

SYMPOSIUM REVIEW

Contribution of intravascular *versus* interstitial purines and nitric oxide in the regulation of exercise hyperaemia in humans

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Abstract The regulation of blood flow to skeletal muscle involves a complex interaction between several locally formed vasodilators that are produced both in the skeletal muscle interstitium and intravascularly. The gas nitric oxide (NO) and the purines ATP and adenosine, are potent vasodilators that are formed by multiple cell types and released into the skeletal muscle interstitium and in plasma in response to muscle contraction. Cellular sources of ATP and NO in plasma are erythrocytes and endothelial cells, whereas interstitial sources are skeletal muscle cells and endothelial cells. Adenosine originates primarily from extracellular degradation of ATP. During exercise the concentrations of ATP and adenosine increase markedly in the interstitium with smaller increases occurring in plasma, and thus the interstitial concentration during exercise is severalfold higher than in plasma. The concentration of NO metabolites (NO_x) in interstitium and plasma does not change during exercise and is similar in the two compartments. Adenosine and NO have been shown to contribute to exercise hyperaemia whereas the role of ATP remains unclear due to lack of specific purinergic receptor blockers. The relative role of intravascular *versus* interstitial vasodilators is not known but evidence suggests that both compartments are important. In cardiovascular disease, a reduced capacity to form adenosine in the muscle interstitium may be a contributing factor in increased peripheral vascular resistance.

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Introduction

In skeletal muscle, several vasoactive compounds, including ATP, adenosine and nitric oxide (NO) contribute to the regulation of skeletal muscle blood flow

at rest and during exercise. These vasoactive compounds can originate from several cellular sources and be present both in the intravascular and the extracellular spaces. The cellular origin of ATP, adenosine and NO

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in the intravascular space is likely to be endothelial cells and erythrocytes, whereas skeletal muscle cells and endothelial cells may be primary sources of these compounds in the muscle interstitial space. Both intravascular and extracellular vasoactive compounds are likely to be important for skeletal muscle blood flow regulation although their relative contributions remain unclear. This review focuses on formation and cellular sources of intravascular *versus* skeletal muscle interstitial ATP, adenosine and NO in humans. It also discusses evidence for their roles in exercise hyperaemia and how these systems may be altered in cardiovascular disease.

Origin of ATP and adenosine

ATP. Mechanical influence, such as shear stress and compression, can induce release of ATP from most cells, including endothelial cells, erythrocytes and skeletal muscle cells (Sprague *et al.* 1996; Burnstock, 1999; Buvinic *et al.* 2009; Mortensen *et al.* 2011). In the intravascular space, ATP released from erythrocytes in relation to off-loading of oxygen from the haemoglobin molecule has been suggested to contribute to blood flow regulation (Bergfeld & Forrester, 1992; Ellsworth *et al.* 1995) (Dietrich *et al.* 2000). The proposed model of release of ATP from erythrocytes is intriguing, in part because it fits the observation that blood flow is more closely related to the amount of O₂ bound to haemoglobin than to the amount of O₂ dissolved in plasma (i.e. P_{O₂}) (Roach *et al.* 1999; González-Alonso *et al.* 2001, 2002). At rest, hypoxia increases leg blood flow and venous ATP levels, suggesting that this mechanism is operating (Mortensen *et al.* 2011), but the role of erythrocyte derived ATP during exercise remains uncertain. Inhibition of erythrocyte ATP release by CO (Jagger *et al.* 2001) does not lower venous plasma ATP levels but increases exercise hyperaemia (González-Alonso *et al.* 2002). Secondly, ATP release from erythrocytes is dependent on the cystic fibrosis transmembrane conductance regulator (Sprague *et al.* 1998), but cystic fibrosis patients who lack this regulator, have normal blood flow responses to exercise (Schrage *et al.* 2005).

