Interstitial and Plasma Adenosine Stimulate Nitric Oxide and Prostacyclin Formation in Human Skeletal Muscle

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

Abstract—One major unresolved issue in muscle blood flow regulation is that of the role of circulating versus interstitial vasodilatory compounds. The present study determined adenosine-induced formation of NO and prostacyclin in the human muscle interstitium versus in femoral venous plasma to elucidate the interaction and importance of these vasodilators in the 2 compartments. To this end, we performed experiments on humans using microdialysis technique in skeletal muscle tissue, as well as the femoral vein, combined with experiments on cultures of microvascular endothelial versus skeletal muscle cells. In young healthy humans, microdialysate was collected at rest, during arterial infusion of adenosine, and during interstitial infusion of adenosine through microdialysis probes inserted into musculus vastus lateralis. Muscle interstitial NO and prostacyclin increased with arterial and interstitial infusion of adenosine. The addition of adenosine to skeletal muscle cells increased NO formation (fluorochrome 4-amino-5-methylamino-2′,7-difluorescein fluorescence), whereas prostacyclin levels remained unchanged. The addition of adenosine to microvascular endothelial cells induced an increase in NO and prostacyclin levels. These findings provide novel insight into the role of adenosine in skeletal muscle blood flow regulation and vascular function by revealing that both interstitial and plasma adenosine have a stimulatory effect on NO and prostacyclin formation. In addition, both skeletal muscle and microvascular endothelial cells are potential mediators of adenosine-induced formation of NO in vivo, whereas only endothelial cells appear to play a role in adenosine-induced formation of prostacyclin. (Hypertension. 2010;56:1102-1108.) • Online Data Supplement

Key Words: cell culture ■ endothelial cells ■ interstitial fluid ■ microdialysis ■ vasodilators

Skeletal muscle blood flow is closely regulated to match O2 delivery to the metabolic demand of the contracting muscle.1 This precise regulation of muscle blood flow is believed to be regulated by a balance between vasoconstrictor activity and locally derived vasoactive substances.2 Three such vasoactive compounds shown to be of importance for muscle blood flow regulation are adenosine, NO, and prostacyclin (PGI2). Accordingly, it has been shown that blockade of adenosine receptors reduces exercise hyperemia by ≈15% to 20%3,4 and simultaneous inhibition of the enzymes NO synthase (NOS), which catalyzes the formation of NO, and cyclooxygenase (COX), which initiates the conversion of arachidonic acid to PGI2, reduces exercise hyperemia in both the forearm and leg by ≈30%.3,5-8 Importantly, these 3 vasoactive compounds show a close interaction in that the vasodilator effect of adenosine primarily appears to be mediated via the formation of NO and PGI2, as evidenced by markedly reduced vasodilatation in response to arterially infused adenosine when NOS3,9 and COX3 are inhibited. Moreover, adenosine receptor blockade does not further reduce exercise hyperemia when combined with inhibition of NOS and COX,3 suggesting that the vasodilator effect of endogenous adenosine is NO and PGI2 dependent. However, because pharmacological inhibition does not discriminate between the vasodilator effect of interstitial and plasma adenosine and only provides indirect evidence for an adenosine-induced formation of NO and PGI2, actual in vivo measurements are still needed to determine whether adenosine in the muscle interstitium and plasma has a stimulatory effect on formation of these vasodilators. There also appears to be a redundancy between NO and PGI2 in that either of the systems appears to compensate for the other when that system is inhibited, thereby maintaining blood flow during exercise.3,6,10 In addition, intra-arterial coinfusion of the NOS inhibitor Nω-monomethyl-L-arginine has been shown to blunt the vasodilation evoked by the PGI2 analog epoprostenol, suggesting that PGI2-induced vasodilation is partly mediated through NO.11 Taken together, this high degree of interdependency suggests that the interaction among these 3 vasodilators may be a major player in the regulation of muscle blood flow and vascular function. Additional knowledge regarding the role of adenosine and its interaction with NO and prostanooid formation is also important for the understanding of mechanisms underlying alterations in vascular...
function in cardiovascular disease and aging. Such information could also be of value for medical treatment of cardiovascular disease by, for example, adenosine. Furthermore, an interdependency of the 3 systems suggests that if one system is impaired, the effect of the other systems may also be altered.

