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SYMPOSIUM REVIEW

ATP as a mediator of erythrocyte-dependent regulation of skeletal muscle blood flow and oxygen delivery in humans

José González-Alonso

Centre for Sports Medicine and Human Performance, Brunel University, Uxbridge, UK

Abstract In healthy human beings, blood flow to dynamically contracting skeletal muscle is regulated primarily to match oxygen (O_2) delivery closely with utilisation. This occurs across a wide range of exercise intensities, as well as when exercise is combined with conditions that modify blood O_2 content. The red blood cells (RBCs), the primary O_2 carriers in the blood, contribute to the regulation of the local processes matching O_2 supply and demand. This is made possible by the ability of RBCs to release the vasoactive substance adenosine triphosphate (ATP) in response to reductions in erythrocyte and plasma O_2, as well as to other adjuvant metabolic and mechanical stimuli. The regulatory role of RBCs in human beings is supported by the observations that, i) exercising skeletal muscle blood flow responds primarily to changes in the amount of O_2 bound to the erythrocyte haemoglobin molecules, rather than the amount of O_2 in plasma, and ii) exercising muscle blood flow can almost double (from 260 to 460 ml min^{-1} 100 g^{-1}) with alterations in blood O_2 content, such that O_2 delivery and $\dot{V}_{O_2}$ are kept constant. Besides falling blood O_2 content, RBCs release ATP when exposed to increased temperature, reduced pH, hypercapnia, elevated shear stress and augmented mechanical deformation, i.e. conditions that exist in the microcirculation of active skeletal muscle. ATP is an attractive mediator signal for skeletal muscle blood flow regulation, not only because it can act as a potent vasodilator, but also because of its sympatholytic properties in the human limb circulations. These properties are essential to counteract the vasoconstrictor effects of concurrent increases in muscle sympathetic nerve activity and circulating vasoconstrictor substances during exercise. Comparison of the relative vasoactive potencies and sympatholytic properties of ATP, other nucleotides, and adenosine in human limbs, suggests that intravascular ATP exerts its vasodilator and sympatholytic effects directly, and not via its degradation compounds. In conclusion, current evidence clearly indicates that RBCs are involved directly in the regulation of O_2 supply to human skeletal muscle during dynamic exercise. Further, intravascular ATP might be an important mediator in local metabolic sensing and signal transduction between the RBCs and the endothelial and smooth muscle cells in the vascular beds of skeletal muscle.

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Corresponding author J. González-Alonso: Centre for Sports Medicine and Human Performance, Brunel University, Uxbridge, Middlesex UB8 3PH, UK. Email: j.gonzalez-alonso@brunel.ac.uk

Abbreviations EDHF, endothelial-derived hyperpolarization factor; MSNA, muscle sympathetic nerve activity; RBC, red blood cell.

José González-Alonso, PhD (University of Texas at Austin) is an integrative physiologist with a particular interest in human cardiovascular regulation. He benefited tremendously from his extensive post-doctoral work at the Copenhagen Muscle Research Centre (University of Copenhagen). His research primarily focuses on the mechanisms underlying the circulatory limitations to exercise capacity and environmental stress in humans and the role of the erythrocytes and intravascular adenosine triphosphate (ATP) on the regulation of the skeletal muscle blood flow and oxygen delivery.

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Skeletal muscle blood flow during exercise

It has long been known that blood flow to working skeletal muscles increases with elevations in exercise intensity. The magnitude of increase in skeletal muscle perfusion from rest to maximal exercise in normal conditions can reach 100- to 160-fold in untrained human beings and in endurance-trained athletes performing single leg dynamic knee-extensor exercise, i.e. an increase from ∼2.5 to ∼250 and ∼400 ml min^{-1} (100 g)$^{-1}$, respectively (assuming that all of the 2.5–3.0 kg of quadriceps muscles are activated at maximal exercise) (Andersen & Saltin 1985; Richardson *et al.* 1993; Saltin 2007). These estimates of maximal perfusion in human skeletal muscle are in agreement with values found in animal studies (Armstrong & Laughlin 1985; Munch *et al.* 1987; Laughlin *et al.* 2012) and demonstrate the enormous capacity of the skeletal muscle vasculature to dilate and increase blood flow, when metabolic demand increases. The delivery of O_2 and nutrients to body tissues and organs, and the clearance of metabolic waste products and heat are a function of blood flow, the blood concentration of gases, substrates and metabolites, and the temperature of the blood. Under normal conditions, the concentration of O_2 and nutrients in the arterial blood is normally maintained or changes only slightly during short duration exercise. Blood flow is therefore the primary determinant of O_2 and fuel delivery to active skeletal muscle during dynamic exercise (Rowell 2004; Saltin 2007; Laughlin *et al.* 2012).

The rate of muscle O_2 utilisation is generally thought to be a key factor that influences the magnitude of increase in blood flow to active skeletal muscle, termed exercise hyperaemia (Saltin 2007; Laughlin *et al.* 2012). Figure 1 illustrates this widely held principle by showing progressive increases in leg blood flow, leg O_2 delivery, leg O_2 extraction and leg $\dot{V}_{O_2}$ in healthy young men during incremental single leg dynamic knee-extension exercise. Notice the striking similarity in the rate of increase in leg O_2 delivery and leg $\dot{V}_{O_2}$ per unit of power (i.e. 16 and 13 ml min^{-1} W^{-1}, respectively) (González-Alonso *et al.* 2008). These circulatory adjustments across the active leg tissues, primarily in the exercising quadriceps muscle in this model (Andersen *et al.* 1985; Richardson *et al.* 1998; González-Alonso *et al.* 2000), are for the most part accompanied by proportional increases in cardiac output and systemic O_2 delivery, but a modest increase in mean arterial and central venous pressure (Fig. 1). Accordingly, a primary aim of the local and central control mechanisms involved in the regulation of cardiovascular function during dynamic steady-state exercise is to maintain a close match between O_2 supply and demand in active muscle (Rowell 2004; Saltin 2007; Laughlin *et al.* 2012). However, close matching of O_2 supply and utilisation is not a universal physiological phenomenon in active skeletal muscle across all exercise modes and intensities. For example, evidence in human models indicates that blood flow and O_2 delivery to active locomotor skeletal muscles is restrained when engaging a large muscle mass, during intense whole-body exercise, e.g. cycling, skiing, rowing or running (Calbet *et al.* 2004; Mortensen *et al.* 2005, 2008). Figure 2 illustrates this concept by showing that leg perfusion (and thus its surrogate O_2 delivery) is the same up to a power output of ∼100 W and is intimately related to leg $\dot{V}_{O_2}$, irrespective of whether power output was generated only by the quadriceps muscles during knee-extensor exercise or spread among the different leg muscles during two leg cycling (Mortensen *et al.* 2008). These observations during single leg knee-extensor and two leg cycling exercise below 100 W are strong evidence of the intimate coupling between O_2 delivery and metabolic demand for O_2 discussed above in relation to the single leg knee-extensor exercise data presented in Fig. 1. However, during cycling exercise above a moderate intensity, the relationship between locomotor limb tissue perfusion/O_2 supply and O_2 utilisation is curvilinear. This indicates a mismatch between O_2 supply and metabolic demand for O_2 in the moderate to maximal intensity domain (Fig. 2) (Mortensen *et al.* 2008). As a consequence, muscle perfusion per unit of power is lower during maximal whole-body exercise than during maximal small-muscle exercise, thereby imposing a circulatory limitation to maximal aerobic capacity (Mortensen *et al.* 2008; Boushel *et al.* 2011). This blunting in skeletal muscle perfusion during intense whole-body exercise may reflect the interaction between an enhanced local vasoconstriction in the active muscle vasculature and the attainment of the limits in cardiac function (Calbet *et al.* 2004; Mortensen *et al.* 2005, 2008; Stöhr *et al.* 2011). However, the majority of human physical or exercise activities do not require near or maximal aerobic capacity; rather, they are performed within the range of submaximal exercise intensities, where muscle O_2 supply and metabolic demand are matched closely. Thus, the consensus view reflected in the literature is that skeletal muscle perfusion reflects primarily the aerobic metabolic rate of the muscle and that factors and signals related to aerobic metabolism are heavily involved in the regulation of active skeletal muscle blood flow.