The cellular source of ATP in the muscle interstitial space is not fully elucidated. Erythrocytes, which may be considered primary sources of ATP in the blood vessels, are unlikely sources of interstitial ATP as the endothelium has been shown to be a solid barrier for nucleotides (Ballard *et al.* 1987). A lack of passage of nucleotides over the endothelium is also supported by the finding in humans that infusion of ATP in the femoral artery does not alter interstitial nucleotide and adenosine concentrations (Mortensen *et al.* 2009a). Instead, likely sources of interstitial ATP are skeletal muscle cells and/or endothelial cells (Fig. 1). Skeletal muscle cells have been shown to

release ATP in response to muscle contraction, possibly through pannexin-1 hemichannels (Buvinic *et al.* 2009) and/or the cystic fibrosis transmembrane conductance regulator (CFTR) (Tu *et al.* 2010). Capillary endothelial cells may release ATP towards both the blood stream and the interstitium in response to shear stress (Burnstock, 1999), via a mechanism shown to be dependent on ATP synthase (Yamamoto *et al.* 2007). However, although these mechanisms have been documented *in vitro*, *in vivo* evidence is lacking. Sympathetic and motor nerves do not appear to greatly contribute to interstitial ATP during muscle contraction (Li *et al.* 2003).

Adenosine. In the skeletal muscle interstitial space, adenosine originates from extracellular dephosphorylation of AMP via ecto AMP 5'-nucleotidase located on the skeletal muscle and endothelial cell membranes (Fig. 1) (Hellsten & Frandsen, 1997). The origin of interstitial AMP is probably ATP released from skeletal muscle and endothelial cells (Burnstock, 1999; Yamamoto *et al.* 2007; Buvinic *et al.* 2009). Intracellular degradation of ATP in the skeletal muscle does not appear to be a likely source as inhibition of nucleoside transport in contracting skeletal muscle cells in culture increases rather than decreases extracellular levels of adenosine, suggesting an uptake rather than release of adenosine during contraction (Lyngé & Hellsten, 2000). The source of intravascular adenosine is probably mainly plasma ATP where soluble and ecto nucleotidases on the endothelium degrade ATP (Marcus *et al.* 2003; Heptinstall *et al.* 2005; Yegutkin *et al.* 2007).

Origin of NO

Intravascular NO may originate from endothelial NO synthase (eNOS) present in endothelial cells, via S-nitrosohaemoglobin (SNOHb; Pawloski *et al.* 2001) and from reduction of nitrite by deoxyhaemoglobin (Hon *et al.* 2010). In the skeletal muscle interstitium, the sources of NO are likely to be neuronal NOS (nNOS), present primarily in skeletal muscle cells, and eNOS in endothelial cells (Frandsen *et al.* 1996). The endothelial cell contribution to interstitial NO concentrations would likely originate from the capillary level. Intraarterially infused adenosine has been shown to increase the concentration of NO in the muscle interstitium, suggesting release of NO towards the interstitium from capillary endothelial cells, as NO is unlikely to traverse smooth muscle cells (Nyberg *et al.* 2010) (Fig. 1). At the arteriolar level, a directional release towards the interstitium would allow for the most localized effect through direct action on the smooth muscle cells, whereas NO released at the capillary level could affect smooth muscle of the upstream terminal arteriole or a nearby arteriole. Release of NO towards the

circulation from the capillary level would appear to be less effective considering the short half-life of this molecule unless NO is carried as for example SNOHb or nitrite, which allows for a circulating source of NO.

The other suggested source of NO in the muscle interstitium, the skeletal muscle cell, contains large amounts of nNOS (Bredt *et al.* 1990) where the predominant isoform is nNOS μ located both in the cytosol and bound to the sarcolemma via the dystrophin complex (Brenman *et al.* 1996). Isolated skeletal muscle (Balon & Nadler, 1994) and skeletal muscle cells in culture (Silveira *et al.* 2003) produce and release NO in response to contraction, and thus

nNOS in skeletal muscle is likely to be a source of muscle interstitial NO. This notion is further supported by the demonstration that NO derived from nNOS μ is important for functional sympatholysis and is consequently likely to be involved in blood flow regulation (Thomas *et al.* 2003).

