral artery for 4 minutes before exercise and during the 1 minute of passive exercise (loading dose) and were then infused at a rate of 60 μg/min per kilogram of leg mass (INDO) and/or 2 mg/min per kilogram of leg mass (l-NMMMA; maintenance dose) during the 4 minutes of active exercise. To test the effectiveness of the NOS inhibition, we infused acetylcholine (160 μg/min) into the femoral artery with and without coinfusion of l-NMMMA and found that NOS inhibition lowered the acetylcholine-induced increase in leg blood flow (LBF) by ~65% (2.5±0.3 versus 0.9±0.1 L/min; n=3). Theophylline (10±1 mg/min) was infused into the femoral vein for 40 minutes (total dose: 399±32 mg, ie, 5 mg/kg of body weight). This dose has been found previously to inhibit the vasodilator response to intraluminal adenosine by ~55%.25 Interstitial theophylline concentrations were assessed by collecting perfusate before and after theophylline infusion from 2 microdialysis probes (CMA 61) inserted into the vastus lateralis muscle, as described previously.6 Interstitial theophylline increased from ~0 to 20±2 μmol/L before and after theophylline infusion, respectively (n=4). Trials were separated by a 45- to 70-minute rest period. Because of the potential long lasting effects of INDO, l-NMMMA, and theophylline, the control trial was always performed first. Blood samples (1 to 5 mL) were drawn simultaneously from the femoral artery (nonexperimental leg) and vein before and during (3.5 minutes) exercise. LBF was measured before and after blood sampling.

**Study 2: Role of Prostaglandin and NO in Adenosine-Induced Vasodilation**

In study 2, adenosine (ITEM development AB) was infused into the femoral artery during 2 stepwise 3-minute infusion periods (0.15±1 and 0.3±0.1 μmol/min per kilogram of leg mass, respectively) under the following conditions: (1) control, (2) INDO (n=7) or l-NMMMA (n=8), and (3) INDO+l-NMMMA (n=6). All of the tests were separated by a 30-minute rest period. Saline, INDO, and/or l-NMMMA were infused into the femoral artery for 5 minutes before adenosine infusion (loading dose) and at a lower rate (maintenance dose) during the 6 minutes of adenosine infusion. Blood samples (1 to 5 mL) were drawn simultaneously from the femoral artery (nonexperimental leg) and vein before and during (2.5 and 5.5 minutes) adenosine infusion.

**Study 3: Leg Plasma Adenosine Concentrations Before, During, and After Exercise**

In study 3, the subjects (n=8) performed one-leg knee-extensor exercise for 10 minutes at 16±1 and 32±2 W (ie, 20% and 40% of peak workload). Microdialysate was collected for 10 minutes before the start of exercise, during exercise, and during the 10-minute recovery period. LBF was measured 5 minutes before exercise, 5 minutes into exercise, and 5 minutes into the recovery period. In study 2, LBF was measured with ultrasound Doppler (Vivid 7, GE Healthcare), whereas LBF in the 2 studies that involved exercise (studies 1 and 3) was measured by the constant-infusion thermodilution method.26,27 Heart rate was obtained from an ECG, whereas arterial pressures were monitored with transducers positioned at the level of the heart (Pressure Monitoring kit, Baxter). Blood gases, hemoglobin, and lactate concentrations were measured using an ABL725 analyzer (Radiometer) and were corrected for temperature obtained in the femoral vein. Leg mass was calculated from whole-body dual-energy x-ray absorptiometry scanning (Prodigy, GE Healthcare). The microdialysis probes were perfused with isotonic saline (0.9% NaCl) at a rate of 5 μL/min. [2-14C]adenosine (<0.1 μCi/mL) was added to the perfusate, and the obtained data were corrected for probe recovery, as described previously.6 Dalteparin (25 IU/mL; Fragmin, Pfizer) was also added to the perfusate to avoid clotting on the membrane. Adenosine and theophylline in perfusate and dialysate were analyzed with high-pressure liquid chromatography without previous treatment of samples.28

**Statistical Analysis**

A 2-way, repeated-measures ANOVA was performed to test significance within and between trials. After a significant F test, pairwise differences were identified using Tukey’s honestly significant difference posthoc procedure. Differences between the single-and double-blockade trials during adenosine infusion were assessed with a t test. The significance level was set at P<0.05, and data are mean±SEM unless otherwise indicated.
Results

