Interaction of Nitric Oxide and Muscle Contraction in Adenosine-Induced Muscle Vasodilation in Humans

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ABSTRACT

Interaction of Nitric Oxide and Muscle Contraction in Adenosine-induced Muscle Vasodilation in Humans

Amanda Rossi

Adenosine (ADO) is one of several endogenous substances known to mediate muscle vasodilation during exercise. The purpose of this study was to examine 1) the role of nitric oxide (NO) in ADO-mediated vasodilation; 2) the magnitude of muscle blood flow (MBF) responses to ADO infusion during exercise; and 3) the influence of ADO in regulating heterogeneous MBF distribution. In healthy, young participants (N=14), local MBF was measured using near-infared spectroscopy and indocyanine green (ICG) dye at two locations on the vastus lateralis (VL). Cardiac output was quantified using a dye-dilution technique with ICG dye. Participants were tested at rest and during 1-leg knee extension exercise (25W) under different conditions: control, ADO infusion (150mg•kg body mass^{-1}•min^{-1}) and ADO+NO blockade (L-NMMA; 1mg•kg body mass^{-1}•min^{-1}). At rest and exercise, we found that mean VLBF increased from control to ADO and decreased significantly from ADO to ADO+L-NMMA infusion; exercising BF values were always significantly higher than at rest. Additionally, with ADO infusion, a peak increase in oxy-hemoglobin concentration (O_2Hb) was reached within 2 minutes of initiating infusion; however the magnitude differed between

muscle regions, indirectly indicating ADO-induced metabolic heterogeneity. O_2Hb subsequently decreased over time, reaching a stable level at 5 min. Also, we observed that when MBF is elevated through ADO infusion, usual exercise-mediated vasodilator signals are not subsequently down-regulated during contraction demonstrating a lack of autoregulation. We conclude that NO does play a role in mediating ADO-induced vasodilation and there is a lack of autoregulatory feedback when ADO is infused during exercise.

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Otherwise, don't do it at all.'

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Mariann Rossi
whose spirit and charm remind me every day of how life should be

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In loving memory of Alla cara memoria di

~ *Maria Magliano*~

nato il 17 ottobre, 1932- deceduto il 18 dicembre, 1986

&

~ *Remo Rossi* ~

nato il 29 dicembre, 1929- deceduto il 14 gennaio, 2003

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E spero che giorno per giorno, in ogni momento siete fieri di me
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Siete stati una parte della mia vita

~ Viva Bene, Spesso L'Amore, Di Risata Molto ~

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~ Sogni d'oro ~

La vostra prima nipote

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LITERATURE REVIEW

Introduction

In the field of cardiovascular physiology, a major emphasis is placed on research aimed at elucidating what factors regulate blood flow to skeletal muscle during exercise. It is known that neural, humoral, metabolic and mechanical factors contribute to the regulation of this complex system (Olsson, 1981; Segal, 2005). Blood functions to transport and deliver oxygen, nutrients and hormones to the body's tissues and subsequently remove a multitude of metabolic by-products. The former role is indeed an important physiological function since almost all cells in the body require oxygen for viability. All tissues are capable of regulating their own local blood flow in proportion to their own metabolic needs (Wolff, 2007). During exercise, for example, blood flow is increased in the active skeletal muscle in order to accommodate increases in metabolic activity (Saltin, 1985). Simultaneously, blood is shunted away from other organs, such as the kidneys and spleen (Rowell, 1986), enhancing the efficiency with which the cardiovascular system can supply blood to the tissues with a higher metabolic demand.

Blood flow increases as a result of vasodilation, which is simply an increase in the inner diameter of blood vessels. Several mechanisms regulate the degree of muscle vasodilation, and therefore how blood flow is controlled. In this review, various mechanisms of blood flow regulation; namely the vascular

myogenic reflex, autonomic sympathetic neural signaling and the muscle pump effect will be briefly described, followed by a more detailed discussion of the main focus; understanding the actions of and interactions between putative biochemical substances currently thought to play a major role in skeletal muscle vasodilation during exercise.

The myogenic mechanism of vascular control maintains that tension receptors present in arterial and arteriolar walls (which contain smooth muscle) sense and respond to pressure exerted on the vascular wall, induced by luminal stretch or abluminal compression forces induced by muscle contraction (Delp, 1999; Guyton & Hall, 2000). The myogenic response is thought to be one of the key regulators of basal vascular tone (Davis & Hill, 1999). In general, the in-vivo myogenic response is evoked by 1) a rapid and transient stretch of the vessel wall during increased pulsatile flow that enhance transmural pressure, resulting in a vascular smooth muscle contractile response (Davis & Hill, 1999; Schubert & Mulvany, 1999) which is considered protective against over distention, and 2) a mechano-chemical response via gap junctions to effect conducted vasodilation (Figueroa & Duling, 2009) when the vessel is compressed abluminally by muscle contraction. The muscle pump system is another proven mechanism whereby rhythmic contractions of skeletal muscle propel blood perfusing the muscle vasculature into the venous system, therefore assisting venous return to the

right heart, which ultimately increases cardiac output via the Frank-Starling mechanism and hence systemic blood flow is enhanced (Delp, 1999).

Another regulator of vascular conductance during exercise is the sympathetic branch of the autonomic nervous system (SNS). It has been proposed that through sympathetic cholinergic action, the SNS has the ability to induce vasodilation; however this effect is minor and inconsequential for exercise induced hyperemia. Conversely, alpha adrenergic signals cause vessels to vasoconstrict (Buckwalter *et al.*, 1997, 1998a; Buckwalter *et al.*, 1998b; Buckwalter & Clifford, 1999) and account in large part for the redistribution of blood flow away from inactive vascular beds during exercise, increasing the direction of the central blood volume to the most metabolically active tissues (Christensen & Galbo, 1983). The influence of the SNS on preferentially enhancing blood flow to contracting muscle is exemplified by studies showing that exercise induces large increases in sympathetic outflow systemically measured by plasma norepinephrine levels and norepinephrine spillover from vessels draining major organ systems (Christensen & Galbo, 1983; Leuenberger *et al.*, 1993) and direct recordings of sympathetic discharge from sympathetic nerves measured by microneurography (Seals, 1989). In actively contracting muscle, local sympatholytic signals emitted from the skeletal muscle act to curtail the vasoconstrictive activity of the SNS (Hansen *et al.*, 2000), whereas in tissues and organs which remain inactive (at a stable metabolic state) the release of sympatolytic mediators also remain constant,

and in this case the sympathetically mediated vasoconstriction will predominate. Precisely how this coordination is manifested is subject of debate and is in part based on the not so clearly defined distribution patterns of alpha 1 and 2 receptors for the maintenance of vascular tone and the impact of motor unit recruitment linking metabolic events to oppose vasoconstriction (Delp, 1999; Segal, 2005).

During the contraction phase of dynamic exercise and during isometric muscle contractions, blood flow may be impeded as a result of the mechanical compression imposed on the vessels. Laughlin (1987) proposed several mechanisms by which the skeletal muscle contraction-relaxation cycle may produce or contribute to the exercise hyperaemia; this is known as the muscle pump. Firstly, the muscle contraction causes venous emptying as a result of the direct compression on the venous vessels. Secondly, it was suggested that blood may be propelled through a muscle by a 'milking' action produced, again, as a result of the mechanical constriction cause by muscle contraction. Additionally, venous filling is facilitated by very low to negative luminal pressures in the veins produced by muscle relaxation, thus increasing the pressure gradient across the skeletal muscle vascular bed and hence driving flow across the muscle. Muscle contraction may also pass on kinetic energy to blood flow; however the accuracy of this theorem is not yet known (Laughlin, 1987). Tschakovsky and Sheriff (2004) put forth the notion that mechanical disruption of the vascular smooth muscle cells may dislocate the

cross-bridging, resulting in vasodilation. Also, the authors speculate that negative venous pressures may 'charge' arterioles, increasing pressure higher than normal during contraction, and subsequently upon relaxation this discharge pressure would drive the blood into the venous circulation.

As mentioned previously, the magnitude of blood flow to organs and tissue regions follow closely with the metabolic needs of each tissue, which implies there exists a system of communication between signals of tissue metabolism and signals controlling vascular tone, which defines tissue O_2 delivery. A prevailing argument is that this matching of blood flow to metabolic demands results from the production of local vasodilatory metabolites. Sources of these dilator substances include the endothelial lining of the vessels, the vascular smooth muscle, and possibly skeletal muscle itself (Clifford & Hellsten, 2004). There are several different substances which are known to increase in the vicinity of the interstitial space which is the interface between the vessel and muscle tissue. Examples of these substances considered to play a role in regulating vascular tone include nitric oxide (NO), prostacyclin (PGI_2), adenosine (ADO), adenosine triphosphate (ATP) and epoxyeicosatrienoic acids (EETs), among others.

The vascular endothelium, which is in direct contact with the blood, is of particular interest in cardiovascular physiology because of abundant evidence for its major role in sensing and responding in a mechano-metabolic fashion

to signals induced luminally to alter flow dynamics, and by local diffusible metabolic signals released from adjacent tissues. When stimulated by shear stress endothelial cells produce both NO, which will be discussed later in this review, and PGI_2 (Boushel, 2003). Prostaglandins, and more specifically PGI_2, which is formed from cyclooxygenase oxidation of arachidonic acid in the lipid bilayer of the vascular wall by activation of phospholipase A2 and released from the endothelial cell membrane inward to elicit vasodilation of underlying smooth muscle, are also considered important endothelial vasoactive substances (Boushel *et al.*, 2004). The skeletal muscle may also be a source of PGI_2 (Clifford & Hellsten, 2004). Concentrations of PGI_2 have been shown to increase in the circulation (Wilson & Kapoor, 1993) and in muscle interstitial fluid (Young & Sparks, 1980) during exercise. Additionally, based on studies conducted by various groups of researchers, it has been confirmed that PGI_2 does indeed influence vascular tone during rest, reactive hyperaemia and during exercise recovery periods (Boushel, 2003). The vascular endothelium also produces an endothelial derived hyperpolarizing factor (EDHF), which is thought to be a member of the EETs. EETs are also formed from arachidonic acid, specifically through cytochrome P-450 epoxygenase pathways (Boushel, 2003) and have been shown to induce vasodilation of coronary microvessels in dogs (Clifford & Hellsten, 2004). Studies examining the involvement of the EETs cascade on exercise hyperaemia have found reduced muscle blood flows during exercise when pharmacologically blocking the EET pathway with sulphaphenazole; however,

significant decreases in blood flow are only noted when EET blockade is combined with nitric oxide synthase (NOS) inhibition with L-NMMA (Passauer *et al.*, 2003). The capacity for EETs to exert a major independent role in muscle vasodilation is obscured by the inability to experimentally detect either 12-13 or 14-15 EET isoforms in the interstitial fluid samples collected from human muscle (Lattereur *et al.*, In preparation).

Also derived from the endothelium are ADO and NO, both of which are considered to be very important vasodilators. The remainder of this review will be dedicated to the role of these two latter vasodilation mediators during exercise and how they may potentially interact with each other to co-ordinate blood flow regulation by their actions on the vasculature.