Intravascular and interstitial ATP and adenosine levels

Determinations of plasma levels of adenosine and ATP are crucial for the understanding of the role of these purines in the regulation of skeletal muscle blood flow and to elucidate which physiological stimuli that can

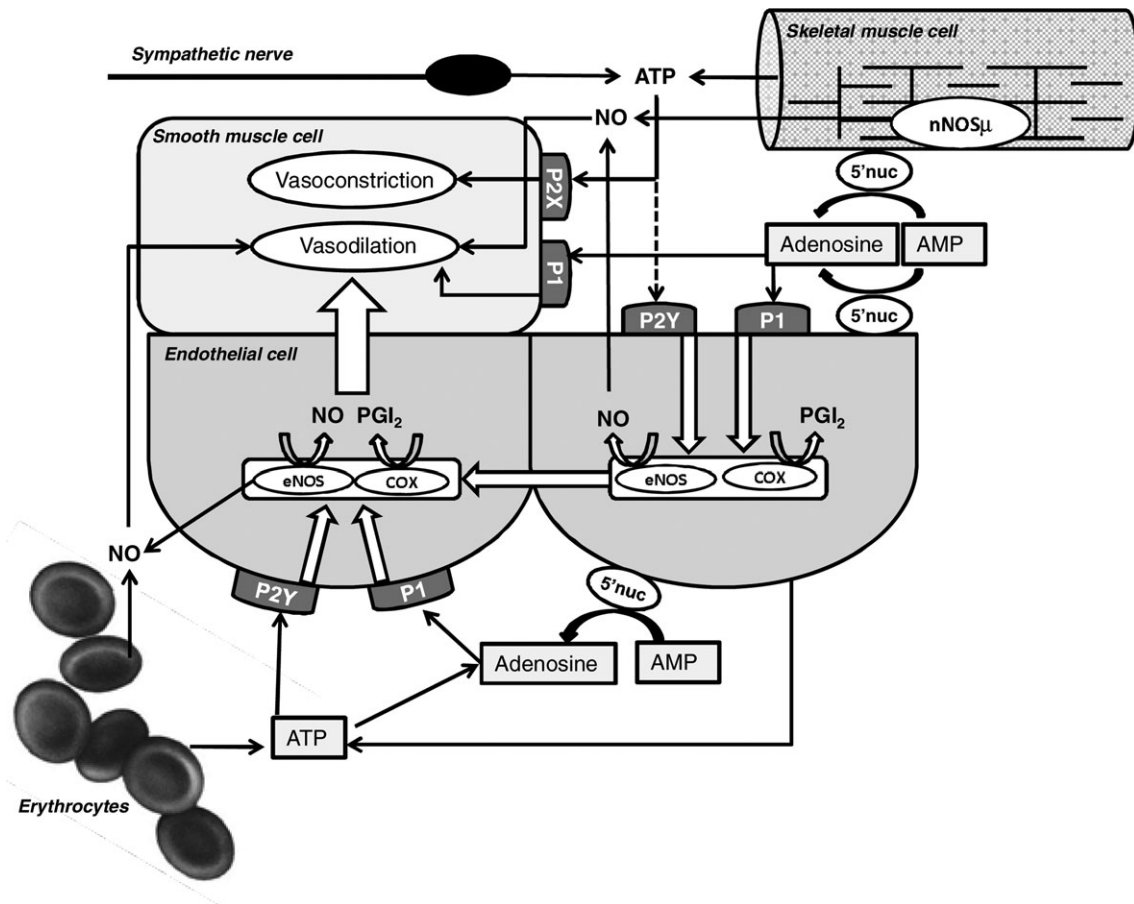


Figure 1. Putative mechanisms by which intravascular and interstitial ATP, adenosine and NO contribute to regulation of skeletal muscle blood flow