Locally, skeletal muscle perfusion is determined by vascular conductance and perfusion pressure gradient, i.e. arteriovenous pressure difference. During dynamic incremental exercise, the increases in active limb muscle blood flow are primarily the result of increases in vascular conductance. The latter is indicative of vasodilatation of the vascular beds irrigating the active muscle, as the perfusion pressure gradient does not change during mild intensity exercise, or increases slightly, in comparison to flow during intense exercise (Fig. 1). In exercising limbs, the diameter of the large conduit vessels such as the femoral and brachial arteries, does not change significantly, or changes little, in comparison to the increases in arterial

blood velocity (Rådegran 1997; Shoemaker *et al.* 1997). Hence, the enhanced vasodilatation, indicated by the global increases in limb vascular conductance during exercise, is attributable largely to increases in the diameter of small arteries and arterioles perfusing active muscle fibres (Segal 2005). A long-standing question is, what are the mechanisms underlying the apparently precise regulation of flow and O_2 supply to active skeletal muscle during the majority of submaximal exercise conditions? Over more than a century of extensive investigation of this question, a number of local and central control mechanisms have been proposed to regulate active muscle blood flow. These include metabolic, myogenic, mechanical, humoral and neural mechanisms, which under *in vivo* conditions involve the interaction of multiple intravascular, interstitial and intracellular signalling pathways (Rowell 1993, 2004; Laughlin *et al.* 2012). Discussion of each of these mechanisms and their relative contributions to exercise hyperaemia in humans is beyond the scope of this symposium review and the reader is referred to the following excellent comprehensive review articles for a more extensive discourse on these topics: Shepherd *et al.* (1983), Rowell (1993), Saltin *et al.* (1998) and Laughlin *et al.* (2012). The current review will focus on recent evidence from human studies providing insight into the influence of red blood cell (RBC) signalling. In particular, the review will focus on the role of erythrocyte-derived ATP, as a local vascular control mechanism contributing to the matching in O_2 supply and demand in skeletal muscle during dynamic exercise

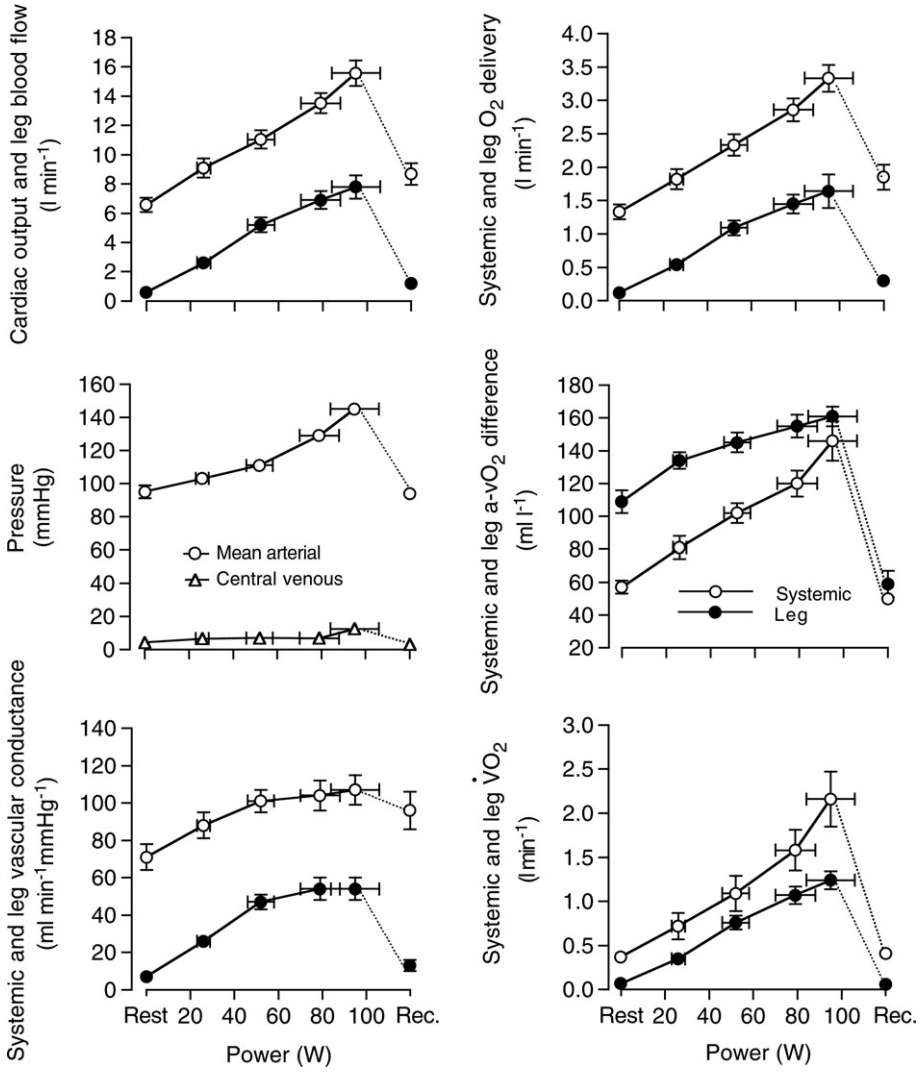

Figure 1. Leg and systemic haemodynamics during incremental single leg exercise
Cardiovascular variables were plotted against the increases in power output during single leg knee-extensor exercise. Note the similar rate of increase in leg O_2 delivery and leg $\dot{V}_{O_2}$ (16 and 13 ml min^{-1} W^{-1}, respectively). In all graphs, open circles represent the systemic responses and the filled circles represent the leg responses. Central venous pressure is depicted in empty triangles. Reproduced from González-Alonso *et al.* (2008).

