SYMPOSIUM REVIEW

Vasodilator interactions in skeletal muscle blood flow regulation

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Abstract During exercise, oxygen delivery to skeletal muscle is elevated to meet the increased oxygen demand. The increase in blood flow to skeletal muscle is achieved by vasodilators formed locally in the muscle tissue, either on the intraluminal or on the extraluminal side of the blood vessels. A number of vasodilators have been shown to bring about this increase in blood flow and, importantly, interactions between these compounds seem to be essential for the precise regulation of blood flow. Two compounds stand out as central in these vasodilator interactions: nitric oxide (NO) and prostacyclin. These two vasodilators are both stimulated by several compounds, e.g. adenosine, ATP, acetylcholine and bradykinin, and are affected by mechanically induced signals, such as shear stress. NO and prostacyclin have also been shown to interact in a redundant manner where one system can take over when formation of the other is compromised. Although numerous studies have examined the role of single and multiple pharmacological inhibition of different vasodilator systems, and important vasodilators and interactions have been identified, a large part of the exercise hyperaemic response remains unexplained. It is plausible that this remaining hyperaemia may be explained by cAMP- and cGMP-independent smooth muscle relaxation, such as effects of endothelial derived hyperpolarization factors (EDHFs) or through metabolic modulation of sympathetic effects. The nature and role of EDHF as well as potential novel mechanisms in muscle blood flow regulation remain to be further explored to fully elucidate the regulation of exercise hyperaemia.

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Introduction

Blood flow to skeletal muscle is highly dynamic and increases markedly with exercise at a rate closely related to the oxygen demand of the muscle (Andersen & Saltin, 1985). Overall, muscle blood flow is regulated through a balance between, on the one hand, sympathetic activity and vasoconstrictors and, on the other hand, vasodilators and compounds modulating the effect of sympathetic activity. These vasodilating compounds are formed locally in the skeletal muscle tissue and are released from end-othelial cells, red blood cells and skeletal muscle cells as a result of signals primarily related to the balance between oxygen delivery and demand. Several vaso-dilators, including nitric oxide (NO), prostacyclin, ATP, adenosine, potassium and compounds associated with the endothelium derived hyperpolarizing factor (EDHF) concept, such as 11,12-eicosatrienoic acid (11,12-EET), have been proposed to be of importance for muscle blood flow regulation. For review on this topic see Clifford & Hellsten (2004) and Sarelius & Pohl (2010). Evidence for the role of these vasodilators in exercise hyperaemia stems from studies showing that the vasodilators are formed in exercising muscle and from studies using pharmacological interventions to either inhibit or promote the vaso-dilator systems. None of the proposed vasodilators seem to operate independently or to be essential for reaching adequate blood flow during exercise, but they show a close interaction with other vasodilator systems. Vaso-dilator interactions may serve two purposes, where one is a redundancy mechanism whereby one vasodilator can take over when the formation of another vasodilator is impaired, and the other is activation of other vasodilator systems. The redundancy interaction may occur either chemically, by direct interactions between the vasodilator systems, or be functional and coupled to the demand for oxygen. Redundancy is a physiologically important concept as it can secure adequate oxygen supply despite impairments in vasodilator function. It is important to keep in mind that functional redundancy only becomes apparent in experimental settings when there is a demand for oxygen in the tissue, such as during exercise or hypoxia, whereas it is lacking in *in vitro* set-ups and experiments utilizing infusion of vasodilators. The other kind of vaso-dilator interaction serves to promote the formation of one or several other vasodilating systems, thereby potentially enhancing the vasodilator effect. NO and prostacyclin appear to be central in both of these interactions as they share a redundancy interaction and as they both are activated by multiple compounds and mechanical signals.

It should be emphasized that several other compounds than mentioned in this review have been proposed to contribute to exercise hyperaemia, e.g. potassium and lactate. Due to restrictions in length of this review we have chosen to discuss only selected compounds and their interactions and with a focus on human studies.