Intravascular ATP released from endothelial cells and red blood cells interact with P2Y receptors located on the luminal side of endothelial cells, which leads to the formation of NO and prostanoids that relaxes adjacent smooth muscle cells. Similarly, intravascular adenosine, generated from AMP via AMP 5' nucleotidase, may act on P1 receptors to induce vasodilatation via endothelial formation of NO and prostacyclin. Interstitial ATP released from skeletal muscle cells and sympathetic nerves may interact with P2X receptors on smooth muscle cells to induce vasoconstriction but may also be degraded to adenosine and thereby induce vasodilatation. Furthermore, muscle interstitial ATP may potentially induce vasodilatation via action on endothelial P2Y receptors and consequent formation of NO and prostacyclin. Interstitial adenosine induces vasodilatation by interacting with P1 receptors on smooth muscle cells and/or by acting on P1 receptors on the abluminal side of capillary endothelial cells, which leads to dilatation upstream via formation of NO and prostacyclin. Formation of intravascular NO may occur via release from SNOHb in red blood cells, reduction of nitrite or formation in endothelial cells. In the skeletal muscle interstitium, sources of NO are skeletal muscle cells and endothelial cells. Abbreviations: eNOS, endothelial NO synthase; COX, cyclooxygenase; PGI₂, prostacyclin; 5'nuc, 5'-nucleotidase; P1, P2Y, P2X, purinergic receptors.

induce their release. ATP and adenosine are short-lived substances, which continuously are released, taken up by cells (Forrester, 1972; Milner *et al.* 1990, 1996; Wang *et al.* 2005) or degraded by soluble (Yegutkin *et al.* 2007) and membrane bound (Marcus *et al.* 2003; Heptinstall *et al.* 2005) enzymes. The short half-life of ATP and adenosine is essential as it allows for a precise regulation of vascular tone within the muscle, but it imposes a challenge for accurate measurements of these purines. The actual ATP and adenosine levels in the vascular beds, supplying and draining skeletal muscles in humans, therefore remain controversial with resting venous plasma ATP concentrations ranging from 30 to 1000 nM depending on the method and sampling site (Gorman *et al.* 2007). Similarly, data on the ATP response during exercise are conflicting with some studies reporting unchanged (Rosenmeier *et al.* 2004; González-Alonso *et al.* 2006; Dufour *et al.* 2010) and others increased (González-Alonso *et al.* 2002; Mortensen *et al.* 2011) plasma concentrations of ATP with exercise. To overcome the problems with a continuous release and degradation of nucleotides, intravascular microdialysis probes (Fig. 2) have been used to determine ATP and adenosine levels in arterial (Mortensen *et al.* 2009b, 2011) and venous blood (Riksen *et al.* 2005; Mortensen *et al.* 2009b, 2011). Intravascular microdialysis probes allow ATP and adenosine to be separated from cells and degrading enzymes *in vivo*. With this method, plasma ATP levels of 100–150 nM at rest and of 360–560 nM in arterial and venous blood during exercise (Mortensen *et al.* 2011) have been reported (Fig. 3). The similar elevation in arterial and venous concentration of ATP during contraction, in combination with the estimated half-life of ATP of <1 s (Mortensen *et al.* 2011), provides evidence that ATP is continuously released from the arterial to the venous side in the vasculature. Although release of ATP in response to haemoglobin deoxygenation may contribute to capillary and early venous ATP concentrations, other signals for ATP release may be more important at the

arterial level. Arterial ATP concentration increase only in the exercising leg and not the resting leg (Mortensen *et al.* 2011), and thus mechanical compression, which has been shown to increase arterial ATP concentrations (Mortensen *et al.* 2011), is an important stimulus, whereas shear stress, induced by either passive exercise or arterial infusion of acetylcholine, appears to be less important (Mortensen *et al.* 2011).

The plasma adenosine concentration at rest are similar to that of ATP, e.g. around 20–50 nM; however, in contrast to ATP, the adenosine levels do not increase in concentration with exercise (Mortensen *et al.* 2009b), indicating that plasma adenosine is not a major contributor to exercise hyperaemia. Nevertheless, intravascular adenosine formation appears to be increased during exercise as the adenosine concentration is maintained despite the large increases in blood flow that occur during exercise (Mortensen *et al.* 2009b). Compared to plasma concentrations of purines, interstitial purine levels during exercise are many-fold higher, further supporting a lack of diffusion of ATP and adenosine across the endothelium. Muscle interstitial ATP concentrations are approximately 200–400 nM at rest and increase in relation to increasing exercise intensity and levels of several micromolar have been observed (Fig. 4) (Hellsten *et al.* 1998; Mortensen *et al.* 2009b; Hansen *et al.* 2011). The concentration of adenosine in the muscle interstitium increases from approximately 200 nM at rest, and the concentration increases at a low rate with increasing exercise intensity up to $\sim 2 \mu\text{M}$ during intense exercise (Fig. 4) (Hellsten *et al.* 1998). The fact that muscle interstitial concentrations are so relatively high is intriguing and underscores a potential role for extracellular purines as signalling compounds for various physiological events such as blood flow regulation and angiogenesis (Høier *et al.* 2010).