Another major unresolved issue in muscle blood flow regulation is that of the role of circulating versus interstitial vasodilatory compounds. During muscle contraction, adenosine increases in the muscle interstitial fluid at a rate closely associated with the increase in blood flow,12 and this adenosine is likely to originate from AMP 5’-nucleotidase located on the extracellular membrane of skeletal muscle and endothelial cells.13 Because pharmacological inhibitors affect both intravascular and interstitial systems, such designs cannot reveal the role or mechanisms of action of interstitial adenosine. Potentially, interstitial adenosine might induce vasodilation by acting directly on smooth muscle adenosine receptors14,15 or by acting on adenosine receptors located on endothelial cells16 and/or skeletal muscle cells, thereby inducing formation of NO and PGI2. The latter pathway may be more important in humans, as evidenced by the above-mentioned lack of additional reduction in exercise-induced blood flow when adenosine receptors and the NO and PGI2 systems are inhibited simultaneously.3 Moreover, the concentration of adenosine is also ~35-fold higher in the interstitium than in the circulation during moderate-intensity exercise. Whether interstitial adenosine leads to formation of NO and PGI2 is not known, because direct measurements of these compounds in response to adenosine infusion have never been performed previously.

Although never tested in humans, the endothelium has been described to function as an effective barrier for adenosine in other species,17 suggesting that the vasodilator effect of plasma adenosine is mediated via formation of vasodilators released from vascular endothelial cells and not through a direct action on vascular smooth muscle cells. Given this scenario, an interesting aspect is to what extent circulating adenosine affects interstitial levels of NO and PGI2 at the capillary level. In theory, adenosine acting on capillary endothelial cells should enhance the release of NO and PGI2 in both directions unless the enzyme systems are located solely on the luminal side.

In the current study we hypothesized that both interstitial and intra-arterial adenosine stimulate the formation of NO and PGI2 and that microvascular endothelial cells and skeletal muscle cells release NO and PGI2 on adenosine exposure. To test these hypotheses, a combination of experiments was performed in human subjects using microdialysis in skeletal muscle and the femoral vein and in cultures of rat skeletal muscle cells and microvascular endothelial cells isolated from the rat skeletal muscle.

Methods

Human Study

Ten moderately trained male subjects with a mean (±SD) age of 26±5 years, body weight of 76±11 kg, height of 183±6 cm, and VO2max relative to body mass of 56.9±6.9 mL·min⁻¹·kg⁻¹ participated in the study. The purpose, nature, and potential risks were explained to the subjects before they gave their informed, written consent to participate in the study. The study was approved by the Ethics Committee of Copenhagen and Frederiksberg (H-KF 11289201) and conducted in accordance with the guidelines of the Declaration of Helsinki. The subjects were informed to abstain from caffeine, alcohol, and exercise for 24 hours before the experiment. An expanded Methods section is available in the online Data Supplement (see http://hyper.ahajournals.org).

Experimental Protocol: Microdialysis

Before the experiment, the subjects visited the laboratory and were screened to ensure that they had a body mass index <25, were normotensive, nonsmokers, and not taking any medications. During this visit, the subjects also performed an incremental bicycle ergometer exercise test in which pulmonary maximal oxygen uptake (1 min⁻¹, VO2max) was determined online (Quark b2 system, Cosmed). On the day of the experiment, the subjects arrived at the laboratory at 8:30 AM after a light breakfast. After 30 minutes in the supine position, catheters were placed into the femoral artery and vein of the experimental leg under local anesthesia (Lidocaine, 20 mg·mL⁻¹). One microdialysis probe (CMA Microdialysis AB) was inserted in the distal direction of the femoral vein of the experimental leg, and 2 microdialysis probes were inserted into the thigh muscle (musculus vastus lateralis) of the experimental leg. Thirty minutes after insertion of the probes, the subjects performed a 10-minute exercise bout at 10 W with the purpose of minimizing the tissue response to insertion trauma.18 To re-establish resting conditions, the subjects rested for another 30 minutes, and dialysate was then collected for 20 minutes during the following conditions: (1) rest (baseline); (2) infusion of a low dose of adenosine (ITEM development AB; 0.16±0.01 μmol·min⁻¹·kg⁻¹ of leg mass⁻¹) into the femoral artery; and (3) infusion of a high dose of adenosine (0.31±0.01 μmol·min⁻¹·kg⁻¹ of leg mass⁻¹) into the femoral artery. Infusion of the 2 doses of adenosine was separated by 10 minutes of rest. After 45 minutes of rest, dialysate from the probes inserted into the thigh muscle was collected for 20 minutes during rest (baseline) and infusion of adenosine (0.09±0.01 μmol·min⁻¹) through the probes (interstitial adenosine infusion). Leg blood flow (LBF) was measured after 10 minutes of collection of dialysate for each of the above conditions and before infusion of the high dose of intra-arterial adenosine. Blood samples (1 to 5 mL) were drawn simultaneously from the femoral artery and vein of the experimental leg at the same time that LBF was measured.