Adenosine

Evidence for ADO formation and action in the skeletal muscle extracellular matrix and vascular endothelium has greatly contributed to fortifying a role for ADO as one of the major vasodilators. Previously, it was thought that ADO was produced from the degradation of adenine nucleotides (adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP)) within contracting skeletal muscle. More recently, it has been shown that in fact ADO is formed extracellularly from AMP by the membrane bound ecto-enzyme 5'-nucleotidase (ecto-5'N) (Cheng *et al.*,

2000). ADO can be produced both intra- and extracellularly and although other non-specific phosphotases are also capable of de-phosphorylating AMP to ADO, it was shown that ecto-5'N is the main enzymatic pathway in its formation. The rate of ADO production was found to be dependent on pH. Subsequently, other factors including 1) affinity of ecto-5'N for AMP and 2) AMP concentration were found to be important driving forces for ADO formation under acidic (1 & 2) and alkalinic (2 only) conditions (Cheng *et al.*, 2000). Further support for the extracellular formation of ADO was reported by Lynge *et al.* (2001) who showed that inhibition of ecto-5'N resulted in a 70% reduction in the rate of extracellular ADO accumulation when compared with control cells. It is true that ADO can still be formed in locations other than the extracellular space; however, based on this evidence, it has been accepted that the extracellular space is one of the key sites for ADO production.

Another important piece of evidence which supports ADO being a potent vasodilator candidate is the location where ADO exerts its action; the vasculature itself. Lynge and Hellsten (2000) were the first to localize ADO receptors in skeletal muscle and vasculature cells. Using immunohistochemical techniques, the researchers identified A_{2A} and A_{2B} receptors in the cytosol and sarcolemma of skeletal muscle cells biopsied from human quadriceps muscles. Furthermore, A_1, A_{2A} and A_{2B} receptors were found to co-exist on the membrane of both smooth muscle and vascular endothelial cells. Of importance is the fact that these results were obtained

from human skeletal muscle tissue, and were supported by parallel cell culture studies (Lynge & Hellsten, 2000). Therefore, these findings are specific to humans and transferability/generalization of the results between species is not a concern. Missing still is a full understanding of the precise physiological role these receptors may play.

Recent work has shown that ADO plays some role in regulating the distribution of microvascular blood flow during exercise in humans. A Finnish team used positron emission tomography (PET) to quantify blood flow while infusing theophylline, a competitive non-specific ADO receptor antagonist, and found that blockade increased blood flow heterogeneity (Heinonen *et al.*, 2007). Additionally, using novel biological tracers which specifically bind to A_{2A} ADO receptors, it has been shown that the density of A_{2A} receptors is markedly increased in the myocardium of trained athletes compared to untrained individuals (Heinonen *et al.*, 2008).

ADO has been shown to accumulate in human skeletal muscle during contraction. Hellsten et al. (1998) were the first to report interstitial ADO concentrations in human skeletal muscle. From their analysis, resting interstitial fluid ADO concentration was measured to be 220 ± 100 nmol/L at a corresponding femoral artery blood flow of 0.19 ± 0.02 L/min. The authors also showed that interstitial ADO concentration increased with muscle contraction, which in this case was achieved using the dynamic knee

extension model (Andersen & Saltin, 1985). A significant increase in ADO concentration was seen between rest and exercise; even at a low intensity. Moreover, the interstitial fluid concentrations of ADO increased at a rate associated with the observed increases in blood flow and exercise intensity (Hellsten *et al.*, 1998; Langberg *et al.*, 2002). These findings clearly demonstrate that ADO does indeed accumulate with contraction in human skeletal muscle and more importantly that this increase in ADO concentration is directly related to augmentation of blood flow and exercise intensity.

Studies quantifying the blood flow response to ADO infusion and blockade are also meaningful when examining the role of ADO as one of the key vasodilator substances. Radegran and Calbet (2001) infused theophylline and found that the hyperaemic response to exercise was approximately 20% lower under ADO receptor blockade when compared to control conditions. Several animal studies have shown similar results, where the hyperaemic response is significantly diminished with specific ADO receptor blockade; A_1 receptor antagonist DPCPX, A_{2A} receptor antagonist ZM 241385 (Bryan & Marshall, 1999; Ray & Marshall, In press-a). Meanwhile, adenosine infusion revealed the opposite effect. In fact, infusion of ADO at submaximal exercise intensities elicited blood flow values previously seen at peak effort. Continuous infusion of adenosine (up to 220 $\mu g \cdot kg$ body $weight^{-1} \cdot min^{-1}$) into the femoral artery of healthy humans at rest increases leg blood flow to peak values of ~6-8 L $\cdot min^{-1}$ measured by ultrasound Doppler (Radegran & Calbet,

2001). Using constant infusion thermodilution, the vasodilatory response of similar subjects to infusion of both (maximal dose response) adenosine (150 μg.kg body weight^{-1} min^{-1}) and ATP (80 μg·kg^{-1} body weight^{-1} min^{-1}) elicit leg blood flows of similar levels (Calbet *et al.*, 2006). The findings reported therefore demonstrate an essential role for endogenous ADO in sustaining hyperaemic response during exercise. Furthermore, the significant increase in blood flow up to maximal levels brought forth the concept that there exists a vasodilator reserve which is potentially mediated by ADO.

It also appears that ADO plays a role in peripheral vasodilation during systemic hypoxia. ADO has the capacity to dilate both arterioles and venules under hypoxic conditions in rats (Mian & Marshall, 1991). Using microdialysis to collect interstitial fluid samples and applying plethysmography to measure blood flow in humans, MacLean *et al.* (1998) showed that interstitial concentrations of ADO significantly increased from 0.44±0.08 μmol·L^{-1} to 1.03±0.15 μmol·L^{-1} and 0.85±0.09 μmol·L^{-1} at 15 and 30 minutes of hypoxia, respectively. These values have been similarly replicated in recent studies (Lattereur *et al.*, In preparation).

ADO was also proposed to be one of the key regulators of insulin-mediated vasodilation. However, after much debate arose from animal studies, Dela and Stallknecht (1999) showed that there was no change in ADO concentration when comparing interstitial fluid samples during rest, under

control and hyperglycaemic and hyperinsulinemic conditions in humans. This study was conducted in both healthy and spinal cord injury patients, so as to eliminate any controversial effects of ADO or adenine nucleotides potentially released from nerve terminals. However, in a follow-up study it was shown that combined infusion of ADO + insulin increases muscle blood flow to a level higher than either substance alone, indicating a synergistic action of these substances for increasing muscle blood flow (Scheede-Bergdahl *et al.*, Submitted). Other studies have shown a great deal of support for ADO as a regulator of the coronary circulation (Berne, 1980). However, most of these studies have been conducted on animals. Nonetheless, it is quite clear that ADO does play a role here with comparable quality evidence to that seen in human skeletal muscle concerning ADO formation in the myocardium and ADO action (Berne, 1980).

Nitric Oxide

NO is also considered to be a ubiquitous regulator of tissue vasodilation. Nitric oxide synthase (NOS) is the main catalyst of NO production and is found in several different isoforms; for example, endothelial (e), neuronal (n) and inducible (i) NOS. NOS acts to catalyse the hydrolysis of L-arginine to L-citrulline and NO in the presence of oxygen (Boushel, 2003). nNOS and μNOS, the skeletal muscle-specific isoform, were found to be located in the sarcolemma and cytosol of skeletal muscle cells. Their presence is highly

associated with mitochondrial density and also dependent on fiber type. Greater quantities of nNOS have been observed in type I muscle fibers in comparison to type II fibers (Stamler & Meissner, 2001). In human skeletal muscle, eNOS is only found in the vascular endothelium, as opposed to observations in rats, where eNOS has also been located within type I muscle fibers (Stamler & Meissner, 2001).

There are both similarities and differences between NOS isoforms regarding which factors regulate their activity. All isoforms can be transcriptionally regulated by hypoxia and upregulated by cytokines (Stamler & Meissner, 2001). Increased nNOS expression is seen with crush injuries, muscle activity and ageing; and decreased after denervation. Vascular eNOS is upregulated by shear stress. Chronic exercise can also promote vascular and skeletal muscle eNOS expression (Stamler & Meissner, 2001). NOS expression and hence activity is also regulated by phenotypic factors (myotube vs. fused myoblast), in addition to other developmental factors beyond the scope of this review (Stamler & Meissner, 2001). These factors influencing NOS expression, however, are important because ultimately, they are the regulators of NO production.

It is well known that NO regulates basal vascular tone. It does so via activation of guanylate cyclase in smooth muscle cells of the vasculature (Clifford & Hellsten, 2004). NG-monomethyl-L-arginine (L-NMMA) a non-

specific, competitive NOS inhibitor has been frequently used in studies to determine the role of NO for tissue vasodilation. During resting conditions under NOS blockade, vascular resistance is increased significantly (Gilligan *et al.*, 1994), leading to increased mean arterial pressure (Jimbo *et al.*, 1994). Significant physical reduction of vessel diameter has been shown in animal models measured by in-vivo microscopy and isolated microvessels ex-vivo with NOS blockade (Clifford & Hellsten, 2004). The rate of blood flow, as measured by plethysmography in humans is also decreased at rest when L-NMMA is infused (Gilligan *et al.*, 1994). Moreover, this decrease has been shown to be dose-dependent (Vallance *et al.*, 1989) and reversible when L-arginine (the substrate from which NO is formed) is infused either after cessation or in combination with infusion of L-NMMA (Vallance *et al.*, 1989; Tagawa *et al.*, 1994). L-NMMA has also been used in studies examining the coronary vessels. During resting conditions, it has been shown that a significant decrease in vessel diameter and increase in vascular resistance occurs (Duffy *et al.*, 1999) as well as significant decreases in blood flow (Clifford & Hellsten, 2004).

Several studies have found a lack of evidence to support NO in an obligatory role contributing towards the initiation or maintenance of reactive hyperemia in response to cuff occlusion. Peak hyperaemic flow after 3 and 10 minutes of arterial occlusion did not decrease in response to intra-arterial infusion of L-NMMA (Tagawa *et al.*, 1994). Engelke et al. (1996) also used a forearm

occlusion model to elicit hyperaemic conditions, in combination with L-NMMA administration and found that NO was not essential for reactive hyperaemia. Taken together with other data collected in this study concerning PGI_2, the authors concluded that endothelial factors do play some role in reactive hyperaemia, however, the endothelial component of vasodilation can only account for part of the large increase in blood flow observed. Other metabolites, such as H^+, K^+, PO_4, PGI_2, ATP, ADO and myogenic factors (Boushel, 2003; Clifford & Hellsten, 2004) are thought to contribute more to reactive hyperaemia than NO.