Functional role of NO, prostanoids and EDHF in exercise hyperaemia

In 1969 prostanoids were proposed to be involved in muscle blood flow regulation based on the findings that infusion of prostanoids into the brachial artery increased blood flow (Bevegård & Orö, 1969). A role for prostanoids in exercise hyperaemia was later supported by the findings that both plasma (Wilson & Kapoor, 1993) and interstitial (Frandsen *et al.* 2000) prostacyclin and prostaglandin E_2 concentrations were increased during muscle contractions in the forearm and leg, respectively. However, infusion of cyclooxygenase (COX) inhibitors to inhibit the formation of prostanoids, has shown no effect on blood flow at rest or during exercise in the human forearm (Shoemaker *et al.* 1996) or leg (Fig. 1) (Mortensen *et al.* 2007; Schrage *et al.* 2010).

In the late 1980s, Vallance and co-workers blocked NO synthase (NOS) by infusion of N^G-monomethyl-L-arginine (L-NMMA) and showed a 50% reduction in resting forearm blood flow (Vallance *et al.* 1989). The importance of NO for resting blood flow, blood flow in recovery from exercise, and blood flow during passive movement has since been widely confirmed in the leg (Rådegran & Saltin, 1999; Mortensen *et al.* 2009*b*; Heinonen *et al.* 2011) and in the forearm (Panza *et al.* 1993; Gilligan *et al.* 1994; Dyke *et al.* 1995). During exercise, however, inhibition of NO formation has been shown not to reduce blood flow to the leg (Rådegran & Saltin, 1999; Bradley *et al.* 1999; Frandsen *et al.* 2001; Kingwell *et al.* 2002; Heinonen *et al.* 2011), whereas a transient effect has been observed in the forearm (Schrage, 2004). Thus, at least in the leg, neither NO nor prostanoids appear to be obligatory for exercise hyperaemia (Fig. 1). However, experiments in which the synthesis of NO and prostanoids have been inhibited simultaneously have demonstrated a clear reduction in leg blood flow during exercise (Boushel *et al.* 2002; Mortensen *et al.* 2007; 2009*b*; Heinonen *et al.* 2011). The observation that single inhibition of a system has no effect on exercise hyperaemia whereas combined inhibition markedly lowers blood flow suggests that there is a compensatory formation of the other vasodilator so that adequate blood flow is achieved. Direct interactions between the vasodilator systems may explain this redundancy, as described below.