(Ellsworth *et al.* 1995; González-Alonso *et al.* 2002). Evidence supporting the involvement of erythrocytes in the control of O_2 delivery to active skeletal muscle will be reviewed first.

Role of blood oxygen in the regulation of skeletal muscle perfusion and oxygen supply during exercise

Under normal exercise conditions, where arterial O_2 content is stable, oxygen delivery to skeletal muscle is determined by blood flow. However, the magnitude of exercise hyperaemia is influenced greatly by large increases or decreases in blood O_2 content, even when the metabolic demand for O_2 remains unaltered. Accordingly, when human beings are exposed to alterations in arterial blood O_2 content due to separate or combined manipulations of inspired O_2 and carbon monoxide (CO), as well as anaemia, plasma volume expansion or polycythaemia, exercising skeletal muscle blood flow changes in relation to blood O_2 such that O_2 supply is well-preserved (Rowell *et al.* 1986; Richardson *et al.* 1995; Koskolou *et al.* 1997; Saltin *et al.* 1998; Roach *et al.* 1999; González-Alonso *et al.* 2002, 2006; Casey & Joyner 2011) (Fig. 3). Indeed, the capacity of the muscle circulation to respond to changes in blood O_2 content appears to be substantial since perfusion to exercising quadriceps muscles almost doubles, with large alterations in blood O_2 content during constant power submaximal knee-extensor exercise; consequently, O_2 delivery and $\dot{V}_{O_2}$ remain constant (González-Alonso *et al.* 2006). For example, muscle perfusion increases from 260 ml min^{-1} (100 g)$^{-1}$ during exercise with combined polycythaemia and hyperoxia to 460 ml min^{-1} (100 g)$^{-1}$ during exercise with combined anaemia, plasma volume expansion and hypoxia, when total leg blood flow increases from 4.5 to 8 l min^{-1} (Fig. 3A). Notice that the difference in active limb tissue perfusion associated with changes in blood O_2 content during moderate intensity small muscle mass exercise in trained individuals (i.e. 50% peak power) is 200 ml min^{-1} (100 g)$^{-1}$, a value that is close to the reported maximal muscle perfusion values of untrained individuals (Andersen & Saltin 1985). Although these data from exercising human limb are unable to provide information about the precise site(s) of O_2 sensing and signal transduction mechanisms, they nonetheless provide

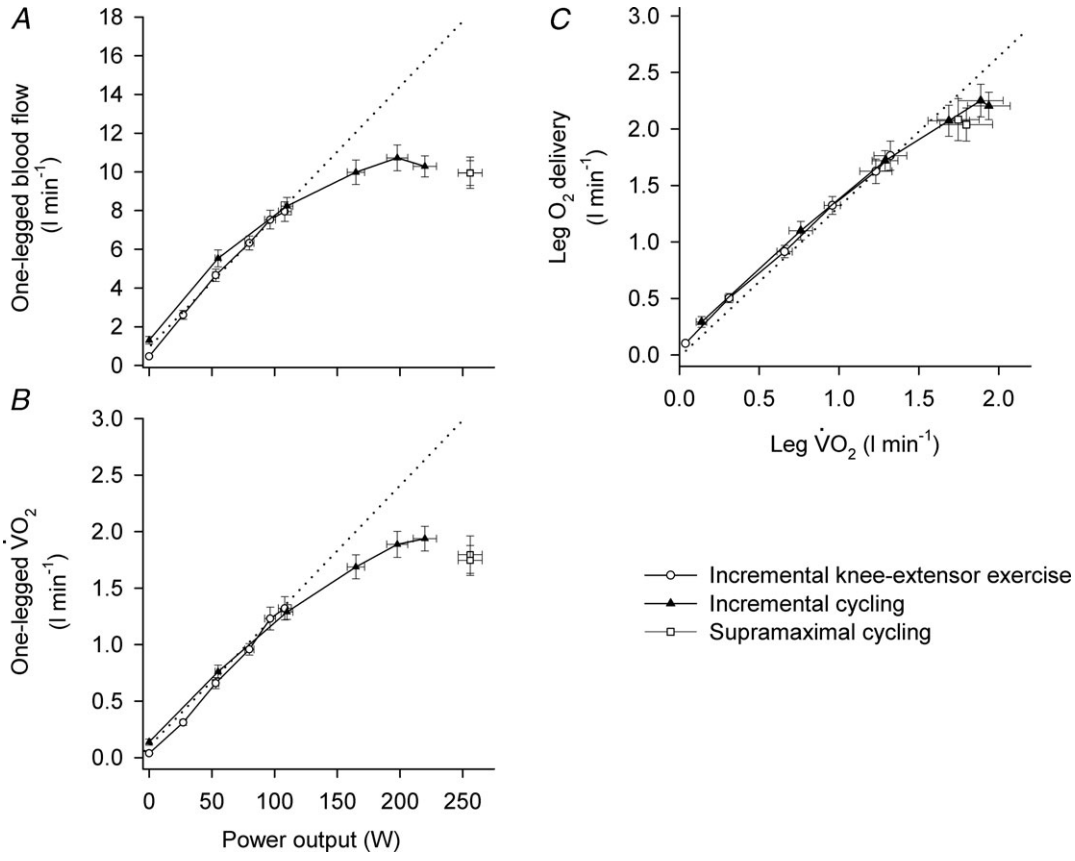

Figure 2. Leg haemodynamics during cycling and knee-extensor exercise
Note the similar absolute values and rate of increase in leg blood flow and $\dot{V}_{O_2}$ until 100 W during both two leg cycling and single leg knee-extensor exercise, becoming attenuated thereafter during cycling in association with a blunting in leg blood flow and vascular conductance (*A* and *B*). Close relationship between leg O_2 delivery and $\dot{V}_{O_2}$ responses during incremental exercise (*C*). Modified from Mortensen *et al.* (2008).

strong support for a critical role of blood O_2 content in the regulation of active skeletal muscle blood flow.

Blood O_2 content is largely determined by O_2 bound to the highly abundant haemoglobin molecules in the RBCs (250 million per RBC), but there is also a small amount of O_2 dissolved within plasma (termed partial pressure of O_2, P_{O_2}, and which accounts for 1.5% of the arterial blood O_2 content). The P_{O_2} is widely thought to act as an important signal for the control of the cardiovascular system, particularly in the control of ventilation and heart rate in conditions that alter arterial blood O_2 content (Jackson 1987; Baron *et al.* 1990; Pries *et al.* 1995). The question here is the role of P_{O_2} in local muscle blood flow regulation. Under conditions of systemic hypoxia or hyperoxia that are created by reducing or enriching the O_2 content of inspiratory gases, respectively, arterial and venous levels of both oxyhaemoglobin and P_{O_2} are affected. Accordingly, studies using systemic hypoxia and hyperoxia interventions cannot discriminate between the regulatory influences of O_2 bound to haemoglobin and of P_{O_2} on muscle perfusion. In recent years, the theory has been advanced that vasoactive signals/substances released from the circulating erythrocytes during the offloading of O_2 from the haemoglobin molecules (reflected by reductions in venous oxyhaemoglobin) contribute to the regulation of the supply of O_2 and blood to vascular beds