Whether or not the same can be said for NO during exercise conditions is still questionable. There is a lack of consistency amongst findings concerning hemodynamic changes induced by L-NMMA infusion between rest, exercise and recovery from exercise. Mean arterial pressure has been found to either remain the same or increase during L-NMMA infusion (Gilligan *et al.*, 1994; Koller-Strametz *et al.*, 1998). Discrepancies were also seen between studies measuring vascular resistance during NO blockade (Gilligan *et al.*, 1994; Koller-Strametz *et al.*, 1998). In addition, decreased blood flow has also been observed between control and L-NMMA infusion during exercise (Endo *et al.*, 1994; Gilligan *et al.*, 1994); however authors have attributed this decrease to the fact that basal blood flow is lowered with L-NMMA infusion (Endo *et al.*, 1994) prior to beginning exercise. Hence, evidence exists both to support and

contradict NO's role as an independent vasodilator during exercise, and most researchers have attributed only minimal involvement of NO during exercise. When we consider studies that have blocked NO production by pharmacological NOS inhibitors in addition to other vasodilator substances (i.e. multiple blockade), we see that there is a significant reduction in blood flow to the working muscles during exercise. Boushel *et al.* (2002) were the first to report the results of double vasodilator blockade; NO and PG with L-NMMA and indomethacin, respectively. It was shown that microvascular blood flow was significantly reduced during exercise under double blockade when compared to control conditions. The report reinforces the concept of synergy and redundancy amongst vasodilators. Mortensen *et al.* (2007) also observed similar blood flow decreases during double blockade; however additional blockade of EDHF, by inhibiting calcium activated K^+ (K_{Ca}) channels with tetraethylammonium chloride (sulphaphenazole), revealed no further decrease in blood flow. The authors therefore concluded that EDHF is not able to compensate for reduced NO and PG production (Mortensen *et al.*, 2007). This evidence indicates that NO does indeed play a part in exercise hyperaemia; however, other vasodilation mediators have the capacity to compensate for the blocked NO during exercise, displaying the redundancy between mechanisms.

It seems NO also exerts some role in maintaining vasodilation post-exercise. L-NMMA has been shown to attenuate peak flow and limit decreases in

vascular resistance immediately post-exercise (Endo *et al.*, 1994; Gordon *et al.*, 2002). L-NMMA also significantly reduced hyperaemic volume post-exercise (Gordon *et al.*, 2002).

Interactive Mechanisms

As previously mentioned, there exists redundant vasodilator pathways for regulating muscle blood flow (Boushel & Kjaer, 2004). Therefore, there must be some mechanism(s) by which the synergistic actions of the metabolically derived dilation mediators are regulated. It is possible that they interact via second messenger systems or even through feed forward or feed back loops. In keeping with the focus of this review, the mechanism proposed for the interaction between ADO and NO will be discussed.

Earlier it was shown that ADO does indeed have some influence on vascular tone based on measurements of blood flow and interstitial ADO concentration under different conditions. However, these studies tell little about how ADO exerts its effects at the cellular level. A reductionist approach is necessary to determine the means by which ADO provokes a vasodilatory response. In doing so, it has been uncovered that both A_1 and A_{2A} ADO receptors located on the endothelium are important in the ADO-mediated vasodilation cascade (Olsson & Pearson, 1990; Danialou *et al.*, 1997; Ray & Marshall, 2006). A_1 receptor agonists induced concentration dependent dilation of rat

diaphragmatic arterioles (Danialou *et al.*, 1997). Moreover, A_1 antagonists both attenuated ADO-induced dilation (Danialou *et al.*, 1997) and reversed hemodynamic changes and vasodilatory effects generated by A_1 selective agonists (Bryan & Marshall, 1999). Stimulation and inhibition of A_{2A} receptors showed similar results as those seen for A_1 ADO receptors (Danialou *et al.*, 1997; Bryan & Marshall, 1999). However, it seems that in hypoxia, A_1 receptors contribute more than A_{2A} receptors (Danialou *et al.*, 1997; Bryan & Marshall, 1999), whereas during normoxia both receptor types contribute equally to ADO induced vasodilation (Bryan & Marshall, 1999). In pig coronary arteries A_1 agonists, but not A_{2A} agonists, were able to mimic ADO-generated dilation. Conversely, A_1 receptor antagonist inhibited vasodilation resulting from ADO infusion, and A_{2A} receptor antagonist were ineffective (Dart & Standen, 1993). More recently, Ray and Marshall (In press-a) have suggested that in the rat hindlimb during both twitch and tetanic muscle contractions ADO derived from the muscle builds up in the interstitium regardless of the nature of the contraction imposed. Moreover, the authors state that based on the data collected, the released ADO acts directly on the vascular smooth muscle A_{2A} receptors; however the contribution towards exercise hyperaemia is greater for tetanic contractions. This remains to be investigated in humans.

ADO receptors are coupled to ATP sensitive K^+ (K_{ATP}) channels in the vascular endothelium. It has been shown that vasodilation responses induced

by A_1 receptor agonists can be attenuated with simultaneous administration of K_{ATP} channel blockers (Nakhostine & Lamontagne, 1993; Danialou *et al.*, 1997) or even cause vasoconstriction (Nakhostine & Lamontagne, 1993). Kleppisch and Nelson (1995) showed that when arterial smooth muscle K_{ATP} channels are blocked ADO-induced signalling is inhibited. Additionally, electrophysiological recordings revealed that A_{2A} agonists were capable of triggering K_{ATP} channel inhibitory currents; however A_1 agonists were not effective (Kleppisch & Nelson, 1995).

When ATP sensitive K^+ channels are stimulated in endothelial cells causing a K^+ efflux, the membrane becomes hyperpolarized (i.e. increased negative charge within the cell and a corresponding extracellular positive membrane charge). This creates an electrochemical force for Ca^{2+} influx which initiates intracellular activation of cAMP (Busse *et al.*, 1988). ADO has also been shown to increase intracellular concentrations of Ca^{2+} through activation of other K^+ channels (Mehrke & Daut, 1990). Recent work by Ray and Marshall (2006) has shed light on some of the confusion about which endothelial ADO receptors activate receptor-specific K^+ channels for the production of NO. The authors measured the NO response to A_1 and A_{2A} receptor-specific antagonists alone and each in combination with large-conductance K_{Ca}, small-conductance K_{Ca} and K_{ATP} channel inhibitors. Briefly, this study showed that both large- and small-conductance K_{Ca} channels are crucial for A_{2A}-

mediated NO release and that A_1-mediated NO release is dependent on K_{ATP} channels (Ray & Marshall, 2006).

There is strong evidence that A_1 and A_{2A} ADO receptors are involved in ADO-mediated vasodilation and these receptors are dependent on K_{ATP} and K_{Ca} channels, respectively, for NO release. **Figure 1** displays the remaining intermediate steps in ADO-mediated NO production.

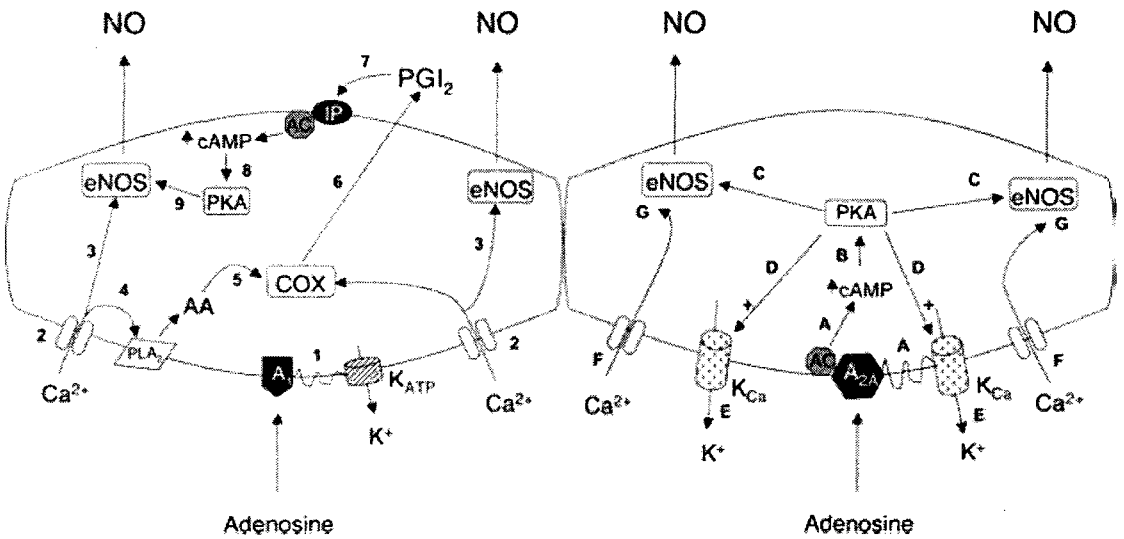

Figure 1. *Potential pathways for ADO-mediated NO production.* A1 stimulated NO production in left endothelial cell, and A2A stimulated NO production in right cell (from Ray and Marshall (2006)).

The figure above represents the pathways for A_1- and A_{2A}-mediated NO release as proposed by Ray and Marshall (2006). Based on their findings and reports by others it is suggested that the cascade begins with A_1 receptor stimulation initiating the opening of K_{ATP} channels resulting in K+ efflux, hyperpolarizing the membrane, which drives Ca^{2+} influx, hence increasing the intracellular concentration of Ca^{2+}. The rise in intracellular Ca^{2+} may directly influence NO production (Dora *et al.*, 1997) via the Ca^{2+}-calmodulin pathway

(Ray & Marshall, 2006). However, the Ca^{2+}-calmodulin pathway does not involve phospholipase A_2 (PLA_2), the activity of which has been shown to increase with stimulation of A_1 receptors (Dickenson & Hill, 1997). PLA_2 cleaves cell membrane phospholipids, yielding arachidonic acid (AA) which stimulates cyclo-oxygenase (COX) to form PGI_2. PGI_2 functions in an autocrine manner acting on endothelial IP receptors coupled to adenylate cyclase (AC) which increases intracellular cAMP, stimulating PKA, which in turn causes phosphorylation and subsequent activation of eNOS.

The proposed pathway for A_{2A}-mediated NO production is somewhat less complicated. Ray and Marshall (2006) explain that previous studies have shown that A_{2A} receptors are positively coupled to AC, and A_{2A}-stimulated release of NO is dependent on intracellular increases in cAMP, which could potentially stimulate PKA and directly phosphorylate eNOS. PKA stimulation would simultaneously activate K_{Ca} channels, and similar to A1-mediated NO release, K+ efflux, membrane hyperpolarization and Ca^{2+} influx would follow. The increased intracellular Ca^{2+} would subsequently activate eNOS and ultimately form and liberate NO.

To our knowledge, there is only one study in humans that has evaluated the effects of endothelial released NO on ADO-induced vasodilation. Smits *et al.* (1995) used venous occlusion plethysmography to measure resting forearm blood flow response to graded ADO infusion both before and during L-NMMA

infusion. Before L-NMMA was infused, ADO produced a dose-dependent increase in forearm blood flow. ADO and L-NMMA together still increased forearm blood flow, however baseline blood flow was significantly decreased compared to measurements without L-NMMA. Additionally, the authors found that during infusion of either K_{ATP} or K_{Ca} channel blockers there was no attenuation of blood flow (Smits *et al.*, 1995). This study, however, does not address how the pathway may be influenced during reactive or exercise hyperaemia, immediately post-exercise and during recovery. More work in humans needs to be conducted before judging whether or not this pathway is that by which ADO exerts its vasodilatory effects.