EDHF is a concept derived from the observation that acetylcholine induces hyperpolarization of smooth muscle cells in the presence of NOS and COX inhibitors (Busse *et al.* 2002). The EDHF concept, which has not yet been fully elucidated, entails multiple compounds and the identity of these compounds varies according to tissue
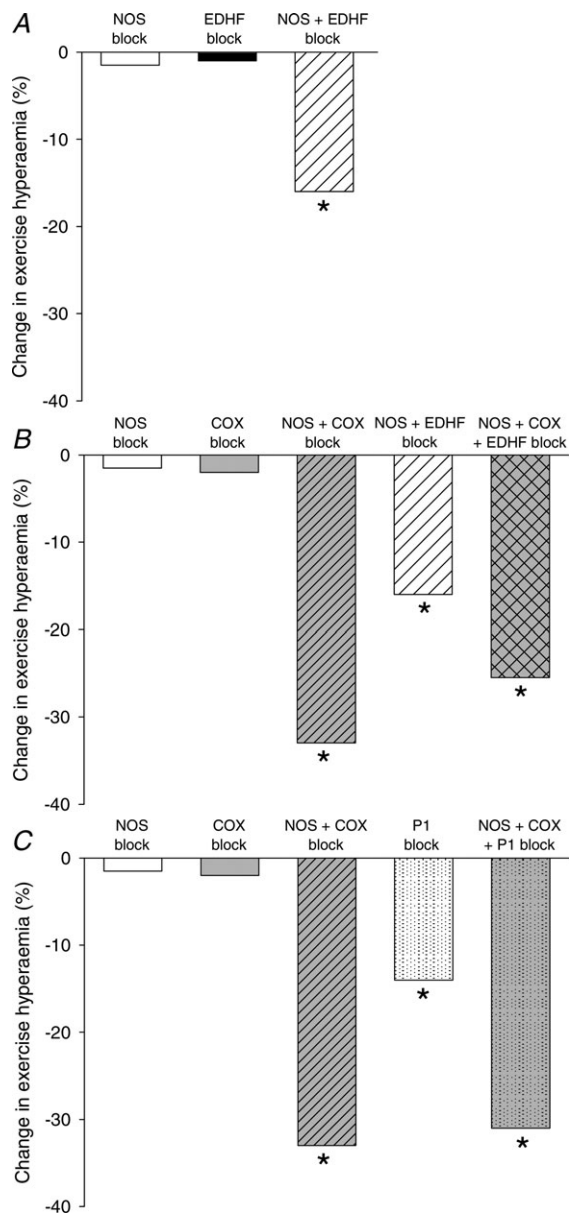
Figure 1. Change in exercise hyperaemia in the leg during inhibition of nitric oxide, prostaglandins, EDHF and the purinergic P1 receptor in young healthy male subjects
A, relative change in leg exercise hyperaemia with single or combined inhibition of nitric oxide synthase (NOS) and the endothelial derived hyperpolarizing factor (EDHF) cytochrome P450 2C9. *B*, relative change in leg exercise hyperaemia with single, double or triple blockade of NOS, EDHF and cyclooxygenase (COX). *C*, relative change in leg exercise hyperaemia with single, double or triple blockade of NOS, COX and the adenosine P1 receptor. In all experiments the exercise performed was single leg knee extensor exercise. Inhibition was achieved by arterial or venous infusion: NOS inhibition by N^{G}-monomethyl-L-arginine (L-NMMA) or N^{G}-nitroarginine methyl ester (L-NAME), COX inhibition by indomethacin, CYP 2 C9 by sulfaphenazole, EDHF by tetraammoniumchloride, and P_{i} receptor blockade by theophylline. Blood flow was determined by either thermodilution or ultrasound Doppler technique. Adapted from Frandsen *et al.* (2000), Hillig *et al.* (2002), Mortensen *et al.* (2007) and Mortensen *et al.* (2009).

and blood vessel type (Busse *et al.* 2002). In coronary and skeletal muscle resistance arteries, the product of CYP 2C9, 11,12-eicosatrienoic acid (11,12-EET), as well as other eicosatrienoic acids (EETs), such as 8,9- and 14,15-EETs, have been identified as EDHFs (Fisslthaler *et al.* 1999; Bolz *et al.* 2000). In the human forearm, bradykinin has been shown to elevate blood flow during NOS and COX blockade, indicating the presence of an EDHF mediated mechanism (Halcox *et al.* 2001). In the human leg, single blockade of CYP 2C9 by arterial infusion of sulphaphenazole has no effect on exercise hyperaemia but combined inhibition of NO synthesis and CYP 2C9 lowers blood flow by ∼15% (Fig. 1) (Hillig *et al.* 2003). This finding suggests an interaction between these two vasodilator systems, similar to the redundancy interaction observed between NO synthase and COX. Interestingly, when EDHF blockade by infusion of the non-specific potassium channel blocker tetraammonium chloride (TEA) is added to combined inhibition of NO synthase and COX, exercise hyperaemia is not reduced beyond that of NO synthase and COX inhibition combined (Mortensen *et al.* 2007). The immediate interpretation could be that EDHF induced vasodilatation cannot compensate for the impaired NO and prostanoid systems, but an equally possible explanation is that TEA infusion is not sufficiently specific to examine an EDHF effect.