Effect of Theophylline on Leg and Systemic Variables Before and During Exercise

Theophylline infusion increased resting LBF, vascular conductance, and O₂ delivery (P < 0.05), whereas there was no difference in mean arterial pressure (MAP; Figure 2). During exercise, LBF, vascular conductance, and leg O₂ delivery were lower after theophylline infusion (P < 0.05), whereas there was no difference in MAP. The lower O₂ delivery during exercise was paralleled by an increase in a-vO₂ difference (P < 0.05), resulting in a maintained leg VO₂. Blood variables are displayed in Table S1 (available in the online data supplement at http://hyper.ahajournals.org).

Effect of INDO, L-NMMA, and Theophylline on Leg and Systemic Variables Before and During Exercise

At rest and during exercise, LBF, vascular conductance, and leg O₂ delivery were lower with double and triple blockade compared with control (P < 0.05), whereas there was no difference between double and triple blockade. Blood pressures remained unchanged between trials. The lower O₂ delivery during double and triple blockade was paralleled by an increase in a-vO₂ difference, but leg VO₂ remained lower during exercise in both trials compared with the control (P < 0.05).

Effect of INDO on Leg and Systemic Variables at Baseline and During Adenosine Infusion

Resting LBF was 0.30 ± 0.03 L/min during control conditions and did not change with infusion of INDO (0.23 ± 0.01 L/min; Figure 3). Adenosine infusion increased LBF to 1.75 ± 0.22 and 2.58 ± 0.27 L/min, but it was ≈48% lower with coinfusion of INDO (0.89 ± 0.10 and 1.31 ± 0.16 L/min; P < 0.05). There was no change in arterial pressure when adenosine was infused alone (90 ± 2 versus 88 ± 3 and 89 ± 3 mm Hg) or in combination with INDO (93 ± 2 versus 93 ± 2 and 93 ± 2 mm Hg). Therefore, leg vascular conductance (LVC) was lower when adenosine was coinfused with INDO during the low (20 ± 2 versus 9 ± 1 mL/min per mm Hg in control versus INDO, respectively) and high (29 ± 3 versus 14 ± 2 mL/min per mm Hg, respectively) adenosine infusion rates. Adenosine infusion increased leg VO₂ during INDO (P < 0.05), but there was no difference between trials (Table 2).

Effect of L-NMMA on Leg and Systemic Variables at Baseline and During Adenosine Infusion

Infusion of L-NMMA lowered basal LBF from 0.32 ± 0.04 to 0.22 ± 0.04 L/min (P < 0.05). Adenosine infusion increased
LBF to 1.93±0.40 and 2.71±0.46 L/min during control conditions, but it was lower with coinfusion of l-NMMA (0.85±0.11 and 1.58±0.22 L/min; P<0.05). Accounting for the change in blood flow during basal conditions, the adenosine-induced increase in LBF was reduced by 44±10% and 35±7% with coinfusion of l-NMMA during the low and high adenosine infusion rates, respectively. MAP increased from 89±3 mm Hg during baseline conditions to 91±3 and 93±3 mm Hg when adenosine was coinfused with l-NMMA and was higher than when adenosine was infused during control conditions (P<0.05). LVC was lower with l-NMMA inhibition during basal conditions (4±0 versus 3±1 mL/min per mm Hg; P<0.05). During adenosine infusion, LVC was lower with l-NMMA (9±2 and 18±3 mL/min per mm Hg, respectively) compared with control (25±4 and 32±3 mL/min per mm Hg, respectively; P<0.05). There was no difference in leg VO2 with adenosine and/or l-NMMA infusion.

Effect of Combined INDO and l-NMMA on Leg Blood Flow and Systemic Variables at Baseline and During Adenosine Infusion

Combined infusion of INDO and l-NMMA lowered basal LBF from 0.36±0.02 to 0.20±0.01 L/min (P<0.05). Adenosine infusion increased LBF to 1.80±0.26 and 2.42±0.26 L/min during control conditions, but it was lower with coinfusion of INDO and l-NMMA (0.50±0.04 and 0.94±0.13 L/min; P<0.05). Accounting for the change in blood flow during basal conditions during combined INDO and l-NMMA infusion, the adenosine-induced increase in LBF was reduced by 69±6% and 59±7% compared with control. MAP increased from 89±3 mm Hg at baseline to 91±3 and 93±3 mm Hg when adenosine was coinfused with l-NMMA (P<0.05) and was higher compared to when adenosine was infused during control conditions (P<0.05). l-NMMA infusion reduced LVC at baseline (4±0 versus 3±1 mL/min per mm Hg; P<0.05) and was lower during adenosine infusion (9±2 and 18±3 mL/min per mm Hg) compared with control conditions (25±4 and 32±3 mL/min per mm Hg; P<0.05). There was no difference in leg VO with adenosine and/or INDO+l-NMMA infusion.