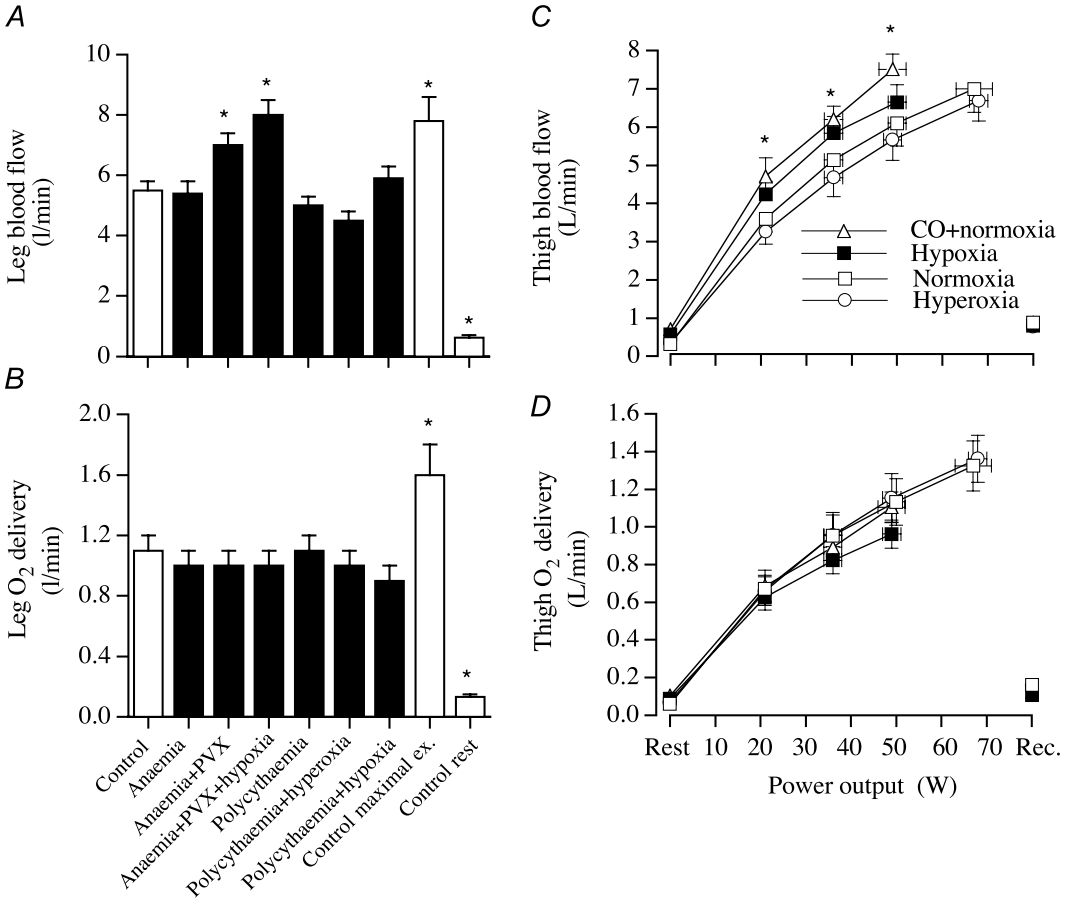

Figure 3. Limb blood flow and O_2 delivery during exercise and altered blood O_2 content
Leg blood flow (*A*) and O_2 delivery (*B*) during submaximal one-legged knee-extensor exercise (~50 W) in normocythaemia (control), anaemia, anaemia combined with plasma volume expansion (anaemia + PVX), anaemia combined with plasma volume expansion and hypoxia (anaemia + PVX + hypoxia), polycythaemia, polycythaemia combined with hyperoxia (polycythaemia + hyperoxia) and polycythaemia combined with hypoxia (polycythaemia + hypoxia). For comparison, haemodynamics and oxygenation data at rest and during peak one-legged knee-extensor exercise (95 ± 11 W) in the control condition are depicted. *Significantly different from exercise control, $P < 0.05$. Modified from González-Alonso *et al.* (2006). Thigh blood flow (C) and O_2 delivery (D) at rest and during incremental knee-extensor exercise and after 10 min of recovery with exposure to normoxia, hypoxic hypoxia, hyperoxia and carbon monoxide hypoxia in normoxia. *Values during hypoxia and carbon monoxide in combination with normoxia are significantly different from normoxic control, $P < 0.05$. Reproduced from González-Alonso *et al.* (2002).

experiencing an augmented demand for O_2 (Ellsworth *et al.* 1995; Stamler *et al.* 1997; Gladwin *et al.* 2000; González-Alonso *et al.* 2002). Accordingly, the level of oxyhaemoglobin in RBCs is thought to be a critical stimulus for the release of vasoactive substances underpinning metabolic vasodilatation in skeletal muscle.

Important insight into the role of RBCs and O_2 tension in the regulation of active limb muscle blood flow has emanated from experiments in human beings using CO inhalation in combination with the breathing of normoxic or hyperoxic gas mixtures (González-Alonso *et al.* 2001, 2002; Richardson *et al.* 2002; Hanada *et al.* 2003). CO inhalation offers the opportunity to independently manipulate arterial P_{O_2} and oxyhaemoglobin without altering RBC count. The affinity of haemoglobin for CO is some 200-fold greater than its affinity for O_2 (Piantadosi 1987). Consequently, arterial oxyhaemoglobin and O_2 content can be reduced with CO inhalation to the same extent as with systemic hypoxia exposure. Furthermore, arterial and venous P_{O_2} can be manipulated independently of CO-induced changes in oxyhaemoglobin by breathing either normoxic or hyperoxic gas mixtures. Using this approach, we compared human exercising limb muscle blood flow, limb vascular conductance, circulating noradrenaline and muscle sympathetic nerve activity (MSNA) under two reduced O_2 content conditions. Change in blood flow (relative to control) was similar during CO inhalation in combination with hyperoxia, and during hypoxic hypoxia, despite an 11-fold difference in arterial P_{O_2} between the two hypoxic conditions (550 *vs.* 40 mmHg; Fig. 4) (González-Alonso *et al.* 2001; Hanada *et al.* 2003). To put these findings in perspective, it is important to realise that the high arterial P_{O_2} (~500–600 mmHg) and oxyhaemoglobin values produced with pure systemic hyperoxia are generally associated with a reduced MSNA and exercising limb blood flow (Welch *et al.* 1977; Seals *et al.* 1991; González-Alonso *et al.* 2002) (Fig. 3). We observed the opposite when inhalation of a hyperoxic gas mixture was combined with inhalation of CO. Furthermore, since sympathetic activation can diminish limb muscle perfusion (Joyner *et al.* 1992; Buckwalter *et al.* 1997), it is intriguing that the up to 2- to 4-fold elevations in MSNA and circulating noradrenaline fail to reduce resting limb blood flow, or to prevent a greater increase in exercising limb tissue blood flow in either hypoxic condition compared to normoxia (Hanada *et al.* 2003). These findings suggest the existence of compensatory vasodilator mechanisms linked to the oxygenation state of haemoglobin that are capable of overriding the elevated neural and humoral vasoconstrictor stimuli in resting and exercising limb muscles under hypoxic and submaximal exercise conditions.