Stimulators of nitric oxide and prostanoid formation in endothelial cells

NO produced from eNOS and prostacyclin produced by the COX pathway play critical roles in normal vascular biology and pathophysiology and regulate vascular conductance, platelet aggregation, angiogenesis and vascular smooth muscle proliferation (Dudzinski & Michel, 2007; Félétou *et al.* 2011). eNOS is activated by increases in intracellular Ca^{2+} with concurrent binding of calmodulin (CaM) to the enzyme (Nathan & Xie, 1994) and by protein phosphorylation at several sites (Fleming & Busse, 2003; Mount *et al.* 2007). The formation of prostacyclin is stimulated by increases in intracellular Ca^{2+} in endothelial cells that lead to liberation of arachidonic acid and activation of the COX pathway (Fig. 2) (Carter *et al.* 1988; Ray & Marshall, 2006; Domeier & Segal, 2007). Numerous chemical and mechanical stimuli including ATP, adenosine, ACh, insulin, bradykinin, histamine, thrombin, stretch and shear stress have been shown to activate eNOS through increases in Ca^{2+} and/or phosphorylation status (de Wit *et al.* 1997; Fleming & Busse, 2003; Ray & Marshall, 2006; Domeier & Segal, 2007; Dudzinski & Michel, 2007; da Silva *et al.* 2009; Nyberg *et al.* 2010; Raqeeb *et al.* 2011). Similarly, prostacyclin formation has been shown to be increased by several of the same stimuli, e.g. ACh, ATP, adenosine, bradykinin, histamine
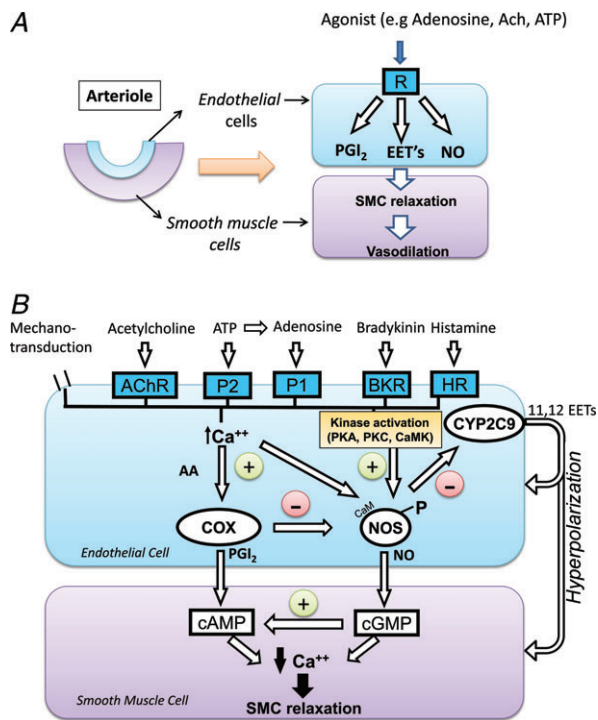
Figure 2. Schematic illustration of vasodilator interactions in skeletal muscle arterioles