Experimental Protocol: Cell Cultures

Before experiments were performed, the cells were washed once with PBS containing 5 mmol/L of glucose, and then PBS containing 100 μmol/L of l-arginine and 5 mmol/L of glucose was added. After 30 minutes of incubation, the cells were washed once with PBS containing 5 mmol/L of glucose, and then PBS containing 10 μmol/L of NO-sensitive fluorochrome 4-amino-5-methylamino-2’-7-difluorescein (DAF-FM) and 5 mmol/L of glucose was added.

To investigate the role of adenosine for microvascular endothelial and skeletal muscle cell NO and PGI2 formation, either adenosine (20 μmol/L, 100 μmol/L, or 1 mmol/L) or PBS containing 5 mmol/L of glucose (control) was added to culture medium. The adenosine concentration of 1 mmol/L was chosen because it has been shown previously to elicit the maximal adenosine-evoked release of NO from rat aortic endothelial cells.16 Medium for determination of DAF-FM fluorescence was collected after 5 minutes of incubation and transferred to microplates, and fluorescence was measured immediately with a fluorescence microplate reader (Fluoroscan Ascent, Thermo Labsystems) calibrated for excitation at 485 nm and emission at 520 nm. Medium for determination of PGI2 (6-keto PGF1α) was collected after 10 minutes of incubation and immediately stored in a freezer (−80°C) for later analysis.

Statistical Analysis

A 1-way repeated-measures ANOVA was performed to test significance within trials in the microdialysis study. Effect of interstitial adenosine infusion was determined by a paired t test. Values from
cells treated with adenosine were expressed as formation rate per milligram of protein. A 1-way ANOVA on ranks was used to test the effect of adenosine on cells. After a significant F test, pairwise differences were identified using the Tukey honestly significant difference post hoc procedure. The significance level was set at \( P < 0.05 \), and data are mean ± SE unless otherwise indicated.

**Results**

**Leg and Systemic Variables During Arterial and Intersitial Adenosine Infusion**

Arterial adenosine infusion increased \( (P < 0.05) \) LBF in a dose-dependent manner from \( \approx 0.4 \) L·min\(^{-1} \) to \( 1.3 \pm 0.1 \) and \( 2.2 \pm 0.4 \) L·min\(^{-1} \) during the low and high dose of adenosine, respectively (Figure S1, available in the online Data Supplement at http://hyper.ahajournals.org). Mean arterial pressure remained unchanged during both doses of adenosine. Leg vascular conductance increased \( (P < 0.05) \) from \( \approx 4 \) mL·min\(^{-1} \)·mm Hg\(^{-1} \) to \( 15 \pm 1 \) and \( 24 \pm 4 \) mL·min\(^{-1} \)·mm Hg\(^{-1} \) during the low and high dose of adenosine, respectively. Leg arteriovenous oxygen difference decreased \( (P < 0.05) \) during both adenosine infusions such that leg oxygen consumption remained unchanged. Intersitial adenosine infusion had no effect on LBF, mean arterial pressure, leg vascular conductance arteriovenous oxygen difference, or leg oxygen consumption.

**Muscle Intersitial NO, 6-Keto PGF\(_{1α}\), and Adenosine Levels With Arterial Infusion of Adenosine**

Muscle interstitial NO (NOx) was \( 27 \pm 6 \) µmol/L at baseline, and the level increased \( (P < 0.05) \) 2- and 3-fold during the low and high dose of adenosine, respectively (Figure 1). Intersitial 6-keto PGF\(_{1α}\) levels increased \( (P < 0.05) \) 1.7- and 1.9-fold from a baseline value of \( 1570 \pm 635 \) pg·mL\(^{-1} \) during the low and high dose of adenosine, respectively. Interstitial adenosine was not affected by either of the 2 adenosine infusions.