Systemic hypoxia not only reduces blood O_2 content, but also intracellular O_2 markers (Richardson *et al.* 1995). Thus, it is still possible that the greater increases in skeletal muscle blood flow with hypoxic hypoxia and CO-hypoxia could also be due to reductions in intracellular P_{O_2}. However, intracellular O_2 markers such as P_{O_2} and MbO_2 saturation cannot explain the elevation in exercising limb muscle blood flow with CO inhalation-mediated hypoxia compared to normoxia since quadriceps muscle P_{O_2} and MbO_2 saturation are the same during exercise in normoxia and the CO trials in line with a normal venous P_{O_2} (Richardson *et al.* 2002). Thus, exercising leg blood flow responses in these experimental conditions are unrelated to arterial, venous or muscle P_{O_2} (González-Alonso *et al.* 2001, 2002; Richardson *et al.* 2002; Hanada *et al.* 2003). Collectively, these observations suggest that the main vascular O_2 sensor locus for the control of blood flow lies in the erythrocyte itself, rather than in the P_{O_2}-sensitive areas of the vascular endothelium, vascular smooth muscle or skeletal muscle. A fundamental question is then how do the RBCs signal to the vascular endothelium and smooth muscle to increase or decrease skeletal muscle blood flow in relation to the changes in oxyhaemoglobin. To date, three O_2-dependent signalling mechanisms have been proposed: (1) the release of ATP from erythrocytes (Ellsworth *et al.* 1995), (2) the formation of *S*-nitrosohaemoglobin (Jia *et al.* 1996; Stamler *et al.* 1997), and (3) the reduction of nitrite to vasoactive NO by deoxygenation (Gladwin *et al.* 2000). Evidence in humans of the role of erythrocyte-derived ATP in the regulation of limb muscle blood flow is discussed below. The cellular and molecular mechanisms of erythrocyte-derived ATP release, its role in blood distribution in skeletal muscle, and the role of intravascular NO and nitrite in the control of circulation are discussed in companion reviews (Ellsworth & Sprague 2012; Hellsten *et al.* 2012; Owusu *et al.* 2012).

The role of erythrocyte-derived ATP in the control of skeletal muscle perfusion

Four decades ago, Forrester & Lind (1969) and Forrester (1972) showed that venous plasma [ATP] increases during human forearm exercise compared to rest. The ATP source, however, was not identified. Twenty years later, Bergfeld & Forrester (1992) demonstrated that human RBCs release ATP when exposed to hypoxia and hypercapnia *in vitro*. This original finding highlighted that RBCs could be an important source of ATP in the vasculature of active tissues. In 1995, Ellsworth *et al.* demonstrated that low P_{O_2} and low pH are strong stimuli for ATP release from RBCs and that ATP placed into an arteriole causes a significant conducted arteriolar vasodilatation (which is really important as a local change in diameter has little impact on flow) and that ATP applied to capillaries results in an increase in red blood cell supply rate in the

capillaries (Ellsworth *et al.* 1995). In this classic study, Ellsworth and co-workers first proposed the hypothesis that 'RBC is not only the major O_2 carrier but also serves as an O_2 sensor and affecter of changes in O_2 delivery via its release of ATP, which subsequently binds to P_2Y receptors on the vascular endothelium, altering vessel calibre'. We tested this hypothesis a few years later in healthy human beings, by measuring femoral venous and arterial plasma [ATP], blood gases and haemodynamics during incremental single leg knee-extensor exercise under conditions of normoxia, hypoxia and hyperoxia, and during CO inhalation in combination with normoxia (González-Alonso *et al.* 2002). These interventions produced reciprocal alterations in arterial O_2 content and thigh blood flow, but equal thigh O_2 delivery and $\dot{V}_{O_2}$ (Fig. 3). In support of Ellsworth and co-workers' hypothesis, plasma [ATP] in the effluent blood from the exercising thigh increased in all conditions; however, the magnitude of increase was greater during intense hypoxic exercise than during the corresponding normoxic exercise. Plasma [ATP] also tended to be attenuated during hyperoxic exercise and during exercise with CO inhalation combined with normoxia (Fig. 5). Interestingly, venous plasma [ATP] from the non-exercising limb remained low, but was closely related to changes in $(O_2 + CO)$Hb fraction, as in the exercising leg. The observation that exercising limb blood flow was similarly elevated during the CO and hypoxic hypoxia trials suggests that CO and/or other vasodilator substances mitigated the effects of a reduced plasma ATP during the CO compared to hypoxia trial.

Surprisingly, plasma [ATP] in the femoral artery increased with the rise in exercise intensity. Although there is still controversy about the absolute values (Gorman *et al.* 2007), the increases in venous and arterial plasma [ATP] during incremental and constant high intensity exercise