A, illustration of a simplified view of endothelium-dependent induction of vasodilatation. Vasodilator agonist, such as adenosine or acetylcholine (ACh), acts on specific receptors on endothelial cells lining the luminal side of an arteriole. The receptor activation leads to the formation of compounds which cause relaxation of smooth muscle cells situated adjacent to the endothelial cells resulting in vasodilatation of the arteriole. *B*, detailed illustration of how vasodilator systems in the vascular wall can be activated and of proposed interactions between vasodilator systems. In vascular endothelial cells, several compounds, including acetylcholine, ATP, adenosine and bradykinin as well as mechanical signals including shear stress, activate both endothelial NO synthase (eNOS) and the arachidonic acid pathway leading to formation of prostacyclin (PGI$_2$) and eicosatrienoic acids (EETs). Activation of NOS and the arachidonic acid pathway occurs via an increase in intracellular calcium but eNOS activity is also regulated by protein phosphorylation at different sites. Redundancy exists between the NO and the prostacyclin systems where one described mechanism is inhibition of NOS by prostacyclin. Redundancy also exists between the NO system and cytochrome P450 2C9 (CYP 2C9), which produces 11,12-eicosatrienoic acid (11,12-EET) that can induce smooth muscle cell (SMC) relaxation by hyperpolarization. During normal conditions, NO exerts an inhibitory effect on CYP 2C9 but when NO formation is inhibited, the activity of CYP 2C9 increases. In the smooth muscle cell cyclic guanosine monophosphate (cGMP) can promote cyclic adenosine monophosphate (cAMP) levels by inhibiting phosphodiesterase III, which degrades cAMP. Abbreviations: AChR: acetylcholine receptor; AA: arachidonic acid; ATP: adenosine 5'-triphosphate; BKR: bradykinin receptor; 11, 12 EETs: 11, 12 eicosatrienoic acid; CaM: calmodulin; CaMK: calmodulin kinase; cAMP: cyclic adenosine monophosphate; cGMP: cyclic guanosine monophosphate; COX: cyclooxygenase; CYP 2C9: cytochrome P450 2C9; HR: histamine receptor; PGI$_2$: prostacyclin; PKA, protein kinase A; PKC: protein kinase C; P1: purinergic receptor 1; P2: purinergic receptor 2; SMC: smooth muscle cell.

and shear stress (Baenziger *et al.* 1980; Grabowski *et al.* 1985; Carter *et al.* 1988; Koller *et al.* 1994; Ray & Marshall, 2006; Domeier & Segal, 2007; Nyberg *et al.* 2010). The multiple stimulators of eNOS and the COX pathway highlight the importance of these vasoactive systems for vascular function and illustrate a dynamic control of NO and prostanoid bioactivity. The advantage of this design is that activation can occur even if some activation pathways are weak. On the other hand, impairments in these central vasodlator systems, as can occur in cardiovascular disease (Vanhoutte *et al.* 2009) can have a large impact on vascular function.

Interactions of nitric oxide and prostanoids in endothelial cells

In addition to their effects on smooth muscle cells, NO and prostanoids may also influence autacoid production in the endothelial layer. Inhibition of prostanoid synthesis leads to an increased NO formation in endothelial cells, an effect brought about by a cAMP induced reduction in the intracellular calcium level (Fig. 2) (Bolz & Pohl, 1997). The inhibitory effect of prostanoids on NO formation (Bolz & Pohl, 1997) could explain why exercise hyperaemia is not reduced when COX is inhibited in humans (Mortensen *et al.* 2007; 2009*b*). In this setting, inhibition of prostanoid formation would increase intracellular calcium, leading to a compensating increase in NO formation. In contrast, inhibition of the NO system may not severely affect NO function as this system is already suppressed by the contraction-induced formation of prostanoids (Frandsen *et al.* 2000; Karamouzis *et al.* 2001). This latter finding is supported by a lack of effect of NO synthase inhibition on the exercise induced increase in prostacyclin levels in the skeletal muscle interstitium (Frandsen *et al.* 2000). A redundancy interaction between the prostacyclin and the NO system may also explain the synergistic effect of combined NOS and COX inhibition in reducing ATP induced vasodilatation (Mortensen *et al.* 2009*b*).

As described above, the NOS system also shows an interaction with CYP 2C9 in that inhibition of CYP 2C9 or NOS alone does not lower exercise hyperaemia, whereas the combined inhibition does (Hillig *et al.* 2003). The explanation may lie in the observation that NO can inhibit the activity of CYP 2C so that when NO is inhibited the activity of CYP2C is enhanced and more of the vasodilator 11,12-EET is formed (Bauersachs *et al.* 1996).