**Venous Plasma NOx and 6-Keto PGF\(_{1α}\) Levels and Efflux With Arterial Infusion of Adenosine**

At baseline, venous plasma NOx and 6-keto PGF\(_{1α}\) were \( 43 \pm 3 \) µmol/L and \( 1329 \pm 571 \) pg·mL\(^{-1} \), respectively, and did not change during the 2 adenosine infusions (Figure S2). When accounting for plasma flow, venous plasma efflux of NOx and 6-keto PGF\(_{1α}\) increased 6.4- and 5.4-fold, respectively, during the high dose of adenosine \( (P < 0.05) \).

**Muscle Intersitial NOx and 6-Keto PGF\(_{1α}\) Levels With Intersitial Infusion of Adenosine**

Intersitial NOx was \( 25 \pm 6 \) µmol/L at baseline, and the level increased \( (P < 0.05) \) 2.6-fold during adenosine infusion (Figure 2). Similarly, intersitial 6-keto PGF\(_{1α}\) was \( 675 \pm 133 \) pg·mL\(^{-1} \) at baseline and increased \( (P < 0.05) \) 2.3-fold during adenosine infusion.

**Standard Curve for DAF-FM**

In the DAF-FM, standard curve fluorescence values were found to increase linearly with increasing concentrations of the NO donor s-nitroso-n-acetyl-penicillamine in the range from 5 to 100 nmol/L \( (r=0.99; P < 0.001) \).

**Effect of Adenosine on DAF-FM Fluorescence and 6-Keto PGF\(_{1α}\) Levels**

The addition of 20 µmol/L, 100 µmol/L, and 1 mmol/L of adenosine to microvascular endothelial cells induced an increase \( (P < 0.05) \) in DAF-FM fluorescence, indicating NO formation, and the level of 6-keto PGF\(_{1α}\) (Figures 3 and 4). The addition of 100 µmol/L and 1 mmol/L of adenosine to skeletal muscle cells increased \( (P < 0.05) \) DAF-FM fluorescence, whereas 6-keto PGF\(_{1α}\) levels remained unchanged.

**Discussion**

In the present study, novel information is provided on the mechanisms by which adenosine regulates skeletal muscle...
blood flow in humans. We demonstrate that both plasma and interstitial adenosine stimulate the formation of NO and PGI\(_2\) in muscle interstitium, which we propose to be of importance for vascular function and control of blood flow. Based on cell culture studies, we further show that both skeletal muscle cells and microvascular endothelial cells are potential mediators of adenosine-induced formation of NO in vivo, whereas only endothelial cells appear to play a role in adenosine-induced formation of PGI\(_2\).

It has been shown that the adenosine concentration increases in the exercising muscle interstitium at a rate closely associated with the magnitude of muscle blood flow, indicating that interstitial adenosine could be important for exercise hyperemia. Intersitial adenosine could potentially induce vasodilation through a direct interaction with vascular smooth muscle or via formation of NO and PGI\(_2\). To examine the latter possibility, adenosine was infused directly into the muscle interstitium. We hypothesized that infusion of adenosine would increase the interstitial concentrations of NO and PGI\(_2\). Our findings confirm that interstitial adenosine is a stimulator of NO and PGI\(_2\) formation in humans, supporting the suggestion that interstitial adenosine contributes to local vasodilation during exercise by stimulating the formation of these vasodilators.\(^3\)

To determine which cells in the interstitium are responsible for the increase in NO and PGI\(_2\), experiments were performed on cell cultures of skeletal muscle myotubes and muscle microvascular endothelial cells. Both endothelial cells and skeletal muscle cells are potential sources of NO and PGI\(_2\), because both cell-types express adenosine receptors and contain NOS\(^2\) and COX.\(^2\) In congruence with our hypothesis, the microvascular endothelial cells and the myotubes were found to produce NO in response to adenosine application. The release of NO per milligram of protein from skeletal muscle cells was approximately one eight of that observed from microvascular endothelial cells, suggesting a minor role of skeletal muscle for adenosine-induced formation of NO. However, taking into account that the amount of skeletal muscle cell tissue in vivo is many-fold greater than that of endothelial cells, the interstitial adenosine-evoked release of NO from skeletal muscle may well be of similar importance as that from microvascular endothelial cells. Although it cannot be excluded that cell types other than muscle cells and endothelial cells contribute to muscle interstitial NO levels during contraction, we conclude from our data that these 2 cell types are major sources.