Methodological Considerations

As convincing as some of the evidence presented has been, there are important considerations to be taken into account before drawing any conclusions on the relative importance of specific autocoids for the control of muscle blood flow. Firstly, many studies, and others not included in this review, have been conducted using animal models. There has always been debate surrounding the use of animals and the generalizability of the results to humans. Due to the nature of this fundamental research, it is important to form a strong foundation of knowledge before attempting to measure cardiovascular parameters invasively in humans. In this regard, it is clear that elucidation of the regulatory factors in biochemical control of the human

circulation necessitates invasive procedures. It is important to acknowledge that results from isolated animal tissue or in-vivo animal models may not entirely reflect the processes occurring in the human organism due to differences between species and thus generalizability of regulatory mechanisms in healthy and various physiological states must be taken with caution. It is necessary for prevailing concepts generally accepted as dogma from studies in the animal model be experimentally borne out in human models.

It is also important to consider different blood flow and vasodilator methodologies both in terms of sampling location and technique. Some studies measure concentrations of NO or ADO, for instance, in the blood (Ballard *et al.*, 1987) or interstitial fluid (Hellsten *et al.*, 1998). It is crucial to understand that these measurements are not the same, and therefore can not be compared. Furthermore, blood flow measurements can be taken at different levels of the vascular tree; for example large arteries such as the aorta, brachial and femoral arteries versus microvascular flow in skeletal muscle tissue and other vascular beds. Additionally, different studies will report values using different measurement techniques; ultrasound Doppler, dye-dilution and plethysmography are just a few to mention. Under pharmacological manipulation of the circulatory response, researchers have often used different drug doses and administered them in different manners; bolus injection versus constant infusion. The extent to which these studies

represent physiological conditions is a necessary interpretive consideration for drawing conclusions. These design-related differences between studies make it somewhat difficult to compare results.

It has also been suggested that different types of exercise may produce a different pattern of flow within the vessel lumen and therefore would stimulate the endothelium differently (Green *et al.*, 2005). This may also warrant careful consideration when comparing and drawing conclusions from previous studies, and would also be worth investigating in the future.

Based on the evidence of redundancy that has been shown (Boushel *et al.*, 2002; Boushel & Kjaer, 2004), it would be worthwhile to continue this experimental approach and further clarify how vasodilator substances interact under different physiological conditions. Although this review concentrates principally on the interaction of ADO and NO, the recommendations for future research apply to investigations of all vasodilatory agents. Interventions should be planned such that different design components, including metabolic state, vasodilatory substance availability, exercise intensity and timing, among other parameters, are manipulated and studied simultaneously. While much evidence points to a communicative process between the muscle and the vasculature for matching blood flow to muscle oxidative metabolic demand, how this is regulated remains elusive. Future studies in this area are of high priority.

Rationale

Despite the wealth of knowledge that has been acquired over the last decades, there are still many unknowns concerning the regulation of blood flow during both resting and exercising states. We are now aware that there are many different substances which contribute to this complex phenomenon, for example ADO (Radegran & Calbet, 2001), NO (Joyner & Dietz, 1997), ATP (Ralevic & Burnstock, 1998), PGI_2 (Duffy *et al.*, 1999), and EETs (Oltman *et al.*, 1998), among others. Boushel *et al.* (2002) have also shown that there is an element of synergy and redundancy between vasodilators. The details of how this redundant system operates have yet to be elucidated, especially in humans. In fact, the specifics of the individual vasodilator substances' signalling pathways are not yet clear.

As previously mentioned, researchers have proposed that a few of the substances known to induce vasodilation are, in reality, acting as second messengers or mediating the action of another vasodilator. It has been shown in rat aortic endothelium that ADO initiates a cascade of signals which ultimately leads to the production of NO (Ray & Marshall, 2006). This pathway has also been proposed to be that by which ADO exerts its vasodilatory effects. To our knowledge, this theory has only been tested once before in humans; this trial measured forearm blood flow only under resting conditions (Smits *et al.*, 1995).

In addition, which of the local substances produced preserves a large portion of the blood flow response has not been clearly established in the literature. Furthermore, a great deal of work is still necessary in order to clarify any dependencies or interactions that many exist amongst vasodilator agents carried in blood, produced by vascular endothelium and smooth muscle as well as those potentially produced and released by adjacent skeletal muscle.

This particular study was designed to investigate the role of ADO, NO and potential interactions between the two endogenously produced vasodilator substances in regulating skeletal muscle blood flow at rest and during exercise in humans.

Specific Objectives & Hypotheses

The first objective was to determine the blood flow response to combined ADO and L-NMMA infusion. We hypothesized that muscle blood flow would be lower in ADO+L-NMMA infusion when compared to ADO infusion alone supporting the concept that adenosine-induced vasodilation is partially NO mediated. Secondly, we sought to determine the magnitude of muscle blood flow responses to ADO infusion during exercise compared to the response to control exercise and exercise + ADO infusion. We hypothesized that the magnitude of blood flow during exercise + ADO would be similar to that of ADO infusion alone at rest, reflecting local autoregulation of blood flow during

exercise to match blood flow to metabolic demand. Thirdly, as has been previously shown in one study in human muscle, adenosine may play an important role in regulating blood flow distribution and dispersion linking blood flow to discrete motor unit activation. Therefore the third purpose of this study was to determine the role of ADO for regulating muscle blood flow heterogeneity. It was hypothesized that ADO infusion would result in increased blood flow heterogeneity in muscle reflecting local differences in ADO receptor sensitivity and/or density. Lastly, we collected muscle interstitial fluid by microdialysis in order to evaluate the concentration of the NO metabolite, nitrite, the concentration of ADO and any additional compensatory release of other vasodilator substances. It was hypothesized that muscle interstitial nitrite and ADO concentrations would increase during ADO infusion when compared to control conditions, and that nitrite would be reduced, but not ADO, during L-NMMA infusion.

METHODS

Participants

Fourteen healthy subjects (13 men, 1 woman) participated in this study. Written informed consent was obtained from the participants after having been explained the purpose, nature and potential risks of the study. The project protocol was reviewed and approved by the Ethical Committee of

Copenhagen (H-KF-01-211/95) and the study was performed in accordance with the Declaration of Helsinki. Subjects were asked not to exercise, smoke or consume alcohol or caffeine within 24 hrs prior to testing.

Exercise Protocol

The study protocol is shown in **Figure 2**. Measurements were all performed on the same day for each individual participant. The participants were tested at rest and during exercise, each under 3 different conditions; control, ADO infusion (150 mg • kg body mass $^{-1}$ •min^{-1}) and combined L-NMMA (1 mg • kg body mass $^{-1}$ •min^{-1}) and ADO infusion. Based on experience, the dosage of ADO infused was selected to elicit a blood flow response similar to that expected during the exercise phase alone (exercise conditions described below). Each condition was 30 minutes in duration. The control, ADO infusion and combined infusion phases were divided into a 15 minute resting measurement period and 15 minutes of 1-leg knee extension exercise at 25 Watts. During these collection periods, cardiac output and muscle blood flow measurements were taken at 5 and 10 minute time points. Interstitial fluid was collected for the entire duration of each phase. Prior to administering the combined infusion of ADO+L-NMMA, an L-NMMA primer of the same concentration was infused for 10 minutes. The total testing time was approximately 5 hours.

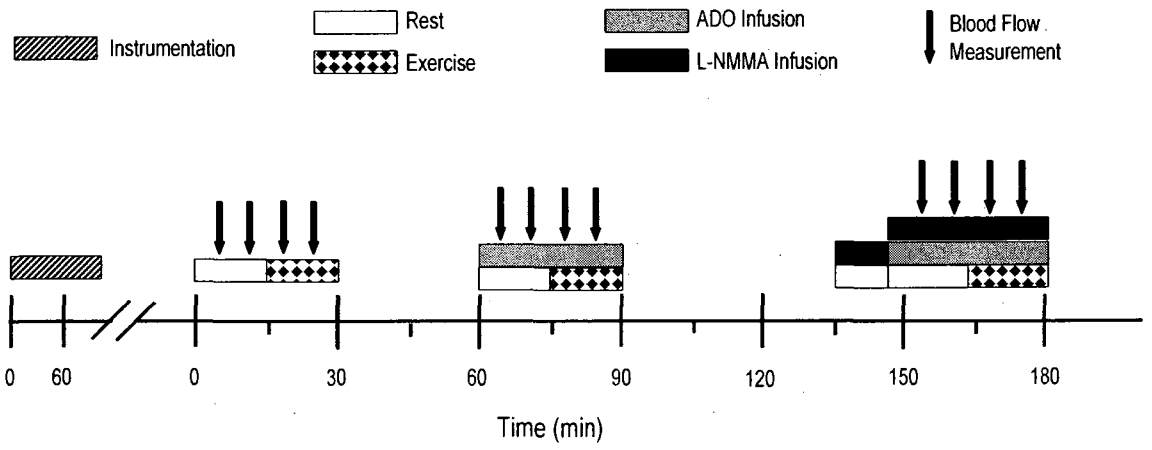

Figure 2. *Study Protocol.*

Arterial blood pressure was constantly monitored using a digital hospital monitoring system which recorded pressure from a transducer placed in the radial artery (Baxter Inc, USA) interfaced with a chart recording device (PowerLab/16SP model ML 795, ADInstruments, Colorado Springs, CO). Heart rate (HR) was determined from the on-line pressure wave recording.

Microdialysis

Prior to beginning instrumentation, thigh length, circumference and skinfolds were measured and used to estimate thigh volume and thigh muscle mass (Andersen & Saltin, 1985). The participants' skin, subcutaneous tissue and underlying muscle fascia was anaesthetised with 1 ml - 1.5 ml of 1% lidocaine, followed by deep tissue perforation with an 18 gauge needle used to guide the insertion of the homemade (described below) or manufactured (CMA 63, CMA/Microdialysis AB, Solna, Sweden) microdialysis probes into the vastus lateralis (VL) muscle of the quadriceps group. Homemade

microdialysis probes were comprised of a 4-cm semipermeable membrane obtained from an artificial kidney dialysis machine (GFS 16-GFE 18; Gambro, Lund, Sweden) with a 20kDa cut-off, connected at either end to hollow nylon tubes. The VL muscles of both the exercising (right leg; 3 homemade and 2 CMA probes) and control (left leg; 1 homemade and 1 CMA probe) legs were instrumented with microdialysis catheters. The homemade probes were inserted into the muscle at a depth of approximately 1 cm and the commercially-made CMA probes were inserted obliquely into the quadriceps muscle at a depth of 4 cm. All microdialysis probes were perfused at a rate of 5 $\mu l \cdot min^{-1}$ with Ringer acetate solution (Pharmacia; Stockholm, Sweden) combined with micro-amounts of radioactive [^{14}C] ethanol (Sigma, Missouri, USA) and [2-^{3}H] Adenosine (GE Healthcare, Amersham, UK). The muscle was perfused at a constant rate throughout the entire duration of the testing day, including recovery periods. Dialysate was collected in capped microvials (CMA/Microdialysis AB, Solna, Sweden) and immediately frozen at -80°C. The dialysate samples will remain frozen until the time of analysis.

Following insertion of the microdialysis probes, the participant rested for 90 minutes, which has been shown to be sufficient to minimize any tissue response from the trauma resulting from the insertion of the microdialysis probe (Bangsbo, 1999; Langberg *et al.*, 2002). During this time, the participant was instrumented with the necessary equipment for cardiac output and blood flow measurements.