Interactions of cAMP and cGMP in smooth muscle cells

The intracellular second messengers involved in smooth muscle cell (SMC) relaxation are the cyclic nucleotides, cyclic adenosine monophosphate (cAMP) and cyclic

guanosine monophosphate (cGMP), generated by the activity of adenylate and guanylate cyclase, respectively. cGMP is considered to be the main mediator of the cellular effects produced by NO (Bina *et al.* 1994), although at least a part of the NO-induced SMC relaxation is cGMP independent (Carvajal *et al.* 2000), whereas the vaso-dilator effects of prostanoids are mediated via elevations in cAMP (Vanhoutte & Mombouli, 1996). Interestingly, cGMP has been shown to have an inhibitory effect on the degradation of cAMP (Maurice & Haslam, 1990), which may explain the synergistic effect of NO and prostanoids on SMC relaxation (Fig. 2) (de Wit *et al.* 1994). This synergism is physiologically interesting as it increases the sensitivity of the system, but on the other hand it also indicates that even a small attenuation in the formation of cGMP could have a large effect on the resulting dilatation. The latter suggestion could explain why the different vasodilator systems that stimulate cGMP and cAMP can be stimulated by so many different compounds. In this context, simultaneous inhibition of NO and prostanoid formation could depress SMC levels of cAMP and cGMP to such an extent that additional inhibition of vasodilator systems coupled to these cyclic nucleotides may not have a further effect.

Vasodilator activation by ATP and adenosine

Adenosine binds to P1 purinergic receptors whereas ATP binds to P2 receptors (Ralevic & Burnstock, 1998). The vasodilator effect of both adenosine (Ray *et al.* 2002; Mortensen *et al.* 2009*b*; Nyberg *et al.* 2010) and ATP (McCullough *et al.* 1997; Hammer *et al.* 2001; Mortensen *et al.* 2009*a*; Crecelius *et al.* 2011) have been shown to be mediated in part via formation of NO and prostanoids (Fig. 3). As ATP is degraded rapidly by membrane-bound and soluble nucleotidases within the vasculature (Gordon, 1986; Yegutkin, 2008), one explanation for the convergence of downstream signalling could be that the vasodilator effect of ATP is mediated via adenosine. However, inhibition of P1 receptors does not reduce the vasodilator response to intra-arterial ATP infusion in humans (Rongen *et al.* 1994; Mortensen *et al.* 2009*b*; Kirby *et al.* 2010), suggesting that the vaso-dilator effect of intravascular ATP is independent of adenosine. This suggestion is also in congruence with observations in isolated endothelial cells demonstrating a similar potency of ATP and the P2Y receptor specific agonist UTP (da Silva *et al.* 2009; Raqeeb *et al.* 2011). Interestingly, in contrast to the adenosine-independent vasodilator effect of intravascular ATP, extraluminal application of ATP to blood-perfused arterioles has been suggested to be dependent on the action of adenosine on P1 receptors (Duza & Sarelius, 2003). This discrepancy between mechanisms underlying interstitial and intra-vascular ATP-induced vasodilatation is likely to reflect

differences in receptor expression and/or the capacity for nucleotide degradation in the two compartments, but more evidence is needed to clarify the interaction of adenosine and ATP in the intravascular and interstitial space.

Forearm and leg: do both models reflect skeletal muscle vasculature?

Experimental considerations in the forearm *versus* leg model. Evaluation of vascular function and blood flow regulation relies on the determination of blood flow and/or changes in arterial vessel diameter which can be evaluated by various methods in the forearm and leg model (Casey *et al.* 2008). Because the forearm volume is low, smaller doses of first-pass pharmacological drugs can be infused into the arterial circulation, which reduces the risk of confounding systemic effects such as an increase in blood pressure. A drawback of the forearm model, however, is that arterio-venous differences across the forearm cannot be obtained, because there is no single vein draining the forearm (Wahren 1966). Consequently, release and uptake of substances across the experimental limb cannot be determined and forearm oxygen uptake ($\dot{V}_{O_2}$) cannot be estimated. Without forearm $\dot{V}_{O_2}$, the physiological importance of differences in blood flow is undisclosed because it is not known if it is secondary to changes in vascular function or a change in metabolic demand. In regards to the latter, it is well known that some infused substances can alter local metabolism (Mortensen *et al.* 2007; Boushel *et al.* 2012).