Plasma Adenosine Concentrations Before, During, and After One-Leg Knee-Extensor Exercise

Exercise increased LBF from 0.2±0.0 to 2.5±0.2 L/min (16 W) and 4.1±0.3 L/min (32 W) and was 0.4±0.0 L/min during the recovery period. Arterial plasma adenosine concentration was 31±3 nmol/L at rest, 30±6 and 38±13 nmol/L during exercise (16 and 32 W, respectively), and 28±9 nmol/L during the recovery period (Figure 4). Venous plasma adenosine concentration was 18±6, 30±12, 28±12, and 26±7 nmol/L, respectively. There was no difference between arterial and venous adenosine concentrations.

Discussion

There were several novel findings in the present study that support a role for adenosine in regulating skeletal muscle
blood flow by increasing prostaglandin and NO formation: (1) adenosine receptor blockade alone, or inhibition of prostaglandin and NO formation combined, lowered vascular conductance during exercise, whereas simultaneous adenosine receptor blockade with inhibition of prostaglandin and NO synthesis did not induce an additive reduction in vascular conductance during exercise; (2) the vasodilatory response to adenosine was inhibited by single blockade of either prostaglandins synthesis or NO synthesis; and (3) combined inhibition of prostaglandin and NO formation produced an additive reduction in the vasodilatory response to adenosine. Furthermore, the unchanged plasma adenosine concentration during exercise indicates that interstitial adenosine may be a more important contributor than luminal adenosine to blood flow regulation.

Adenosine Contributes to Skeletal Muscle Blood Flow Regulation by Stimulating Prostaglandin and NO Formation

The present findings that inhibition of P1 receptors lowered exercise hyperemia by 14% and that simultaneous inhibition of the NO and prostaglandin systems lowered exercise hyperemia by 29% are generally consistent with previous findings.4,5,19,20 Adenosine could, however, explain ≤30% of the exercise hyperemia, because the dose of theophylline used in the present study has been found to inhibit the vasodilatory response to adenosine by 55%.25 A major novel observation was that adding P1 receptor blockade to simultaneous inhibition of prostaglandins and NO did not further reduce vascular conductance. The lack of additive effect suggests that there is not redundancy but rather an interaction among the adenosine, prostaglandin, and NO systems by which the vasodilatory effect of adenosine is mediated by prostaglandins and NO.

Adenosine Vasodilator Response Is Mainly Prostaglandin and NO Mediated

Further investigation into the mechanisms by which adenosine induces vasodilation demonstrated that the prostaglandin and NO systems are mediators of adenosine-induced vasodilation. Our finding of a role of NO in mediating adenosine-induced vasodilation is in agreement with previous findings.
in the human forearm, but this is the first study to demonstrate a close interaction between adenosine and prostaglandins in humans. Interestingly, the inhibition in the adenosine-induced increase in conductance when prostaglandins and NO were inhibited simultaneously (<71%) was lower than the combined effect of single prostaglandin (<56%) and single NO (<41%) inhibition, suggesting that there is an interaction between the prostaglandin and NO systems. Consistent with this observation, an in vitro study clearly demonstrated that prostaglandin inhibition reduced NO formation in response to adenosine stimulation.

This observation, which suggests that prostaglandins promote NO synthesis, is also supported by the greater reduction in vascular conductance with inhibition of prostaglandins compared with inhibition of NOS. Therefore, the present findings suggest that the vasodilator response to adenosine, to a large extent, is mediated by prostaglandins and NO and that part of the increased NO formation could be prostaglandin mediated (Figure 5). The proposed direct action of interstitial adenosine on adenosine receptors on the smooth muscle cells subsequently appears to be less important.