Catheterization

Under local anaesthesia (1% lidocaine), the participants were instrumented with 3 catheters using the Seldinger technique; 2 arterial (femoral and radial arteries) and 1 venous (median antebrachial vein). Insertion of the arterial and venous catheters was, on all occasions, performed by a medical doctor.

Cardiac Output Measurement

Cardiac output was measured using the dye-dilution technique. As described by Dow (1956), this method is based on two concepts. Firstly, if dye is added at a constant rate to flowing blood, the rate of blood flow can be determined by the concentration of the dye which is equilibrated in downstream vessels by first passage and full mixing in the central venous and pulmonary circulations. Alternatively, when a known volume of dye is rapidly injected into any vein, the arterial accumulation of the injected dye over time will result in the quantification of the blood flow which has diluted it (Dow, 1956). Flow is then calculated by the following formula (Hamilton *et al.*, 1948):

$$f = \frac{I}{ct} \qquad \text{Formula 1}$$

where f is the flow in litres·second^{-1}, I is the amount of dye injected into the vein (mg), c is the average concentration of the dye for the duration of the curve (mg/L) and t is the duration of the curve in seconds. When using this technique in animal models, it is necessary to account for recirculation of the

dye (**Figure 3**). To do this, the down slope is extrapolated between points at approximately 75% to 50% of peak arterial dye concentration (Hamilton *et al.*, 1948; Boushel *et al.*, 2000). The resulting constructed curve eliminates recirculation and thus allows for calculation of blood flow based on first passage of the dye.

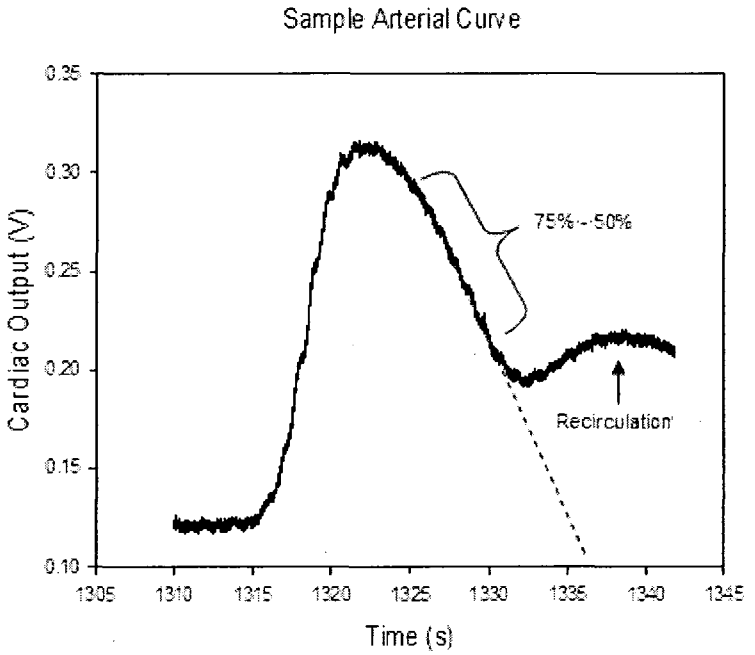

Figure 3. *Sample arterial curve*. Tailing off of the recording represents recirculation of the injected dye. Extrapolation of the downslope (dashed line) of the arterial curve between the points denoted, representing 75% to 50% of peak arterial dye concentration, reconstructs the curve as though recirculation had not occurred.

A bolus of indocyanine green (ICG) dye (ICG-Pulsion, Pulsion Medical Systems, Munich Germany) was quickly injected into the participants' antebrachial vein followed by a flush of saline (approximately 10 ml). Concomitantly, blood was drawn from the radial artery by an automated pump (Harvard Apparatus, Millis, MA, USA) at a rate of 20 ml/min through a linear photodensitometer (Waters CO-10. Instruments Inc., Rochester, MN, USA)

where a voltage signal, representing the light attenuation of the dye, was registered by a chart recorder (PowerLab/16SP model ML 795, ADInstruments, Colorado Springs, CO). The drawn blood was then re-infused into the median antebrachial vein in a close-looped aseptic system. At the end of each experiment, an ICG-voltage calibration curve was derived from 3 samples of the participant's blood spiked with known volumes of ICG. Cardiac output was then calculated using the Formula 1.

Microvascular Blood Flow Measurement

Local blood flow in the exercising VL was measured by near infared spectroscopy (NIRS) using a NIRO 300 spectrophotometer (Hammatsu Photonics, Herrsching, Germany) in combination with bolus injections of ICG dye. This technique has been described in detail by Boushel *et al.* (2000). Two sets of receiving and emitting optodes were placed parallel to fiber direction of the VL muscle at proximal and distal positions. The paired optodes were separated by a distance of 40 mm, which corresponds to a light penetration depth of ~25 mm. ICG dye was injected as previously mentioned for cardiac output measurements.

The ICG bolus enters the venous circulation, travels to the right atrium then ventricle, through the pulmonary circulation and back to the left atrium and ventricle. After having passed though the cardio-pulmonary circulation, the

ICG is sufficiently mixed with the blood, and exits the heart into the arterial circulation, where the ICG dye in the muscle's microcirculatory beds (vessels <200um diameter) will eventually be detected by the NIRS optodes. The peak optimal absorption wavelength of indocyanine green dye (ICG) is 800nm. The light absorption of ICG was thus measured as molar value of light attenuation at the specific wavelengths; 775, 813, 850 and 913 nm using the Modified Beer-Lambert Law incorporating the molecular weight of ICG using a matrix operation programmed in Matlab. Muscle blood flow was then calculated as the ratio of ICG accumulation in the tissue to the average ICG concentration in the arterial blood over the same time frame (Boushel *et al.*, 2000). The Sapirstein principle states that after bolus administration of a tracer, in our case ICG dye, for a certain period of time before reaching peak dye accumulation, the fraction of the tracer distributed to the tissue matches the fraction of cardiac output (otherwise considered the arterial input function in Formula 2) being delivered to the tissue (Sapirstein, 1956). Therefore, blood flow (ml blood · 100 ml tissue^{-1}· min^{-1}) can be calculated by the following formula:

$$\text{Blood Flow} = \frac{\kappa\,[ICG]_m\,t}{{}_0\int^t [ICG]_a\,dt} \qquad \text{Formula 2}$$

where κ is a constant for the conversion of ICG from units of moles to grams·L^{-1}, $[ICG]_m$ is the accumulation of ICG in the muscle tissue (μmoles) over time t and ${}_0\int^t [ICG]_a\,dt$ is the time integral of the arterial concentration of ICG (mg·L^{-1}) (Boushel *et al.*, 2000).

Because the blood was injected into the venous system, we assume it was adequately mixed by the time it had passed though the pulmonary circulation and over to the left side of the heart before entering the systemic circulation. Thus ensuring the accumulated dye concentration and blood flow calculation should be indeed reflective of muscle blood flow. Also, because blood flow was calculated over a time interval less than that to reach peak ICG accumulation, the potential effects of dye recirculation in the muscle are negated.

Statistical Analysis

Power calculations were performed to determine sample size. Based on the literature in the field, a difference of 15% in blood flow was anticipated in order to establish significance. This resulted in a minimum of 10 subjects, whereas we initially recruited 14 in order to account for subject withdrawal. Due to incomplete data sets only 12 of the 14 initially recruited were used in the statistical analysis of the data. Values are presented as mean±SEM for all experiments and measurements. For all statistical evaluations, a P value of < 0.05 was considered significant. Statistical analysis of differences in HR, MAP, SV and CO comparing between rest vs. exercise and conditions were carried out with a two-way repeated measures ANOVA. Because it has been well established that blood flow will significantly increase when shifting from a resting to exercising state, the data has been divided accordingly and the

blood flow analysis was simplified to a two-way repeated measures ANOVA treating condition and optode position as manipulated factors investigated. The oxygenation data was also analysed using a two-way repeated measures ANOVA in addition to regression analysis to determine a time constant for the attenuation of ADO dilation. In the case of a significant main effect and interaction between variables, the Holm-Sidak method was used for post-hoc analysis. SigmaPlot version 11.0 (Systat Software Inc., San Jose, CA, USA) was used for all computational analyses.

RESULTS

Subjects

Table 1 below displays the participant characteristics for the 12 subjects who were included in the analysis.

Characteristic	Mean	±	SE
Age (yr)	24.1	±	1.2
Weight (kg)	78.3	±	3.9
Height (cm)	182.3	±	2.0
BMI (kg/m^2)	23.5	±	1.0
Thigh Volume (L)	9.25	±	0.6
Muscle Mass (kg)	8.81	±	0.6

Table 1. *Subject Characteristics.* Values are Mean ± SE (N=12); BMI=Body Mass Index

Systemic Parameters

Rest

Heart rate (HR) significantly increased between rest and exercise states under control (65.6±2.2 – 84.9±3.5 bpm, $P<0.001$), ADO infusion (79.5±2.7 – 95.2±3.7 bpm, $P<0.001$), and ADO+L-NMMA infusion (74.5±3.4 – 86.8±5.3 bpm, $P<0.01$). See **Figure 4.A**. Moreover, at rest HR was significantly higher ($P\leq0.001$) during ADO and ADO+LNMMA infusions (79.5±2.7 and 74.5±3.4 bpm, respectively) when compared to control (65.6±2.2 bpm). At rest, mean arterial pressure (MAP) was only significantly increased during ADO+L-NMMA infusion when compared to control (104.9±1.8 vs. 94.1±2.6 mmHg, $P<0.01$). No other differences in MAP were observed between conditions at rest (**Figure 4.B**). Stroke volume (SV) remained unchanged between conditions at rest (**Figure 4.C**). CO during ADO infusion was significantly increased when compared to control at rest (9.1±1.1 vs. 6.8±0.8 L • min^{-1}, $P<0.01$) (**Figure 4.D**). There was no significant difference between other conditions.