Limb specific vascular function. Due to the upright post-ure, the human legs are much more exposed to hydrostatic pressure when compared to the forearm (Rowell, 1993). In addition, the use of the leg muscles in locomotion holds the skeletal muscle tissue more active than the arm muscles, even in sedentary individuals. It therefore seems likely that there are limb differences in vascular function and blood flow regulation and studies that have compared the arm and leg have also reported differences in vasodilator responsiveness to endothelium-dependant and –independent substances (Newcomer *et al.* 2004) and α_1-adrenergic responsiveness (Pawelczyk & Levine, 2002). In the forearm, adenosine infusion shows a large difference in vasodilator effect among individuals (Martin *et al.* 2006) whereas the inter-subject variation is less in the leg (Mortensen *et al.* 2009*b*; Nyberg *et al.* 2010; Hellsten *et al.* 2012). Moreover, a large number of studies show that the vasodilator response to ACh is markedly reduced in the forearm in individuals with cardiovascular disease (Virdis *et al.* 2010); however, when measured in the leg, the vasodilator response to ACh has been found to be similar in hypertensive and normotensive individuals, and

in Type II diabetics and healthy controls (Thaning *et al.* 2011; Hellsten *et al.* 2012). This lack of difference in ACh induced vasodilatation also suggests that the finding that ACh infusion induces vasoconstriction in the forearm of individuals with cardiovascular disease, primarily by binding of prostacyclin to TP receptors (Félétou *et al.* 2010), is more evident in the arm. Ageing also appears to affect the endothelial function of the legs more than the arms (Thijssen *et al.* 2011). With regards to blood flow regulation, NO appears to be obligatory for exercise hyperaemia in the forearm of young subjects (Schrage, 2004), whereas it is not in the leg (Rådegran & Saltin, 1999; Frandsen *et al.* 2001; Nyberg *et al.* 2012). Notably, the capacity to form prostacyclin has also been shown to be

lower in plasma and leg skeletal muscle in individuals with hypertension compared to normotensive control subjects (Hellsten *et al.* 2012).