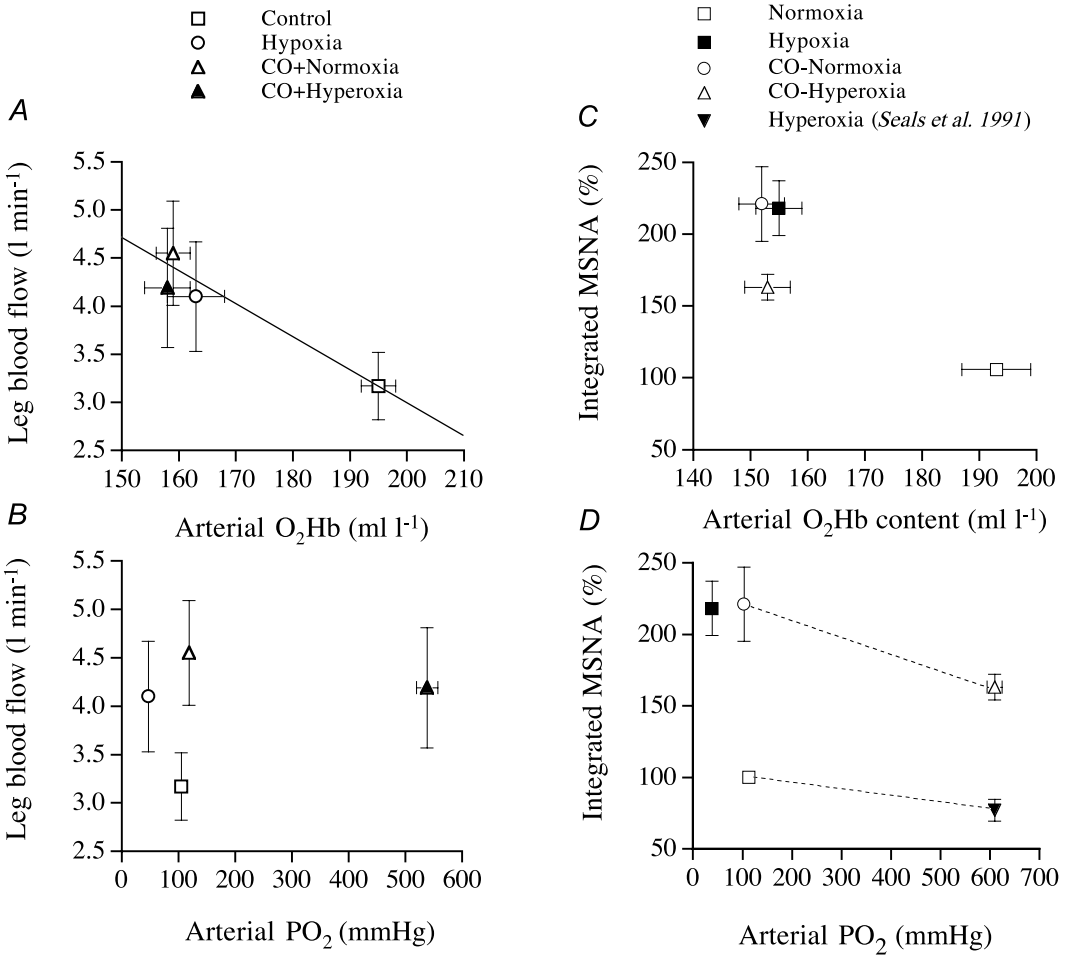

Figure 4. Influence of blood O_2 on leg perfusion and sympathetic nerve activity
Relationship between leg blood flow or total muscle sympathetic nerve activity *versus* arterial oxyhaemoglobin and P_{O_2} in healthy humans exposed to normoxia, hypoxia, carbon monoxide inhalation in normoxia, and carbon monoxide inhalation in hyperoxia. Modified from González-Alonso *et al.* (2001) and Hanada *et al.* (2003). Note that the integrated MSNA *vs.* arterial P_{O_2} graph also depicts data from a published study during hyperoxia (Seals *et al.* 1991).

have been confirmed in several studies (González-Alonso *et al.* 2004; Rosenmeier *et al.* 2004; Yegutkin *et al.* 2007; Mortensen *et al.* 2007), including a recent report using novel intravascular microdialysis probes inserted proximal to the exercising muscles (Mortensen *et al.* 2011). Increases in venous and arterial plasma [ATP] during exercise have been shown in the coronary circulation (Farias *et al.* 2005) and across the human brain during maximal constant load cycling exercise (González-Alonso *et al.* 2004). However, studies using mild constant load single leg exercise have failed to demonstrate significant changes in plasma [ATP] despite significant alterations in blood O_2 content and limb blood flow (González-Alonso *et al.* 2006; Dufour *et al.* 2010). These findings point out some inconsistencies in the literature, which might be due at least in part to differences in the procedures employed to assess plasma ATP. Methodological issues such as the position of the catheter, the use of stop solutions during blood sample collection, the time lapse before ATP measurement and the correction of plasma [ATP] values for haemolysis can all influence the absolute plasma [ATP] (Gorman *et al.* 2007). Placement of the catheter in the direction of the exercising muscles appears to be the most critical issue for detecting small changes in plasma [ATP] occurring during low intensity exercise (Mortensen *et al.* 2011).

Compelling evidence *in vitro* demonstrates that the reductions in haemoglobin O_2 saturation and P_{O_2} are potent stimuli for erythrocyte ATP release (Jagger *et al.* 2001; Ellsworth 2004; Ellsworth *et al.* 2009). *In vivo*, however, there is some evidence that plasma [ATP] increases in the arterial inflow during incremental exercise, but does so in the presence of unchanged blood O_2 content (González-Alonso *et al.* 2002, 2004; Yegutkin *et al.* 2007; Mortensen *et al.* 2011). Likewise, plasma [ATP] increases markedly in the venous and arterial blood of humans with mitochondrial myopathy, who do not display an increase in muscle O_2 extraction during exercise (Jeppesen *et al.* 2012). These observations indicate that during exercise, stimuli other than reductions in blood oxygenation must also increase ATP release from erythrocytes and/or other vascular or interstitial sources. There is evidence that RBCs release ATP *in vitro* when exposed to hypercapnia (Bergfeld & Forrester 1992), reduced pH (Ellsworth *et al.* 1995), augmented mechanical deformation (Sprague *et al.* 1998), elevated shear stress (Wan *et al.* 2008) and increased temperature (Kalsi & González-Alonso 2012), conditions that are all present in the microcirculation of active skeletal muscle. For instance, temperature has recently been shown to be a potent stimulus for erythrocyte ATP release *in vitro* (Kalsi & González-Alonso 2012) (Fig. 6). This finding provides insight into a potential ATP source accounting for the rise in plasma ATP in humans exposed to passive heat stress and heat stress combined with exercise (González-Alonso *et al.* 2004; Pearson *et al.* 2011). Heat may also provide a possible mechanism underpinning the significant increase in skeletal muscle blood flow with heat stress (Keller *et al.* 2010) and combined heat stress and exercise, both conditions being associated with the rise in arterial plasma [ATP] and muscle temperature (Pearson *et al.* 2011) (Fig. 6). Therefore, the rise in plasma [ATP] in conditions where oxyhaemoglobin and P_{O_2} do not decline indicates that increases in erythrocyte-derived ATP release *in vivo* are most likely an integrative response involving several stimuli.

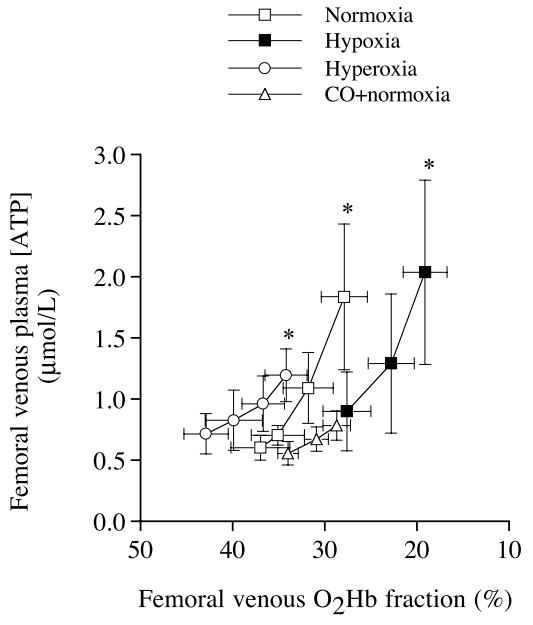

Figure 5. Plasma ATP and erythrocyte oxygenation
Relationship between femoral venous plasma ATP concentration and O_2Hb fraction during incremental knee-extensor exercise with exposure to normoxia, hypoxic hypoxia, hyperoxia and carbon monoxide (CO) inhalation in normoxia. *Significantly higher than mild exercise within a given condition, $P < 0.05$. Modified from González-Alonso *et al.* (2002).