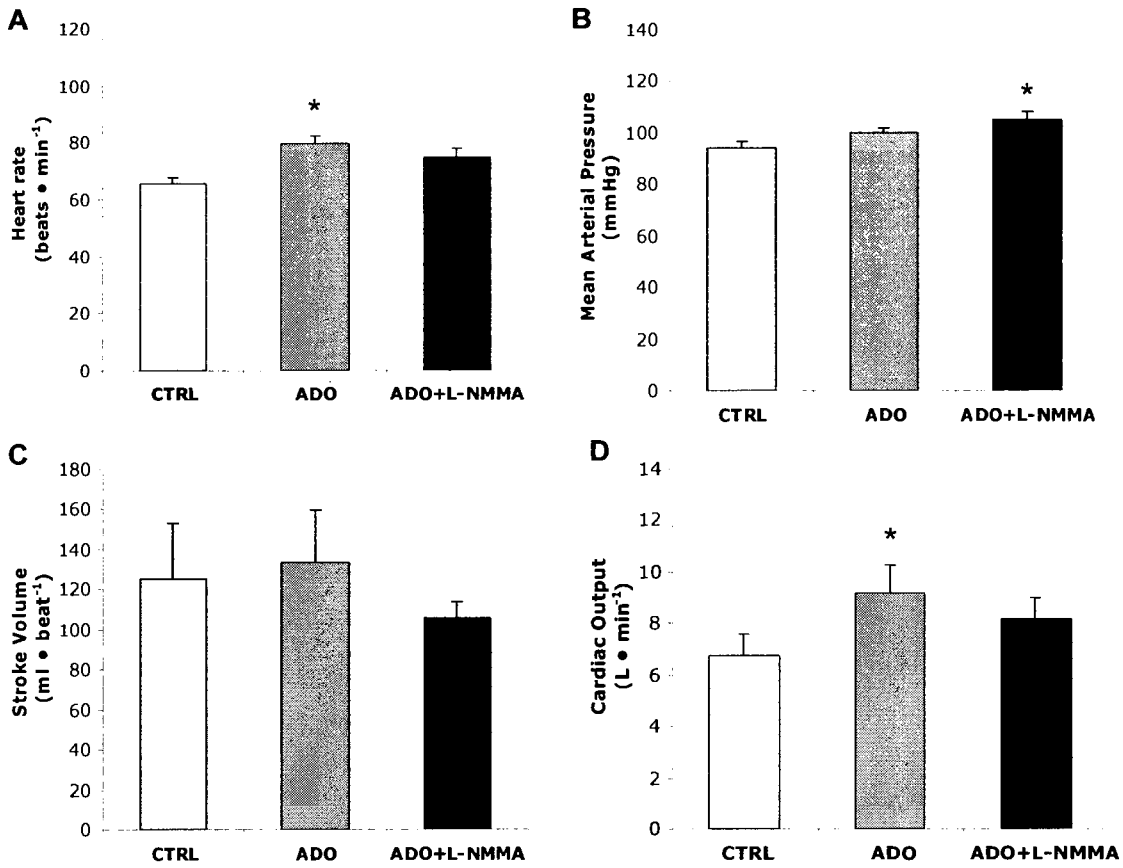

Figure 4. *Systemic parameters at rest.* (A) Heart rate (B) Mean arterial pressure (C) Stoke volume and (D) Cardiac output measurements at rest during; control (CTRL), ADO infusion (ADO) and ADO+L-NMMA infusion (ADO+L-NMMA). *Value different from CTRL, $P< 0.05$; **Value different from ADO, $P< 0.05$.

Exercise

During exercise, HR was greater in ADO compared to control (95.2±3.7 vs. 84.9±3.5 bpm, $P<0.001$) and compared to ADO+L-NMMA infusion (86.8±5.3 bpm, $P<0.01$); however no differences were seen between control and ADO+L-NMMA infusion during exercise (**Figure 5.A**). MAP did not differ between conditions during exercise; however exercising MAP during control and ADO infusion were found to be higher compared to these same conditions at rest (see **Figure 5.B**). SV remained unchanged. Comparison

between SV during rest and exercise for each condition showed no differences (**Figure 5.C**). CO was higher during exercise for each of condition when compared to the same condition at rest. CO during ADO infusion was significantly increased when compared to control during exercise (10.8±0.8 vs. 9.1±1.1 L • min^{-1}, P<0.05) (see **Figure 5.D**).

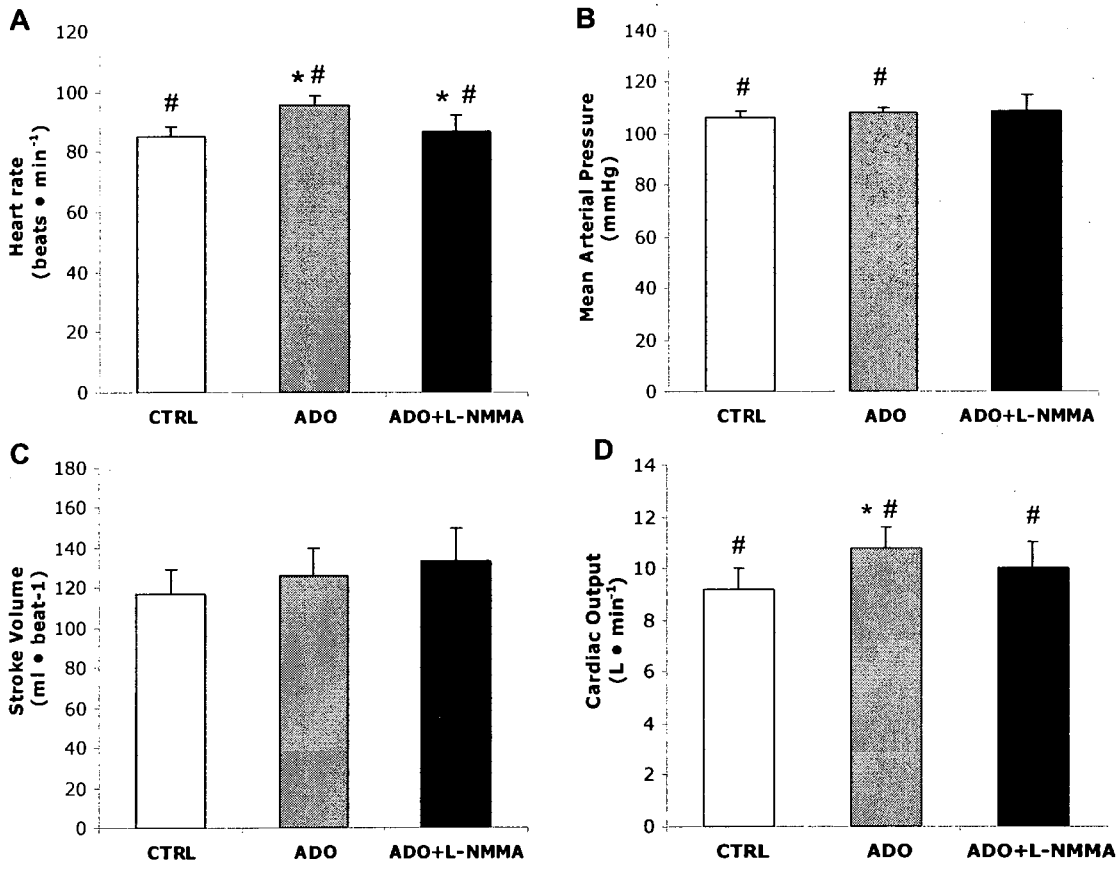

Figure 5. *Systemic parameters during exercise.* (A) Heart rate (B) Mean arterial pressure (C) Stoke volume and (D) Cardiac output measurements at rest during; control (CTRL), ADO infusion (ADO) and ADO+L-NMMA infusion (ADO+L-NMMA). * Value different from CTRL, P< 0.05; ** Value different from ADO, P< 0.05; # Value different from rest, P< 0..05.

Blood Flow

There was no statistical difference between blood flow values calculated for the first (5 minute) and second (10 minute) measurements; therefore the time-dependent data was averaged resulting in a mean blood flow value over the testing periods. It has been well established that blood flow will significantly increase when shifting from a resting to exercising state, and so the data has been divided accordingly as presented here below (**Figure 6**).

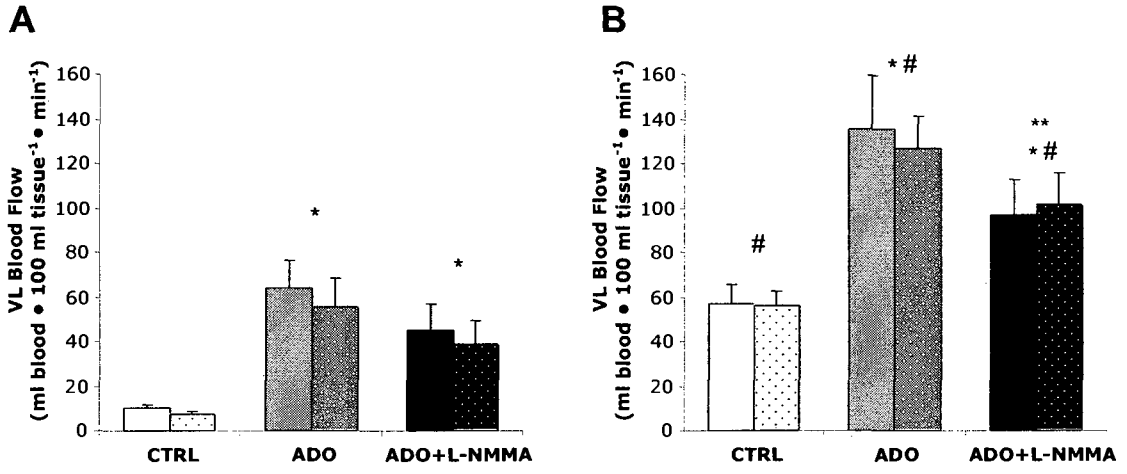

Figure 6. *Proximal vs. distal blood flow.* (A) VL blood flow measured at rest for both proximal (solid bars) and distal (dotted bars) measurement sites. (B) Blood flow measured in VL during 1-leg knee extension exercise; proximal (solid bars) and distal (dotted bars). * Value different from CTRL, $P< 0.05$; ** Value different from ADO, $P< 0.05$; # Value different from rest, $P< 0.05$.

There were no differences in blood flow found between optode locations (i.e. no difference between proximal vs. distal measurements) either at rest or exercise for any of the prescribed conditions (control, ADO, ADO+L-NMMA). Blood flow was significantly increased with ADO infusion when compared to control at rest (proximal: 63.7±12.4 – 9.9±1.7 ml•100ml tissue^{-1}•min^{-1}, $p<0.001$; distal: 55.6±13.1 – 7.0±1.7 ml•100ml tissue^{-1}•min^{-1}, $p<0.001$) and

during exercise (proximal: 135.6±23.5 – 56.7±9.0 ml•100ml tissue^{-1}•min^{-1}, p<0.001; distal: 126.7±14.6 – 55.8±7.0 ml•100ml tissue^{-1}•min^{-1}, p<0.001). The same pattern of significance was observed between ADO+L-NMMA infusion and control for both proximal and distal VL at rest and exercise (see **Figure 6**). Interestingly, there was a significant decrease in blood flow during exercise, for both the proximal and distal VL, when L-NMMA infusion is superimposed on ADO infusion (p<0.01). Additionally, by design, blood flow values during ADO infusion at rest and exercise alone are quite similar in magnitude (see 'Experimental Protocol' in Methods section).

To further investigate the effects of the prescribed conditions, the blood flow data for the 2 measurement sites was pooled, generating a mean blood flow value over time for the VL muscle during testing under each prescribed condition. What we see here is that there is a significant difference of mean VL blood flow between all conditions at rest and during exercise (**Figure 7**).