Thus, existing evidence suggest that there are differences in blood flow regulation in young healthy individuals and that ageing and cardiovascular diseases affect the arms and legs differently (Thijssen *et al.* 2011). Flow mediated dilatation in the forearm is widely used to evaluate endothelial function, but because of the apparent differences in vascular function and relative small mass of the forearm, it is likely that the leg or a combination between evaluation of the endothelial function of the arm and leg is a better indicator of the general cardiovascular system (Thijssen *et al.* 2011; Mortensen *et al.* 2012). More studies comparing
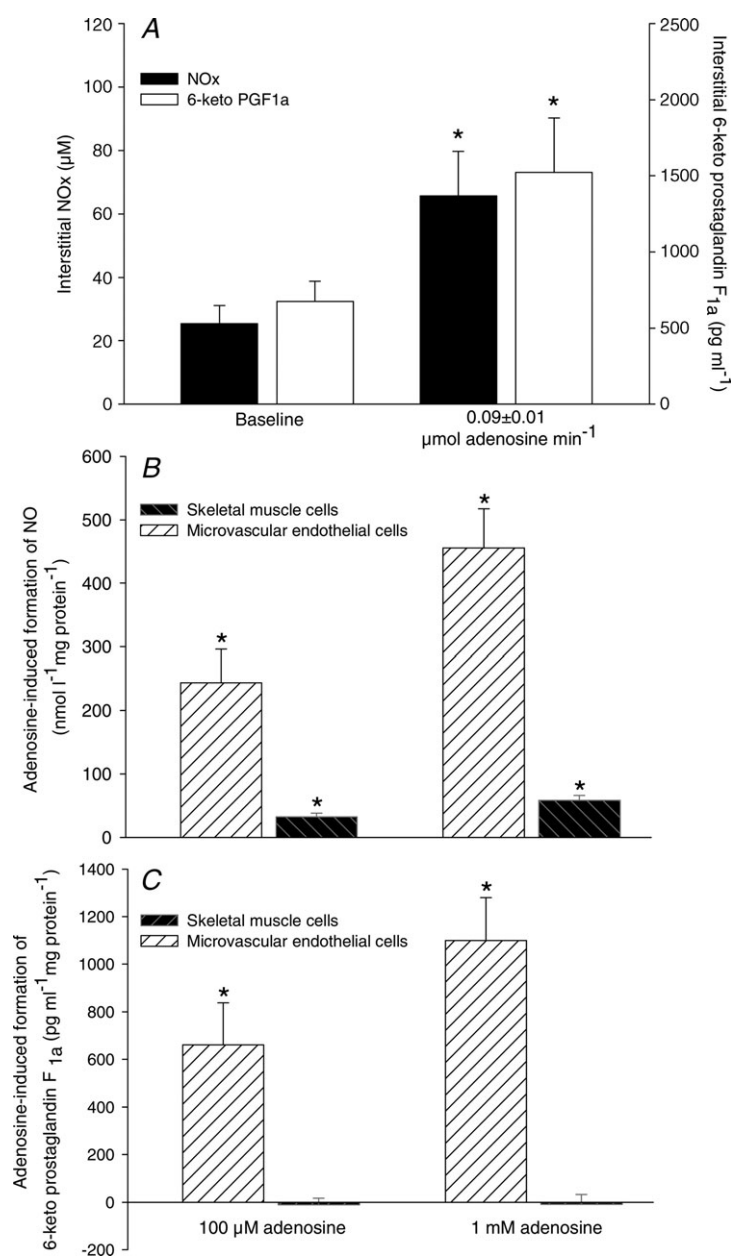

Figure 3. Adenosine induced stimulation of NO and prostacyclin formation
A, muscle interstitial nitrite and nitrate (NOx) and 6-PGF1α concentrations during baseline conditions and interstitial adenosine infusion through microdialysis probes. *Significantly different from baseline ($P < 0.05$). *B*, effect of adenosine on release of NO from skeletal muscle and microvascular endothelial cells. *Significant formation ($P < 0.05$). *C*, effect of adenosine on release of 6-PGF1α from skeletal muscle and microvascular endothelial cells. *Significant formation ($P < 0.05$). Adapted from Nyberg *et al.* (2010).

vascular function and regulation in the arms and legs and evaluating the prognostic value of the endothelial function in the arm and leg in regards to cardiovascular risks are needed, but existing data suggest that observations in one limb cannot be extrapolated to other limbs.

Conclusion

It is clear that regulation of skeletal muscle blood flow is a complex process involving several cellular sources, and many compounds and mechanisms. Most of the vasodilators proposed to be of importance in skeletal muscle blood flow regulation do not appear to be essential as their vasodilator effect can be compensated for by the formation of others. NO and prostacyclin are two central vasodilators which are stimulated by a number of other vasodilator compounds including ACh, ATP, adenosine and mechanical signalling. The many stimulators of NO and prostacyclin can probably be explained by redundancy and fine-tuned control of blood flow that ensures the vital delivery of O_2 even under compromised conditions.

Pharmacological interventions in humans designed to reveal mechanisms underlying the regulation of skeletal muscle blood flow have succeeded in identifying specific vasodilators and vasodilator interactions of importance for exercise hyperaemia. However, regardless of the pharmacological interventions used, exercise hyperaemia has only been partially reduced, suggesting that yet unknown vasodilator mechanisms, potentially independent of cAMP and cGMP formation, remain to be revealed. Future studies should focus on the functional role of EDHF as well as potential novel mechanisms in muscle blood flow regulation.

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