Although L-NMMA alone or in combination with INDO alters baseline flow, we did not infuse a vasodilator to correct for this response, because it has clearly been demonstrated that baseline correction with the NO donor sodium nitroprusside does not alter the blood flow response to adenosine during NOS inhibition. This is consistent with observations during exercise and NOS inhibition, where a change in baseline flow does not alter blood flow during exercise.

**Arterial and Venous Plasma Adenosine Concentrations During Exercise**

To avoid the confounding factors of adenosine degradation and uptake in blood samples, a novel type of intravascular microdialysis probe was used to determine plasma adenosine in vessels supplying and draining active muscle in vivo. The similar arterial and venous adenosine concentrations suggest that adenosine is not released from the interstitium of exercising muscle. This observation is in contrast to observations in the isolated dog limb but in agreement with the observation that the endothelium is an effective barrier for adenosine.

Furthermore, plasma ATP increases during exercise, but adenosine does not play a role in ATP-induced vasodilation, and the breakdown products of ATP are, therefore, likely to be rapidly taken up by erythrocytes. A methodological limitation may be that the rapid uptake and degradation of adenosine require sampling from veins closer to the active muscle.

**Perspectives**

The current finding that adenosine regulates the formation of prostaglandins and NO in the skeletal muscle vasculature and that prostaglandins regulate NO formation suggests that the interaction among these 3 vasodilators may be of importance for proper regulation of peripheral vascular tone. The present results also suggest that interstitial adenosine may be more important than luminal adenosine, and an essential task in the future is to reveal the cellular origin of interstitial adenosine, as well as what physiological stimuli that lead to adenosine formation.

The current finding that the vasodilatory effect of adenosine is mainly mediated by the formation of prostaglandins and NO suggests that, when the formation of NO is impaired in hypertension, this may affect also the ability of adenosine to induce vasodilation. In future studies it will be important to examine the mechanism underlying the impaired vasodilator response to adenosine in patients with essential hypertension, including the aspect of whether formation of NO and prostaglandins in response to adenosine is impaired.
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Disclosures
None.

References
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ADENOSINE CONTRIBUTES TO BLOOD FLOW REGULATION IN THE EXERCISING HUMAN LEG BY INCREASING PROSTAGLANDIN AND NITRIC OXIDE FORMATION

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Short title: Role of adenosine in blood flow regulation

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Tabel S1. Blood variables before and during exercise with and without inhibition of adenosine receptors, prostaglandins and/or nitric oxide

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<tr>
<th>Blood Variable</th>
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<th>TEO</th>
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<td>Rest</td>
<td>Exercise</td>
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<td>26±2†</td>
<td>36±1*</td>
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a. femoral arterial. v. femoral venous. TEO: theophylline. PO2, PCO2 and pH values were corrected for changes in blood temperature.

* Different from control, † different from baseline conditions, ‡ different from double blockade, $P<0.05$. 
Tabel S2. Blood variables before and during adenosine infusion with and without inhibition of prostaglandins and/or nitric oxide

<table>
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<tr>
<th>Blood</th>
<th>Indomethacin</th>
<th>L-NMMA</th>
<th>Indomethacin + L-NMMA</th>
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<td>Control (0.9% NaCl)</td>
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<tr>
<td>v</td>
<td>14.7±0.3</td>
<td>14.5±0.2</td>
<td>14.7±0.1</td>
</tr>
<tr>
<td>O2 saturation (%)</td>
<td>1.9 µmol/min</td>
<td>3.5 µmol/min</td>
<td></td>
</tr>
<tr>
<td>a</td>
<td>98±0</td>
<td>98±0</td>
<td>98±0</td>
</tr>
<tr>
<td>v</td>
<td>61±4</td>
<td>91±1*</td>
<td>94±0*</td>
</tr>
<tr>
<td>O2 content (ml/L)</td>
<td>1.9 µmol/min</td>
<td>3.5 µmol/min</td>
<td></td>
</tr>
<tr>
<td>a</td>
<td>194±4</td>
<td>196±3</td>
<td>198±2</td>
</tr>
<tr>
<td>v</td>
<td>121±10</td>
<td>178±2</td>
<td>187±3</td>
</tr>
<tr>
<td>Leg VO2</td>
<td>21±1</td>
<td>33±3</td>
<td>30±6</td>
</tr>
</tbody>
</table>

- a. femoral arterial. v. femoral venous. PO2 values were corrected for changes in blood temperature.

* Different from control, † different from baseline conditions, $P<0.05$. 