In contrast to our hypothesis, only microvascular endothelial cells were found to produce PGI\(_2\) in response to adenosine. The lack of release of PGI\(_2\) from myotubes is in agreement with the observation that PGI\(_2\) levels remain unaltered when myotubes are electrostimulated (n=9; M Nyberg and Y Hellsten, unpublished results). These observations suggest that the increase in interstitial PGI\(_2\) in response to plasma or interstitial adenosine, or in response to exercise,\(^2\) primarily originates from microvascular endothelial cells in the muscle tissue. By immunohistochemical methods, COX-1 and COX-2 have been found to be present in skeletal muscle cells from humans and rodents, but it has been proposed that the main product of skeletal muscle COX is prostaglandin E\(_2\), which, similar to PGI\(_2\), increases in the human muscle interstitium during exercise.\(^2\)

Several findings have indicated an essential role for endothelial cells in contraction-induced dilation of skeletal muscle vasculature.\(^2\) The findings from the current study showing that interstitial adenosine stimulates the formation of endothelial NO and PGI\(_2\) suggests that this mechanism could constitute one of the endothelium-dependent regulatory pathways leading to changes in skeletal muscle blood flow. How interstitial adenosine activates endothelial cells at the arteriolar level that, in turn, communicate with adjacent smooth muscle cells to produce the dilatory response still needs to be determined. It is, however, unlikely that adenosine diffuses through all of the structures of the vessel wall, in particular, the tight basal lamina. Interestingly, stimulation of adenosine receptors on endothelial cells produces vasodilation that spreads to remote regions with a concomitant increase in endothelial Ca\(^{2+}\) observed along with this response.\(^2\)
Muscle contraction

cAMP

Ca²⁺

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capillary level, thereby inducing a wave of NO and PGI₂

contraction may interact with endothelial cells located at the

ARs located on the abluminal side of capillary endothelial cells, which leads to the activation of Ca²⁺ channels and PKA. The influx of Ca²⁺ activates both COX and eNOS, and PKA phosphorylates and activates eNOS, leading to the formation of PGI₂ and NO. Furthermore, the increase in intracellular Ca²⁺ may spread upstream through gap junctions, thereby inducing a release of NO and PGI₂ from endothelial cells adjacent to smooth muscle cells. Interstitial adenosine also acts on ARs on skeletal muscle cells, which activates PKA and then nNOS, thus increasing the formation of NO. Plasma adenosine may interact with AR located on the luminal side of endothelial cells, evoking a release of NO and PGI₂ from endothelial cells along the vascular tree through a PKA and Ca²⁺-mediated pathway. At the arteriolar level, the generated NO and PGI₂ may diffuse to adjacent smooth muscle cells to induce vasodilation or into the intravas-

cular space. At the capillary level, NO and PGI₂ may diffuse directly into the interstitium or into the intravascular space. AR indicates adenosine receptor; PKA, protein kinase A; eNOS, endothelial NOS; nNOS, neuronal NOS.

Figure 5. Proposed mechanisms by which adenosine contributes to exercise hyperemia via formation of NO and PGI₂. During muscle contraction, adenosine increases in the muscle interstitial fluid, and this adenosine is likely to originate from AMP 5′-nucleotidase located on the extracellular membrane of skeletal muscle cells and microvascular endothelial cells. Interstitial adenosine interacts with ARs located on the abluminal side of capillary endothelial cells, which leads to the activation of Ca²⁺ channels and PKA. The influx of Ca²⁺ activates both COX and eNOS, and PKA phosphorylates and activates eNOS, leading to the formation of PGI₂ and NO. Furthermore, the increase in intracellular Ca²⁺ may spread upstream through gap junctions, thereby inducing a release of NO and PGI₂ from endothelial cells adjacent to smooth muscle cells. Interstitial adenosine also acts on ARs on skeletal muscle cells, which activates PKA and then nNOS, thus increasing the formation of NO. Plasma adenosine may interact with AR located on the luminal side of endothelial cells, evoking a release of NO and PGI₂ from endothelial cells along the vascular tree through a PKA and Ca²⁺-mediated pathway. At the arteriolar level, the generated NO and PGI₂ may diffuse to adjacent smooth muscle cells to induce vasodilation or into the intravas-

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cause conducted vasodilation has been shown to be associ-

ated with increases in Ca²⁺ and release of NO and prosta-

noids,27 interstitial adenosine formed during muscle

contraction may interact with endothelial cells located at the

capillary level, thereby inducing a wave of NO and PGI₂

release from endothelial cells located upstream at the arteri-

olar level (Figure 5).