Despite the uncertainty about the precise stimuli for erythrocyte ATP release *in vivo*, it is clear that the observed increases in plasma [ATP] during incremental exercise and high intensity constant power exercise can induce profound local increases in limb muscle blood flow and proportional elevations in cardiac output. These changes occur independently of the myriad other vasoactive compounds that also rise during exercise. A dose-dependent increase in limb blood flow has been shown repeatedly with infusion of ATP in the femoral and brachial arteries (Duff *et al.* 1954; Rogen *et al.* 1994; González-Alonso *et al.* 2002; Rosenmeier *et al.* 2004; Kirby *et al.* 2008; Mortensen *et al.* 2011), but not with infusion in the femoral vein (González-Alonso *et al.* 2008). The ATP infusion-mediated limb tissue vasodilatation occurs without increases in venous plasma or interstitial [ATP] (González-Alonso *et al.* 2002; Rosenmeier *et al.* 2004; Mortensen *et al.* 2009*b*, 2011), indicating

that ATP acts intraluminally via vascular endothelial pathways. The released ATP binds to P_2Y purinergic receptors in the vascular endothelium, thereby stimulating the production of endothelial NO, prostaglandins and/or endothelial-derived hyperpolarization factors (EDHFs), which in turn act upon the surrounding vascular smooth muscle cells to cause local and conducted vasodilatation (Ellsworth *et al.* 2009; Mortensen *et al.* 2009*a*). Blockade of NO and prostaglandins formation with combined inhibition of NO synthase and cyclooxygenase has been shown to only partially blunt the increase in limb blood flow and vascular conductance evoked by intra-arterial infusion of ATP (Mortensen *et al.* 2009*a*; Crecelius *et al.* 2011). The lack of complete blockade suggests that other, yet to be discovered endothelial factors might also be involved in ATP induced vasodilatation. Importantly, maximal ATP induced vasodilatation in the leg reaches 8 litres min^{-1}, a value similar to that measured during maximal exercise (Rosenmeier *et al.* 2004; González-Alonso *et al.* 2008). Moreover, the vasodilator potency of ATP is much greater than that of its degradative compounds ADP, AMP and adenosine, but similar to UTP (i.e. ATP (100) = UTP (100) ≫ adenosine (5.8) > ADP (2.7) > AMP (1.7)) (Rosenmeier *et al.* 2008). Both ATP and UTP act via the same purinergic P_2Y_2 receptors, which are expressed abundantly in the endothelium of microvessels and smooth muscle cells of human limbs (Mortensen *et al.* 2009*b*). This supports the notion that smaller increases in plasma [ATP] than plasma [ADP], [AMP] or [adenosine] would be necessary to produce the same increase in limb muscle blood flow and suggests that intravascular ATP exerts its vasodilator effect directly and not via its degradation compounds.

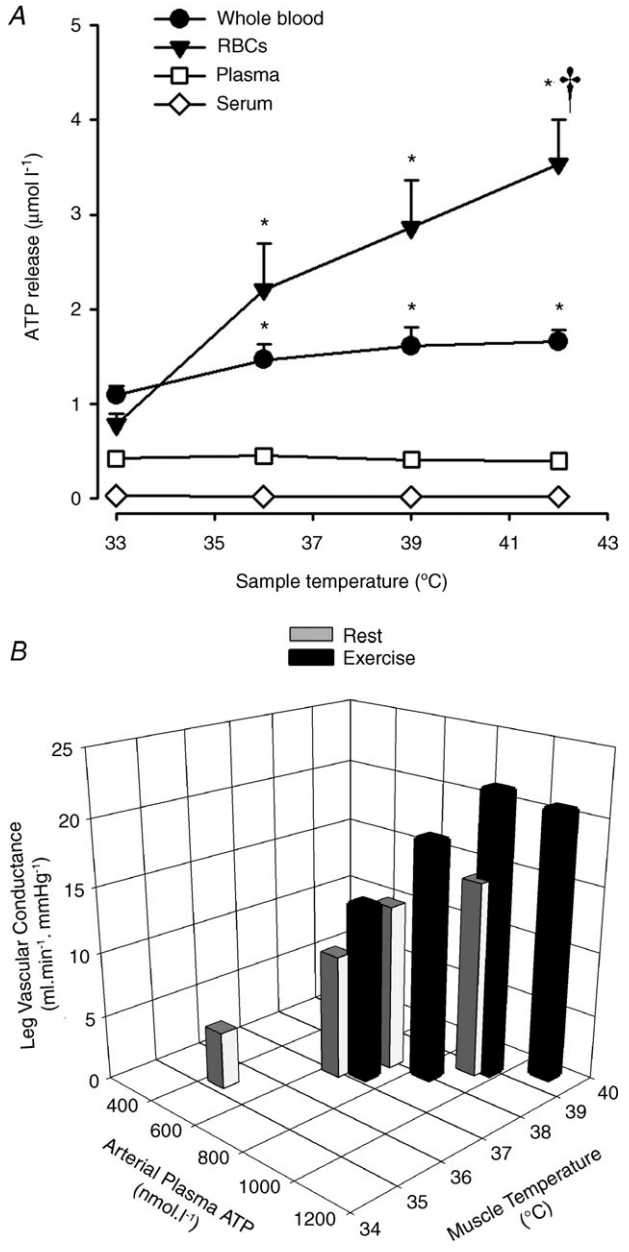

Figure 6. Influence of temperature on ATP release and limb tissue vascular conductance regulation
A, heating whole blood and RBC fraction resulted in significant increases in ATP release, but not in plasma or serum. *Significantly higher than values at 33° C, $P < 0.05$. †Significantly higher than values at 36° C, $P < 0.05$. Modified from Kalsi & González-Alonso (2012). *B*, net leg vasodilatation with passive heat stress and heat stress exercise *in vivo* in human subjects is strongly related to increases in arterial plasma ATP and muscle temperature ($r^2 = 0.87$; $P = 0.001$). Reproduced from Pearson *et al.* (2011).

In understanding the role of erythrocyte-derived ATP on local blood flow regulation, it is important to bear in mind that exercise hyperaemia is the net result of the interplay among multiple changes in vasodilator and vasoconstrictor signals/substances in the vascular beds of active muscles (Clifford & Hellsten 2004), the relative contributions of which vary with increases in the amount of muscle mass engaged during exercise (Fig. 2). In this context, intravascular ATP is an attractive mediator signal for the local regulation of skeletal muscle blood flow because not only can it act as a potent vasodilator at rest and when superimposed during exercise, but it also possesses sympatholytic properties in human limb muscle circulations (Rosenmeier *et al.* 2004, 2008; Kirby *et al.* 2008). The latter is essential to counteract the concurrent local increases in α-adrenergic vasoconstriction during exercise. Using intra-arterial administration of the drug tyramine to evoke endogenous noradrenaline release from sympathetic nerve endings, we provided the first evidence of the sympatholytic effects of circulating ATP (Rosenmeier *et al.* 2004); in contrast to adenosine-mediated hyperaemia, ATP infusion-induced leg hyperaemia persisted during co-infusion of tyramine in a similar manner to that observed during exercise. In a subsequent study, Kirby *et al.* (2008) demonstrated that intravascular ATP modulates both postjunctional α_1- and α_2-adrenergic vasoconstriction and that this response is graded with the rate of ATP infusion,

and thus the magnitude of ATP-induced hyperaemia. In addition, Rosenmeier *et al.* (2008) showed that neither ADP, nor AMP nor adenosine infusion abolishes tyramine-mediated increases in vasoconstrictor drive. This contrasted with the total blunting of sympathetic vasoconstriction produced by ATP and UTP infusion. Therefore, it also appears that the sympatholytic effects of ATP in the skeletal muscle vasculature are largely mediated via ATP itself, which modulates both postjunctional α_1- and α_2-adrenergic vasoconstriction depending upon the magnitude of hyperaemia.