The concentration of NO in humans *in vivo* has only indirectly been assessed, primarily by measurements of

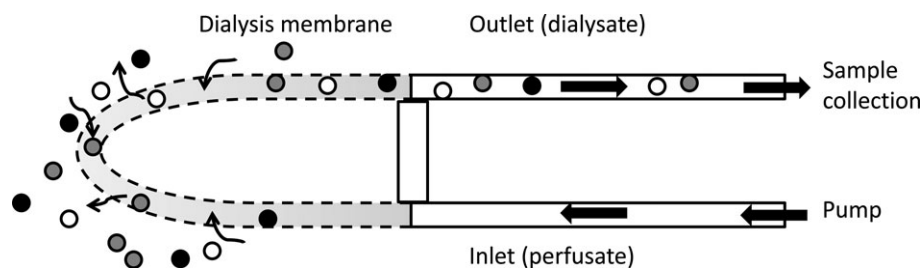


Figure 2. Schematic drawing of a microdialysis probe

The microdialysis probe consists of thin tubing containing a circular dialysis membrane. The cut-off of the membrane can vary and is selected according to the molecular mass of the compound of interest. The microdialysis probe is positioned in a skeletal muscle or, when positioned in large blood vessels, advanced through catheters into the intraluminal space. The tubing is perfused with a buffer (perfusate) by use of a pump, generally at rates of $0.1\text{--}5 \mu\text{l min}^{-1}$. For the intravascular probes, low-molecular heparin is added to the perfusate to avoid blood clotting. At the site of the dialysis membrane, exchange occurs between compounds in the interstitial fluid and the perfusate. The outlet buffer, dialysate, is collected and analysed for compounds of interest.

the stable NO products nitrite and nitrate (NOx), either separately or combined. As concentrations of nitrite and nitrate are the result of formation of NO and reduction of nitrite to NO, the levels are associated with some uncertainties and only provide indirect evidence for NO formation. Resting levels of plasma NOx have been

reported to be in the range of 50 to 80 μM (Nyberg *et al.* 2012) and the levels have been reported to either remain unaltered (Fig. 5) (Nyberg *et al.* 2012) or to be extracted from plasma by skeletal muscle during exercise (Kingwell *et al.* 1997; Lewis *et al.* 1999). Nitrite concentrations, which may provide a more sensitive measure of changes in NO, have been shown to remain unaltered in femoral arterial and venous plasma during exercise (Dufour *et al.* 2010). As *in vitro* studies clearly show release of NO by muscle cells in response to contraction (Silveira *et al.* 2003), the