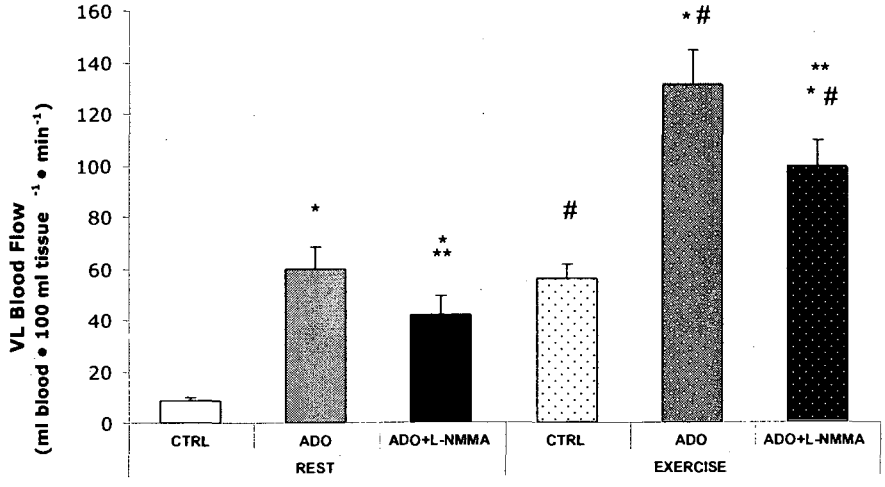

Figure 7. *Mean VL blood flow at rest and during exercise.* Pooled VL blood flow measured at rest (solid bars) and during 1-leg knee extension exercise (dotted bars). * Value different from CTRL, *P*< 0.05; ** Value different from ADO, *P*< 0.05; # Value different from rest, *P*< 0.05.

Vascular Conductance

Vascular conductance (VC; calculated as: blood flow/MAP) increased between control and ADO infusion ($P<0.05$) and between control and ADO+L-NMMA infusion ($P<0.05$). The same observations were made during exercise. Moreover, VC is significantly increased when comparing between rest and exercise for each of the conditions, respectively (**Figure 8**).

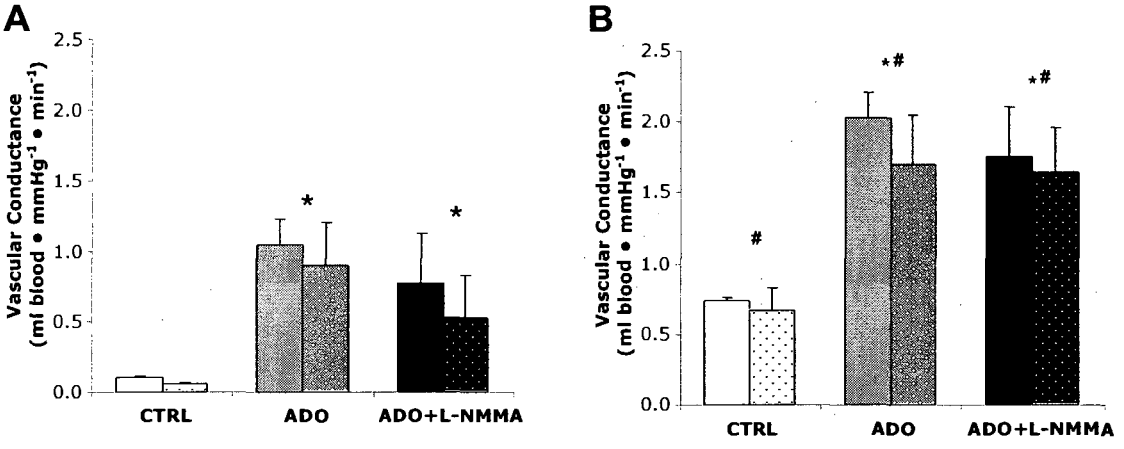

Figure 8. *Proximal vs. distal vascular conductance at rest and during exercise.* (A) Vascular conductance calculated at rest for both proximal (solid bars) and distal (dotted bars) measurement sites. (B) Vascular conductance during 1-leg knee extension exercise for proximal (solid bars) and distal (dotted bars). * Value different from CTRL, $P< 0.05$; ** Value different from ADO, $P< 0.05$; # Value different from rest, $P< 0.05$.

Tissue Oxygenation

The oxygenation data are expressed as the absolute change in oxyhemoglobin concentration ($[O_2Hb]$) with all measurements normalized to baseline concentration. When comparing $[O_2Hb]$ between proximal and distal measurement sites, significant differences were noted at peak concentration ($p<0.05$) and at 2 minutes post-peak ($p<0.05$). Additionally, a clear time

attenuation effect of adenosine was seen from the peak response over the subsequent 5 min (**Figure 9**).

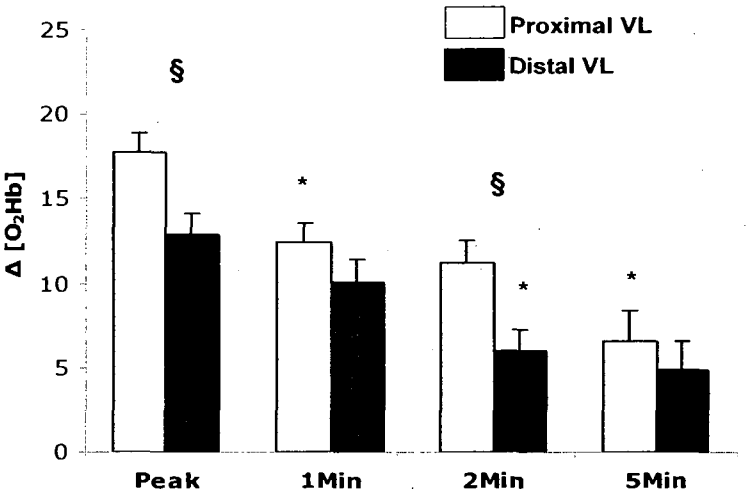

Figure 9. *Δ [O_2Hb] with ADO infusion at rest.* * Value different from measurement at previous time point, $P < 0.05$; § Values significantly different between proximal and distal measurements, $P < 0.05$.

DISCUSSION

The key findings in this study were that 1) ADO-mediated muscle vasodilation is mediated in part by NO in humans, 2) ADO infusion does not generate muscle blood flow heterogeneity in muscle at rest or during exercise, 3) the time course of ADO-induced vasodilation reaches an immediate peak response followed by a gradual attenuation over the course of the next 5 minutes and 4) ADO infusion overrides local autoregulatory blood flow control mechanisms during exercise resulting in an exaggerated blood flow response to muscle contraction and an overperfusion of muscle relative to metabolic demand.

Role of NO in ADO-induced Vasodilation

In this study, we found that there is a significant increase in blood flow at both rest and exercise when ADO is infused, compared to control conditions. Additionally, the results show that the infusion of ADO combined with L-NMMA at rest and during exercise elicits blood flow values lower than those observed with ADO infusion alone, however blood flow is still significantly higher than the control condition. Essentially, when taken together, these findings demonstrate that there is some involvement of NO in the pathway whereby ADO regulates vascular tone. These findings support our main hypothesis and corroborate the results of work done in animals as discussed previously in the literature review (Ray & Marshall, 2006).

Based on previous studies using similar doses of NO blockade as that administered in this study, it can be deduced that approximately 60-70% of NOS enzyme activity was collectively inhibited. Frandsen et al. (2000) measured skeletal muscle NOS activity ex-vivo under systemic pharmacological blockade of NOS with L-NAME and found a 60-70% reduction in NOS activity. Lattereur et al. (In preparation) found a similar 60% lower interstitial concentration of nitrite during blockade of NO with L-NAME administered directly into contracting muscle through a microdialysis probe via infusate solution. In the present study, we found decreases in blood flow between ADO and ADO+LNMMA infusion across the muscle ranging between 35-47% at rest, and 23-30% decrease during exercise. Therefore, while a significant measurable portion of ADO-mediated

vasodilation is clearly triggered by secondary pathways involving NO formation, this value may be an underestimation of the full contribution of NO to this pathway. The precise magnitude of the NO component of vasodilation imparted by endogenous adenosine release under control exercise cannot be estimated from the current data set. However it is with certainty that these vasodilators function in a synergistic manner to regulate blood flow in humans in-vivo.

It is important to acknowledge endothelial-dependent NO production as a contributor to muscle vasodilation, which ultimately influences blood flow. Because ADO is known to be a powerful vasodilator, eliciting blood flow values seen at peak exercise with infusion under resting conditions (Radegran & Hellsten, 2000), sudden and significant increases in blood flow, such as that brought upon with ADO infusion, likely causes an increase in the level of shear stress imposed on the inner luminal wall of any conduit vessel. Furthermore, the resulting increased shear-stress on the endothelial lining is known to initiate an intracellular cascade resulting in augmented production of endothelial-dependent NO. In several studies solely investigating shear stress and its influence on blood flow it has been shown that the endothelial-dependent NO component of shear stress lasts for the first 5 minutes of stimulus (using a cuff-occlusion model) (Pyke & Tschakovsky, 2005). In the context of the present study, if shear stress influences endothelial-dependent NO production then consequential vasodilation would be negligible after 5 minutes of stimulus (i.e. pharmacological agent infusion), then all blood flow measurements recorded following the initial 5

minutes of infusion (as with our study; 5 and 10 minutes) will reflect only the influence of the prescribed conditions (i.e. CTRL, ADO infusion, ADO+L-NMMA infusion). We can be certain that the second blood flow measurement taken at 10 minutes was well beyond the time frame in which endothelial-derived NO exerts its influence on vascular tone via a shear stress effect. Furthermore, because there was no statistically significant difference between the first (5 minute) and second (10 minute) recordings then it can be inferrred that if in fact a shear-induced response was elicited, there was little or no residual influence at the time of the first blood flow measurement, which supports the '5 minute rule' as suggested by Pyke and Tschakovsky (2005). Thus, all of the blood flow values reported do in fact solely reflect the impact of the pharmacological interventions on blood flow, entirely negating the influence of shear stress.

Most recently, Ray and Marshall (In press-b) investigated the reverse pathway; whether or not de novo synthesis of NO required for the release of adenosine. In that study in rats, a series of pharmacological interventions were applied to elucidate the role of adenosine in muscle blood flow regulation. During adenosine receptor blockade by either an A_1 or A_{2A} selective pharmacological agent, it was found that muscle blood flow during electrically evoked muscle contractions was significantly attenuated. The lower blood flow during contraction was observed primarily with ZMA225 blockade of the A_{2A} pathway. No further reduction in blood flow was seen with L-NMMA superimposed on the ZMA225 A_{2A} induced reduction in blood flow. To further confirm that NO was not necessary for the endogenous release and downstream vasodilating effect of adenosine, the

intracellular NO donor 8-bromo cGMP was also administered to increase NO levels, bypassing the NOS blockade effect of L-NMMA, and again no differences in blood flow were observed. It was clearly demonstrated that unlike the dependence of NO for adenosine mediated vasodilation as shown in reverse in the present study, the presence of NO is not necessary for the 'generation' of adenosine during muscle contraction. This is supported by previous work which showed that during NOS blockade with L-NAME (Frandsen *et al.*, 2000) and combined blockade of NOS and cyclooxygenase by L-NAME and indomethacin (Boushel et al., unpublished results), respectively, there is no compensatory increase in adenosine production measured in the interstitium of muscle tissue.

Based on the significant differences observed between muscle blood flow values with ADO infusion and ADO+L-NMMA infusion, the data support NO as a mediator within the ADO-initiated vasodilation cascade. The extent to which NO is involved in ADO signaling system remains to be elucidated.

Role of ADO in Muscle Blood Flow Heterogeneity

It is still not clear whether or not ADO does indeed play a role in heterogeneous distribution of blood flow across a vascular bed feeding skeletal muscle. There is a lack of consistency among the results from studies looking specifically at this question which further complicates the interpretation of the outcomes of the current study. If we consider the 2 different regions of the muscle from which we

recorded (proximal vs. distal) at any of the levels of condition (i.e. CTRL, ADO, ADO+LNMMA.) there is no evidence to support a role for ADO in regulating blood flow heterogeneity.