In contrast to the present findings that indicate that the

vasodilator effect of adenosine is mediated through formation

of NO, it has been suggested previously that adenosine

released during muscle contraction in the rat hindlimb does

not depend on NO synthesis to produce vasodilation.28 This

suggestion was based on the observation that adenosine made

a substantial contribution to muscle vasodilation when NO

was inhibited. Because the vasodilatory effect of adenosine

depends not only on NO formation but also on prostanoid

formation,3 the vasodilatory effect of adenosine during NOS

blockade in the study by Ray and Marshall28 may well reflect

PGI₂-mediated vasodilation.

The present results show that arterial infusion of adenosine

increases the interstitial concentrations of NO and PGI₂.

Because significant increases in blood flow can only be

obtained with substantial dilation of small arteries and arte-

rioles,29 it may be speculated that plasma adenosine acts

primarily on endothelial cells lining arterioles, thereby stim-

ulating formation of NO and PGI₂ that diffuses to adjacent

smooth muscle cells to induce vasodilation. In this setting, the

increase in interstitial NO and PGI₂ during adenosine infusion

suggests that these substances also cross the smooth muscle

cell layer. Another, not mutually exclusive, possibility also

exists, because plasma adenosine most likely also acts on

capillary endothelial cells leading to the formation of NO and

PGI₂ that diffuses directly into the muscle interstitium.

It is well known that exposure of endothelial cells to

laminar shear stress stimulates NO and PGI₂ production.30

Consequently, the observed increase in the formation rate of

NO and PGI₂ during arterial adenosine infusion may have

been caused by a direct effect of plasma adenosine on

vascular smooth muscle cells, because this would have

induced vasodilation and a concurrent increase in blood flow

and shear stress. However, the current study does demon-

strate that arterial infusion of adenosine, sufficient to elevate

LBF ∼5-fold, does not increase the concentration of intersti-

tial adenosine. Therefore, although it cannot be excluded that

the release of NO and PGI₂ was partly related to an increase

in shear stress, a direct effect of plasma adenosine on smooth

muscle cells appears improbable. Furthermore, an increase in

luminal flow has been shown to induce endothelial NOS

translocation from the basolateral membrane and cytoplasm

to the apical membrane,31 suggesting that NO is primarily
released into the luminal space on exposure to shear stress, which is in contrast to the observed increase in interstitial NO observed in the present study.

The concentration of adenosine used to stimulate NO and PGI₂ formation in microvascular endothelial cells was 10- to 20-fold higher than that observed in vivo during contraction as adenosine increases in the human muscle interstitium to \( \sim 1 \) to 2 \( \mu \)mol/L during light to heavy intensity exercise.\(^{12}\) This dose of adenosine was selected to ensure that not all of the adenosine was metabolized or taken up by cells during the time of incubation, because endothelial cells can take up adenosine via transporters and show a high activity of ectoenzymes that metabolize adenine nucleotides and adenosine.\(^{13}\) Thus, when the transport mechanism for adenosine and adenosine deaminase is blocked, the dose-response curve for adenosine in intact endothelium from aorta is shifted leftward by several orders of magnitude.\(^{16}\) Moreover, the concentrations of adenosine in close proximity to the cell membrane and, hence, adenosine receptors are likely to exceed the concentrations measured in microdialysate, because adenosine originates from ecto-AMP 5' nucleotidase located on the extracellular membrane of endothelial cells.\(^{13}\)

**Limitations**

Despite the increase in interstitial concentrations, venous plasma concentrations of NO and PGI₂ remained unchanged. This discrepancy may be related to a methodological limitation, because the interstitial microdialysis probe allowed sampling in the immediate vicinity of microvascular endothelial cells, whereas the probe for plasma sampling was located within the femoral vein. Accordingly, the mean transit time from the arterial infusion site to the sampling site in the femoral vein has been found to be \( \sim 15 \) to 10 seconds at similar LBVs, as observed during the adenosine infusions,\(^{32}\) thereby allowing time for substantial intravascular degradation and uptake of NO, PGI₂, and their metabolites along the vascular tree by endothelial and red blood cells. Furthermore, because plasma flow was elevated during adenosine infusion, the venous plasma efflux was also determined to account for this diluting effect. These results show an increased luminal efflux of NO and PGI₂ during the high dose of adenosine, which cannot solely be explained by the increase in plasma flow, because this increased \( \sim 4,5\)-fold whereas the venous plasma efflux of NO and PGI₂ increased \( \sim 6,5, 5\)-fold, respectively. Hence, the observed increase in plasma efflux suggests that NO and PGI₂ are released into the intravascular space.