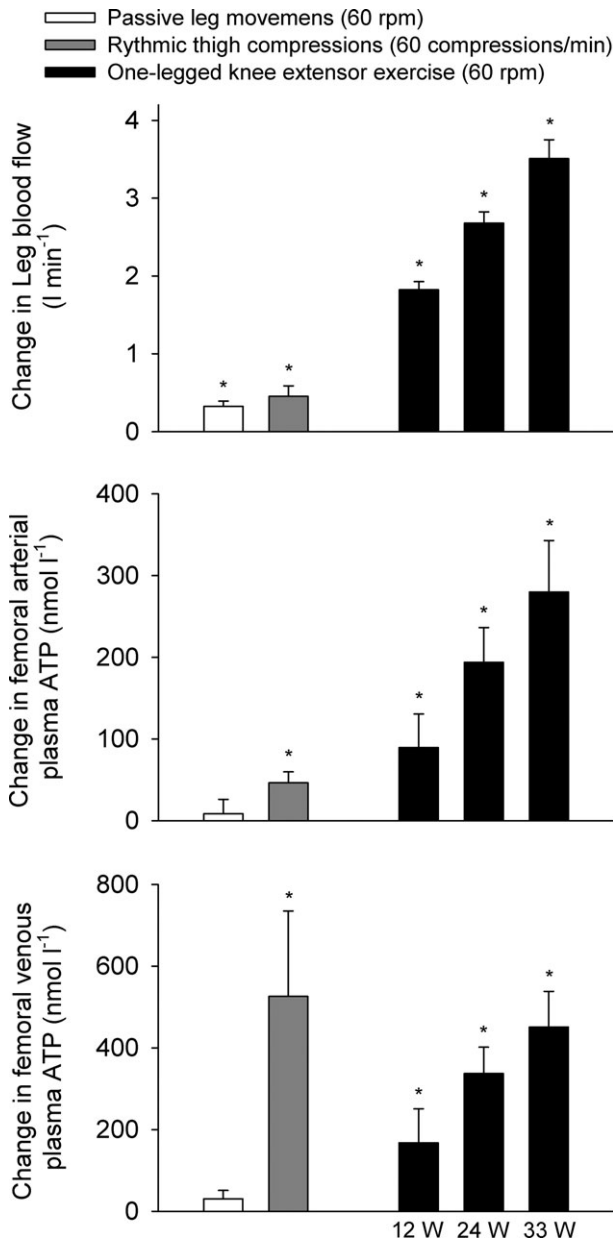


Figure 3. Change in leg blood flow and plasma ATP levels during passive exercise, rhythmic thigh compressions and dynamic exercise

The change in leg blood flow and arterial and venous ATP concentrations with passive movement, rhythmic thigh compressions and exercise are depicted from baseline conditions. The ATP concentrations were obtained by use of microdialysis placed in the femoral artery and vein. Data are means \pm SEM for 6 subjects.

*Different from baseline conditions, $P < 0.05$. Figure modified from Mortensen *et al.* (2011).

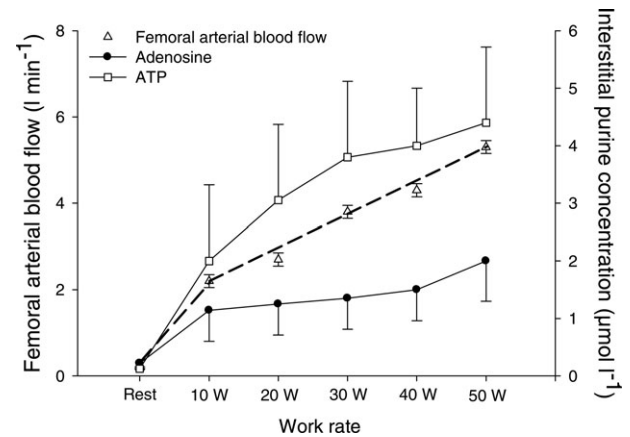


Figure 4. Muscle interstitial concentrations of ATP and adenosine and leg blood flow during exercise

Interstitial concentrations of ATP (open squares) and adenosine (filled circles) were determined in skeletal muscle of seven healthy male subjects by use of microdialysis technique. Leg blood flow was assessed by ultrasound Doppler. Measurements were performed at rest and during bouts of knee-extensor exercise at varying intensities performed in random order. Figure modified from Hellsten *et al.* (1998).

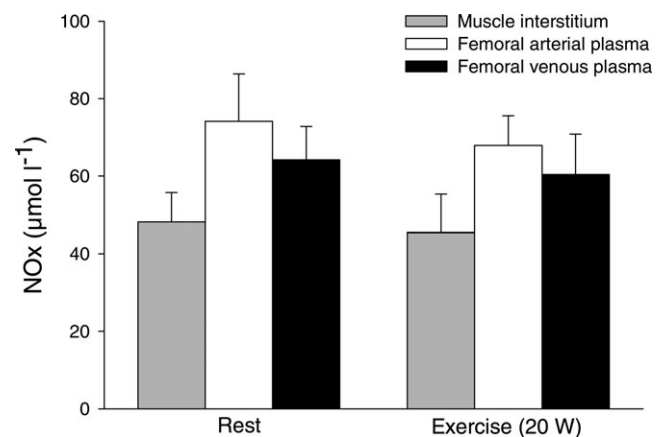


Figure 5. Skeletal muscle interstitial and plasma concentrations of NOx at rest and during exercise