Whether erythrocyte-derived ATP plays a role as a controller of total blood flow to the exercising muscle, a local distributor of blood flow within the active muscle or both remain unknown. However, it is clear that the increase in total blood flow drives most of the rise in $\dot{V}_{O_2}$ in active muscle and that increases in O_2 extraction due to redistribution of blood flow within muscle only accounts for a small part of the haemodynamic response to exercise. This notion is illustrated in Fig. 1 showing that the rise in leg $\dot{V}_{O_2}$ during incremental dynamic knee-extensor exercise to volitional exhaustion is associated with an up to 20-fold elevation in total leg blood flow whereas leg a-v O_2 difference increases by up to 60% compared to resting conditions. ATP infusion into a major human artery increases total limb blood flow during submaximal and maximal in human subjects, yet limb $\dot{V}_{O_2}$ remains unaltered because of a parallel reduction in O_2 extraction (Rosenmeier *et al.* 2004; Calbet *et al.* 2006). The fact that intra-femoral artery ATP infusion results in a reduction in O_2 extraction across the maximally exercising limb (i.e. in a condition where metabolic demand for O_2 is unmet; see Fig. 2) suggests an effect of exogenous ATP on less-active muscle fibres and other non-contracting tissues (Calbet *et al.* 2006). Taken together, these findings indicate that ATP has to act locally at the level of the active muscle microcirculation to be effective in increasing and/or distributing blood flow where it is needed.

Summary

Evidence discussed in this review provides strong support for a pivotal role of circulating RBCs in the regulation of the local vascular processes matching O_2 supply and demand in active skeletal muscle in humans. In humans, RBCs are proposed to contribute to these local regulatory processes, in part, by releasing the vasodilator and sympatholytic substance ATP into the vascular lumen

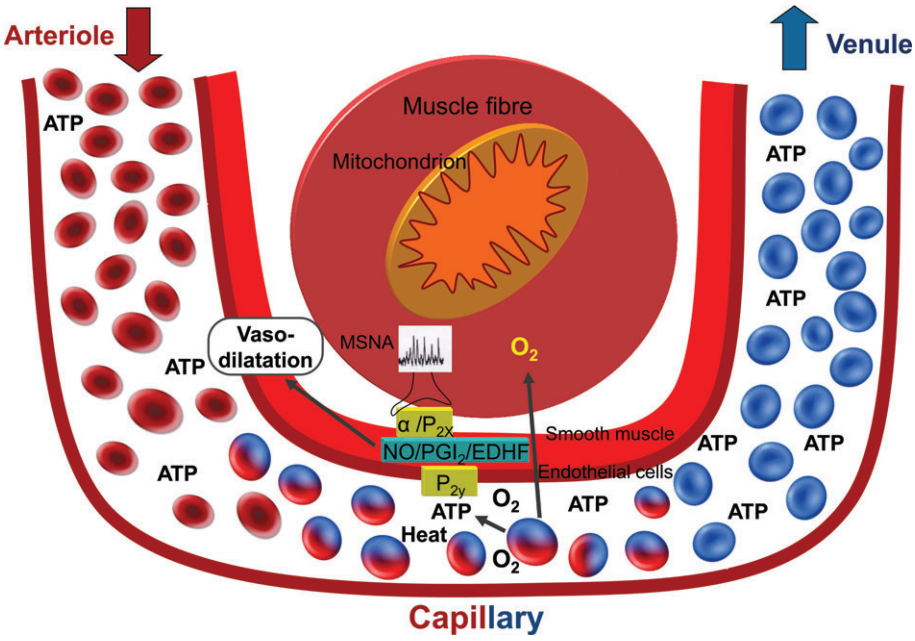

Figure 7. Schematic diagram illustrating how erythrocyte-derived ATP contributes to the local regulation of O_2 supply to active skeletal muscle
The red blood cells contribute to the regulation of the local processes matching O_2 supply and demand, in part by releasing the vasodilator and sympatholytic substance ATP into the vascular lumen. *In vivo* in exercising limb muscle, ATP is released from red blood cells in response to several metabolic and mechanical stimuli, including reduced oxyhaemoglobin, P_{O_2}, and pH and augmented blood temperature, mechanical deformation of RBCs and elevated shear stress. The released ATP binds to vascular endothelial P_2Y purinergic receptors expressed in the endothelium of microvessels and smooth muscle cells of human limbs. In so doing, it induces profound local and conducted vasodilatation by stimulating endothelial NO, prostaglandins, EDHF production and possibly other endothelial vasoactive signals, while also modulating α_1- and α_2-adrenergic vasoconstrictor influences of increases in muscle sympathetic nerve activity (MSNA) during exercise.

in response to several metabolic and mechanical stimuli (Fig. 7). The released ATP binds to vascular endothelial P_2Y purinergic receptors expressed in the endothelium of microvessels and smooth muscle cells of human limbs. In so doing, it induces profound local and conducted vasodilatation by stimulating endothelial NO, prostaglandins, EDHF production and possibly other endothelial vasoactive signals. In human limbs, ATP appears to exert it local vasodilator and sympatholytic effects directly, and not via its degradation compounds ADP, AMP or adenosine. Local heating is suggested as a therapeutic means of increasing blood flow and oxygen and fuel delivery to human limb tissues by stimulating erythrocyte ATP release and other temperature sensitive vasodilator pathways.

Future directions

Whilst scientific and circumstantial evidence in humans exposed to CO inhalation and other interventions that drastically alter blood O_2 content supports a pivotal role of the erythrocyte in O_2 transport, it is as yet unknown whether erythrocyte-derived ATP signalling is essential for the local regulation of total blood flow and its distribution within exercising muscle. Future research should address: (1) the precise time course of the plasma [ATP] responses in relation to the well-established immediate increases in local muscle blood flow during exercise, (2) the relative contribution of different stimuli such as O_2 and temperature on erythrocyte derived ATP during exercise, and (3) the precise signalling mechanisms involved in ATP-induced vasodilatation and sympatholysis and their implications for blood flow regulation in contracting muscle across the full range of exercise hyperaemia. Answering these questions requires the development of new methods to measure ATP in real time in humans as well as the development of specific erythrocyte ATP channels/transporters blockers and downstream purinergic receptor antagonists to block ATP-mediated dilatation and examine whether exercise hyperaemia is compromised.

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