To reduce the effect of tissue damage and consequent inflammatory responses associated with insertion of microdialysis probes, subjects performed a 10-minute exercise bout at 10 W followed by a long resting period. This procedure has been shown to stabilize the tissue and minimize the response to damage.\(^{18}\) Moreover, the interventions were performed during resting conditions, and it is likely that the observed alterations in NOX and PGI₂ in response to the infused adenosine were independent of damage.

**Perspectives**

It is well established that NO and PGI₂ are involved in a wide variety of regulatory and protective mechanisms within the cardiovascular system, and physiological actions include antiproliferative and anti thrombotic effects. The current finding that adenosine in plasma and the interstitium is stimulatory for NO and PGI₂ formation suggests a close interdependency between these systems and also that, if the adenosine system is impaired, NO and PGI₂ levels and thereby vascular function may be affected. Because changes in vascular function are a key early feature in the development of human vascular disease, the current findings raise the possibility of novel approaches to prevention and treatment of vascular dysfunction, for example, by stimulation of the adenosine system.

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**Disclosures**

None.

**References**

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Interstitial and plasma adenosine stimulate nitric oxide and prostacyclin formation in human skeletal muscle

Michael Nyberg\textsuperscript{1,2}, Stefan P. Mortensen\textsuperscript{1}, Pia Thaning\textsuperscript{1}, Bengt Saltin\textsuperscript{1}, & Ylva Hellsten\textsuperscript{1,2}

\textsuperscript{1}The Copenhagen Muscle Research Centre, Rigshospitalet, Denmark, \textsuperscript{2}Department of Exercise and Sport Sciences, University of Copenhagen, Denmark

Running title: Adenosine-induced formation of vasodilators

Supplemental Methods

Microdialysis - data acquisition and analyses

The microdialysis probes were perfused with a microdialysis pump (CMA Microdialysis, Stockholm, Sweden) at a rate of 5 μl min\textsuperscript{-1} and the inflowing perfusate consisted of a isotonic saline (0.9% NaCl). Dalteparin (25 IU ml\textsuperscript{-1}, Fragmin, Pfizer) was added to the perfusate to avoid clotting of blood around the membrane. [2-\textsuperscript{3}H]ATP (<0.1 μCi ml\textsuperscript{-1}) was added to the perfusate and the obtained data were corrected for probe recovery, as described previously\textsuperscript{1}.

LBF was measured with ultrasound Doppler (Vivid 7, GE Healthcare) as described previously\textsuperscript{2}. Heart rate (HR, beats min\textsuperscript{-1}) was obtained from electrocardiogram, while arterial pressure (mmHg) was monitored with transducers positioned at the level of the heart (Pressure Monitoring Kit, Baxter, Deerfield, IL, USA). Arterial and venous blood samples were immediately analyzed for PO\textsubscript{2}, PCO\textsubscript{2}, pH, O\textsubscript{2} saturation and hemoglobin (ABL725, Radiometer, Copenhagen, Denmark). Infusion of adenosine was conducted with a Harvard pump (Model 44, Harvard Apparatus, MA, USA). Leg mass of the experimental leg was calculated from the whole-body dual-energy X-ray absorptiometry scanning (Prodigy, General Electric Medical Systems, WI, USA) and was found to be 12.15±1.29 kg.

The concentration of the final products of NO, nitrate and nitrite (NO\textsubscript{x}), was measured using fluorometric assay kit (Cayman), and the concentration of 6-keto PGF\textsubscript{1α}, a stable metabolite of PGI\textsubscript{2}, was measured using an enzyme immunoassay kit (Cayman). Both kits were used according to the manufacturer’s instructions. Venous plasma efflux was calculated as plasma flow multiplied by the venous plasma concentration of NO\textsubscript{x} and 6-keto PGF\textsubscript{1α}. Adenosine was measured with high-performance liquid chromatography (HPLC) without previous treatment of the samples\textsuperscript{3}.