Muscle interstitial fluid, obtained by microdialysis, and plasma samples obtained from the femoral artery and vein were collected from 8 middle aged subjects at rest and during one leg knee extensor exercise. Figure modified from Nyberg *et al.* (2012).

unaltered interstitial NO_x levels suggest that the rate of NO_x removal is similar to that of NO_x formation from NO. NO_x removal from the interstitium can occur either through release to the blood stream, cellular uptake, or formation of NO from nitrite within the interstitium. However, based on the measurements of a-v differences of NO_x during exercise (Nyberg *et al.* 2012), release of NO_x to the blood stream appears unlikely.

Vasodilator effects of ATP, adenosine and NO

Infusion of ATP or adenosine into the femoral artery leads to a dose-response increase in femoral arterial blood flow with a maximum level of 8–9 litres min⁻¹, which is similar to what is observed during maximal exercise (Rådegran & Calbet, 2001; Rosenmeier *et al.* 2004) although a higher dose of adenosine than ATP is required to reach this level (Rosenmeier *et al.* 2004). Infusion of the NO donor sodium nitroprusside (SNP) into the femoral artery appears to elicit a similar level of vascular conductance as ATP and adenosine although the increase in blood flow with SNP is smaller, approximately 3 litres min⁻¹, due to the parallel large drop in perfusion pressure (Rådegran & Saltin, 1999). The role of physical activity for the vasodilator response to these substances has not been well documented, but inactivity appears to increase the vasodilator response to ATP despite a reduction in muscle P2Y₂ receptor content (Mortensen *et al.* 2012). It therefore appears likely that nucleotidases and/or receptor sensitivity are altered with physical activity and play a key role in purinergic signalling.

Role of interstitial versus intravascular purines in skeletal muscle blood flow

Although indirect evidence for a role for intravascular ATP has been provided through arterial infusion of ATP and measurements of plasma ATP during various conditions (González-Alonso *et al.* 2002; Rosenmeier *et al.* 2004), the actual contribution of intravascular ATP to skeletal muscle blood flow regulation *in vivo* remains unclear. The suggested role of ATP is further supported by the observation that ATP can override sympathetic vasoconstrictor activity (functional sympatholysis) similarly to exercise, although a dissociation between the ability of ATP and exercise to override sympathetic nerve activity has also been reported (Kirby *et al.* 2011; Thaning *et al.* 2011). Selective P₂ receptor antagonists for use in humans are needed to determine the precise role of ATP in blood flow regulation.

In the muscle interstitium, ATP has mainly been attributed a role in vasoconstriction. During muscle contraction, ATP is released into the extracellular space in proportion to the intensity of contraction (Hellsten

et al. 1998; Li *et al.* 2003). Once released, interstitial ATP may contribute to the exercise pressor reflex via activation of P₂X receptors, which leads to sensitization of thin fibre muscle afferents (Li & Sinoway, 2002; Hanna & Kaufman, 2003). This mechanism of action is in accordance with findings from contracting human skeletal muscle demonstrating an intensity-dependent increase in both interstitial ATP and noradrenaline (Mortensen *et al.* 2009a). As the pressor reflex opposes the blood pressure reducing effect of the profound vasodilatation that occurs with exercise, the reflex activation induced by interstitial ATP is likely to be important for maintaining an adequate perfusion pressure across the contracting muscle.

It should also be emphasized that, although mainly a vasoconstrictor role of interstitial ATP has been described so far, a vasodilator effect of extraluminal ATP, primarily via degradation to adenosine, has been shown in the cheek pouch of hamsters (Duza & Sarelius, 2003). This dilatory effect could be mediated through formation of NO and other vasodilators (Nyberg *et al.* 2010). Furthermore, the potential role of interstitial ATP as a mediator of functional sympatholysis remains unexplored. It is an attractive possibility that a single substance could stimulate afferent fibres to increase sympathetic nerve activity, and at the same time locally impair sympathetic vasoconstrictor activity and stimulate the formation of vasodilator substances within the contracting muscle.