As previously mentioned, ADO receptors, namely A_1, A_{2A} and A_{2B}, have been localized to skeletal muscle vasculature (Lynge & Hellsten, 2000) and ADO is locally produced in close proximity to the extracellular space adjacent to endothelial cells (Clifford & Hellsten, 2004). These 2 factors have strongly contributed to the appreciation of ADO as a vasodilator. However, to be able to consider ADO as a factor involved in regulating the distribution of blood flow (whether it be heterogeneous or not), it is important to take into account the pattern of ADO receptor density across a vascular bed. Knowing whether or not one ADO receptor subtype is more dominant in terms of density and affinity, and whether the dispersion of these receptors is uniform within the muscle vasculature will contribute to understanding the capacity of ADO to control blood flow distribution. To date, there is no single study to our knowledge that has directly investigated this question of receptor density specifically in human skeletal muscle and its vasculature. However, one recent study evaluated ADO receptor density in the myocardial microvasculature (Heinonen *et al.*, 2008). A_{2A} receptor volume was found to be much higher in the myocardium of trained athletes compared to untrained controls. When expressed per unit of myocardial mass there was no difference between trained and untrained. Importantly, it was concluded that as the blood flow demands of a trained heart capable of a 70%

larger cardiac output, a relatively higher cardiac work capacity and increased oxygen demand, adenosine receptors known to vasodilate the coronary vasculature were proportionately increased in their expression. A significant secondary conclusion was that the trained heart had a luxury (excess) blood flow per unit heart work which raises the question of vasodilator receptor sensitivity and kinetics. This area of research merits further investigation.

Recent reports evaluating the contribution of ADO to blood flow heterogeneity from a physiological perspective have yielded conflicting results. Heinonen *et al.* (2007) measured blood flow in the 4 compartments (vastus lateralis, medialis, intermedius and rectus femoris) of the quadriceps femoris muscle group during rest and 3 levels of incremental exercise (1-leg knee extension) without and then with non-selective ADO receptor blocking agent, theophylline. Interestingly, the authors observed a decrease in blood flow heterogeneity corresponding to increasing exercise workload both with and without theophylline; however overall and individual muscle blood flow levels increased. Ultimately, the authors concluded that ADO may have a role in contributing to blood flow heterogeneity at low to moderate levels of exercise.

On the basis of the above mentioned and other recent studies employing positron emission tomography using radioactive isotope tracers and magnetic resonance imaging, there is evidence that within a contracting limb, the magnitude of blood flow to discrete regions of muscle is not homogenous

(Mizuno *et al.*, 2003; Heinonen *et al.*, 2007). Extending upon earlier studies which have demonstrated a close matching between muscle metabolic demand and oxygen delivery to limbs engaged in contraction (Andersen & Saltin, 1985), a current avenue in research is to identify how this close matching is regulated.

In the current study, several factors may contribute to the less than robust differences in blood flow heterogeneity observed. Firstly, with prolonged exercise, even at low intensities additional muscle fibers will be recruited in order to continue the exercise task (Ray & Dudley, 1998). These secondarily recruited fibers require blood flow supply to be increased so as to support their elevated metabolic state (Ray & Dudley, 1998). Hence, it could very well be that the time points at which blood flow measurements were recorded in our study were beyond this point of secondary muscle fiber recruitment and blood flow had already leveled out across the muscle.

Furthermore, with the ADO infusion superimposed on exercise, it is possible to have saturated the ADO receptors with the dose given, at which point the receptor activity would be the limiting factor in further dilating the blood vessels. Along these same lines, it is possible that there is a threshold ADO concentration, at which this autocoid would no longer act to extend vasodilation. Further investigation is warranted.

At low exercise intensities such as that prescribed in our protocol, it is possible to meet the metabolic demands of the tissue by means of increasing oxygen extraction and not necessarily increasing the amount of blood delivered to the tissue (Boushel *et al.*, 2002; Mortensen *et al.*, 2007). This may be the case in the present study. Differences observed between proximal and distal HbO_2 reflect differences in metabolic demand between the two sites. However, the data did not reflect any differences when comparing blood flow measured at the two sites. Therefore, if blood flow was not enhanced, then there must exist another system which acts to supply the active tissue with adequate quantities of O_2 to be able to sustain exercise. Thus, even though we observed a metabolic heterogeneity, there was no blood flow heterogeneity because potentially another mechanism, which we suspect is an increase O_2 extraction, was able to maintain a sufficient level of O_2 availability for the active tissue.

It has been suggested that with L-NMMA blockade of NOS, changes in blood flow seen during exercise may be the result of decreased basal flow and not necessarily due to an interaction between NO and exercise (Endo *et al.*, 1994). Prior to beginning the combined infusion of ADO+L-NMMA, we infused a 10 minute primer of L-NMMA so as to ensure NOS was indeed blocked. However, in accordance with the findings of Endo et al. (1994), we likely decreased baseline blood flow, and may have therefore confounded the findings. Throughout the time elapsed during the L-NMMA primer infusion, it is possible that the lack of NO being produced as a result of the NOS blockade was 'sensed' in some manner by the redundant/synergistic vasodilators/systems and thus, additional

vasodilating agents were recruited in a compensatory fashion so as to counteract the lack of vasodilation usually maintained by available NO.

Time Course of ADO-mediated Muscle Vasodilation

Immediately following the initiation of ADO infusion into the femoral artery during resting conditions, the muscle tissue [O_2Hb] significantly increased and reached a peak concentration within 1.5-2 minutes. Subsequently, a gradual decline was observed over the 5 minutes following the point of peak concentration, after which the [O_2Hb] was stable; however still elevated above baseline values. To our knowledge, this is the first time this phenomenon has been reported with ADO infusion.

It is reasonable to speculate that during this initial phase of infusion part of the observed trend would result from the sudden surge of increased endothelial-dependent NO production resulting from the increase shear stress brought on by ADO infusion; however, it is not possible that it is exclusively a shear-induced reaction. Without doubt, there will be some contribution of ADO and the vaso-active substances, with which ADO interacts, in addition to non-endothelial-dependent NO formation.

There are numerous factors which may contribute to the [HbO_2] response observed. Of great interest and importance is the ensuing decline in the [HbO_2].

Why is it that with a constant rate of ADO infusion, and constant metabolic demand (rest), the [HbO_2] is not maintained at the peak (or close to peak) levels throughout the duration of infusion? Unfortunately, we are unable to answer this question based on the evidence collected during this study; however, we propose 3 explanations for this occurrence; 1) desensitization of the local ADO receptors; 2) redistribution of blood flow as a result of opening of new capillary beds; 3) attenuation of reflex neural signals. It is quite possible that the ADO receptors can become desensitized and thus less responsive to the available exogenous ADO when exposed to supranormal levels of exogenous ADO. Additionally, there could be secondary opening of new capillary beds as a consequence of the assault on the initially dilated regions with the overwhelming and immediate increase in flow. Because the metabolic demands of the tissue have remained unchanged (always at rest during this period), there was no need for the excess blood flow being delivered to the muscle regions. Local signals and adjacent networks (activated neurally or from spillover) may have acted to redirect the excess flow to new muscle regions and therefore decreasing [HbO_2] in the initially over-supplied regions. A reflex signal at the time of initial infusion with input to the ventrolateral medulla to evoke increases in blood flow (apart from any local effects) may also be a factor since subjects reported sensations that coincided with the observed rapid increase in HbO_2. Additional research is necessary to support these suggestions.

Influence of ADO on Muscle Autoregulation of Blood Flow

Skeletal muscle tissue and its vasculature have the ability to locally regulate blood flow to match metabolic demands (Andersen & Saltin, 1985). From the control data during exercise, we see that muscle tissue blood flow is increased to approximately 60 ml•100ml tissue^{-1}•min^{-1}. Interestingly, we see that with the same metabolic perturbation (i.e. 1 –leg knee extension exercise at 25W) blood flow by far exceeds the amount necessary to carryout the prescribed activity. There is a significant excess flow when ADO infusion is superimposed onto exercise clearly showing that the tissue has a massive stimulated blood flow reserve. When L-NMMA infusion is added, we still see an excess of flow to the microcirculation. What exactly might cause the ADO infusion to override local autoregulatory mechanisms is unknown.

Limitations

As with any scientific undertaking, our study design and measurement techniques do impose certain limitations on the interpretation of the results. Firstly, while blood flow differences between infusion conditions were found, we cannot confirm the extent to which NO is implicated in ADO-mediated vasodilation because 1) L-NMMA does not block all NOS activity and 2) a dose-dependent response needs to be shown in order to establish the strength of association between NO and ADO induced vasodilation. However, the use of a

repeated measures design, by nature, decreases the inter-participant biological variability and hence the final analyses are more robust. Also, when using the NIRS-ICG technique to quantify blood flow, it is necessary to acknowledge that within the recorded signal, there is some contribution of skin blood flow, although minimal. There is still some debate about what the optimal optode distance is so as to avoid recording signals from the skin and underlying adipose tissue (Quaresima & Ferrari, 2002; Ferrari *et al.*, 2006). Also, the NIRS-ICG technique in and of itself is quite invasive, costly and requires a great deal of experience in performing the technique. However, it is unique in that microvascular blood flow can be measured during exercise, where as other techniques do not have such high spatial and temporal resolution and only flow in major conduit arteries can be assessed. Additionally, imaging techniques cannot be used with an exercise model because it is important for the subject to remain still in order obtain the best quality image possible. Thus while the techniques employed in this study do have some limitations, there are indeed many advantages to using them.

Future Directions

Vascular research has come a long way in the last decades. However, a great deal of information has yet to be uncovered. Perhaps the most significant development in recent times is the concept and acknowledgement of redundancy/synergy between systems and locally produced chemicals regulating vasodilation. The challenge for future studies investigating the control of vascular

tone, especially in exercise hyperemia, is to continue to be innovative in the approach with which hypotheses are tested. Development of new vasodilator blocking or donating agents will be an important advancement in the field. Varying study designs by using different combinations of blockers and donors and varying the order in which they are administered is an example of how to manipulate the experimental setup to approach the same question from a slightly different angle.

When considering the future of the ADO-mediated vasodilation pathway specifically, it will be necessary to learn more about the different receptors, their distribution and sensitivity within skeletal muscle tissue and vasculature from a reductionist perspective. It will also be important to demonstrate a dose-dependent effect between ADO and NO in order to confirm with certainty, and measure to what extent the ADO-initiated vasoactive pathway is mediated by NO.

Conclusions

In summary, the major findings of this study were that 1) ADO-mediated muscle vasodilation is mediated in part by NO in humans, 2) ADO infusion does not generate blood flow heterogeneity in muscle at rest or during exercise, 3) the time course of ADO-induced vasodilation reaches an immediate peak response followed by a gradual attenuation over the course of the next 5 minutes and 4)

ADO infusion overrides local autoregulatory blood flow control mechanisms during exercise, resulting in an exaggerated blood flow response to muscle contraction and an overperfusion of muscle relative to metabolic demand. While this work does help to shed light on a very complex question, a great deal of research is a still warranted to further elucidate the role of various vasoactive substances.

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