Cell culture experiments - materials, cell cultures and analyses
Dulbecco’s modified Eagle medium (DMEM), fetal calf serum (FCS), horse serum (HS), Dulbecco’s Phosphate buffered saline (DPBS), penstrep (penicillin [10 000 U/mL], streptomycin [10 000 U/mL]), and trypsin were all from Life Technologies. Serum Growth Supplement (LSGS) containing fetal bovine serum, fibroblast growth factor, heparin and epidermal growth factor were from Cascade Biologics Inc. DNAse, trypsin/EDTA solution, glucose, L-arginine, adenosine, and s-nitroso-n-acetyl-penicillamine (SNAP) were all products from Sigma. Collagenase (type II) was from Worthington Biochemicals. TriReagent from the Molecular Research 96 Center and Biotinylated Griffonia Simplicifolia Lectin and *ulex europaeus* agglutinin I from Vector Lab. fluorochrome 4-amino-5-methylamino-2’,7-difluorescein (DAF-FM) and Dynabeads were from Invitrogen and Medium 131, microvascular growth supplement, and attachment factor were from Cascade Biologies.

Skeletal muscle cells were prepared from male Wistar male rats as described previously (Høier et al. 2010). Rat microvascular endothelial cells were isolated from soleus, gastrocnemius, and quadriceps muscle of male Wistar rats by digestion with 0.2% collagenase II, 0.01% DNAse, and 0.25% trypsin in DMEM containing 1% penstrep for 1.5 hour at 37°C with rotation. After centrifugation at 200 x g for 15 min pellet was incubated with rotation in a solution of 0.2% collagenase II, 0.01% DNAse, and 0.25% trypsin in DMEM containing 1% penstrep for 30 min at 37°C. 10 ml of the cell suspension was then extracted and 50 µl of Dynabeads coated with Lectin I was added. After 25 min of incubation a magnet was applied to separate Dynabeads and bead-bound microvascular endothelial cells. Cells were then resuspended in medium 131 containing microvascular growth supplement and counted and seeded onto 35 mm dishes coated with attachment factor. After 4 -5 days medium 131 and growth supplement was changed and after 1-3 additional days cells were passaged with trypsin/EDTA solution. After being seeded onto 35 mm dishes coated with attachment factor cells were ready for experiments 3 – 5 days later. Phase-contrast light microscopy and immunohistochemical staining with the endothelium specific *ulex europaeus* agglutinin I was used to ensure that the isolated cells were microvascular endothelial cells. This approach showed intense fluorescence in almost all cells (Fig. S3), illustrating that the majority of the cells were endothelial cells.

All treatment of animals complied with the European Convention for the protection of Vertebrae Animal Used for Experimental or other Scientific Purposes (Council of Europe No. 123, Strasbourg, France, 1985).

NO production in microvascular endothelial and skeletal muscle cells was assessed by measuring extracellular nitrosation of NO-sensitive DAF-FM. Linearity and sensitivity of DAF-FM was assayed using PBS, DAF-FM (10 µM) and four concentrations of the NO donor SNAP (5, 25, 50 and 100 nM). Fluorescence was measured using a fluorescence microplate reader (Fluoroskan Ascent, Thermo Labsystems) calibrated for excitation at 485 nm and emission at 520 nm. The fluorescence intensity for DAF-FM nitrosation was adjusted to the protein concentration and expressed as formation of NO mg of protein⁻¹. The concentration of 6-keto PGF₁α, a stable metabolite of PGI₂, was measured using an enzyme immunoassay kit (and expressed as formation of 6-keto PGF₁α mg of protein⁻¹).

Total protein concentrations of microvascular endothelial and skeletal muscle cells were determined by BCA protein assay using BSA as the standard (Pierce Reagens).

Supplemental References


Figure S1. Leg blood flow during baseline conditions and arterial and interstitial adenosine infusion.

Data are mean ± SE for 7 subjects. * denotes significantly different from baseline, # denotes significantly different from the low dose of adenosine, P<0.05.
Figure S2. Venous plasma NOx and 6-keto PGF$_{1\alpha}$ levels and efflux with arterial infusion of adenosine.
Venous plasma NOx and 6-keto PGF$_{1\alpha}$ concentrations (A,B) and efflux (C,D) during baseline conditions and arterial adenosine infusion. Data are mean ± SE for 5 subjects. * denotes significantly different from baseline, $P<0.05$. 

Figure S3. Phase-contrast light micrograph and immunohistochemical image of microvascular endothelial cells.

Cultures microvascular endothelial cells isolated from rat skeletal muscle visualized with phase contrast light microscopy (A) and corresponding cells histochemically stained with the endothelial specific lectin *ulex europaeus* agglutinin I coupled to FITC (B). The staining shows that the isolated cells were primarily microvascular endothelial cells.