Inhibition of adenosine receptors has been shown to reduce exercise hyperaemia in the leg by 15–20% (Rådegran & Calbet, 2001; Mortensen *et al.* 2009b), suggesting that adenosine is essential for regulation of skeletal muscle blood flow. The observation that adenosine increases in the muscle interstitial fluid at a rate associated with the intensity of contraction (Hellsten *et al.* 1998; Costa *et al.* 2000), whereas exercise does not influence plasma adenosine concentrations (Mortensen *et al.* 2009b), does indicate that it is mainly interstitial adenosine that is responsible for the adenosine component of exercise hyperaemia. The vasodilator effect of interstitial adenosine could occur through direct activation of adenosine receptors on smooth muscle cells (Herlihy *et al.* 1976; Marshall, 2007) and/or by activation of receptors on the abluminal side of endothelial cells (Ray *et al.* 2002; Nyberg *et al.* 2010) and on skeletal muscle cells (Nyberg *et al.* 2010), as these cell types are likely to be responsible for the formation of NO and prostacyclin induced by infusion of adenosine into the interstitium (Nyberg *et al.* 2010). In human skeletal muscle, the A₁, the A_{2A} and the A_{2B} receptors have been found present on endothelial and smooth muscle cells (Lynge & Hellsten, 2000), but their respective roles for adenosine induced vasodilatation in human skeletal muscle have not been elucidated.

Studies in which NO synthase has been inhibited in parallel with at least one other vasodilator system have

clearly shown that NO contributes to exercise hyperaemia in humans, although it does not appear to be an essential vasodilator (Hillig *et al.* 2003; Mortensen *et al.* 2007). The finding that NOx in plasma and the muscle interstitium remain unaltered during exercise should probably not be interpreted to contradict a role for NO in exercise hyperaemia. The direct measurements of NO formation *in vitro* that show that NO is released upon muscle contraction (Silveira *et al.* 2003) suggest that a more direct method for determination of NO *in vivo* could reveal increased production also in humans.

Adenosine and NO formation in essential hypertension

We have recently shown that blood flow to contracting muscles in the leg is lower in humans with essential hypertension (Nyberg *et al.* 2012). This attenuated response to exercise was associated with a lower increase in interstitial adenosine, but not a reduced vasodilator response to arterially infused adenosine nor to NO mediated vasodilatation (Hellsten *et al.* 2012). Interestingly, in the same studies, 8 weeks of aerobic high-intensity training normalized the exercise induced increase in interstitial adenosine but did not affect eNOS levels (Nyberg *et al.* 2012). These observations, combined with a report of a reduced interstitial concentration of ATP during muscle contraction after a period of exercise training in individuals with mild hypertension (Hansen *et al.* 2011), suggest that alterations in the formation of interstitial adenosine and ATP may contribute to the impaired leg exercise hyperaemia observed in essential hypertension.

Conclusion

The marked increase in blood flow to the muscle which occurs when the muscle tissue is activated, is associated with an increase in both intravascular and muscle interstitial ATP and adenosine concentrations. A role for adenosine in exercise hyperaemia has been demonstrated, but the importance of ATP remains uncertain because of the lack of selective antagonists for human use. Thus, although the increase in these purines in the muscle interstitium is many-fold greater than in plasma, the precise contribution and role of intravascular *versus* interstitial ATP and adenosine to the regulation of skeletal muscle blood flow remains unclear. Although skeletal muscle cells and erythrocytes clearly release NO during conditions of muscle activity, the concentration of NO metabolites remains unaltered in the human muscle interstitium as well as in plasma during exercise. This suggests that removal of NO metabolites is similar to NO formation or that measurements of NO metabolites may not be a sufficiently sensitive method for the detection of small changes in NO production. Sensitive methodology

for direct measurements of NO in humans *in vivo* is thus warranted. Cardiovascular disease appears to be associated with a reduced increase in interstitial adenosine with exercise but not in the vasodilator response to infused adenosine, suggesting that poor capacity to form adenosine rather than a decreased sensitivity to adenosine may contribute to the increased peripheral resistance in these individuals.

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