Erythrocytes and the regulation of human skeletal muscle blood flow and oxygen delivery: role of erythrocyte count and oxygenation state of haemoglobin

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Blood flow to dynamically contracting myocytes is regulated to match O_2 delivery to metabolic demand. The red blood cell (RBC) itself functions as an O_2 sensor, contributing to the control of O_2 delivery by releasing the vasodilators ATP and S-nitrosohaemoglobin with the offloading of O_2 from the haemoglobin molecule. Whether RBC number is sensed remains unknown. To investigate the role of RBC number, in isolation and in combination with alterations in blood oxygenation, on muscle and systemic perfusion, we measured local and central haemodynamics during one-legged knee-extensor exercise (∼50% peak power) in 10 healthy males under conditions of normocythaemia (control), anaemia, anaemia + plasma volume expansion (PVX), anaemia + PVX + hypoxia, polycythaemia, polycythaemia + hyperoxia and polycythaemia + hypoxia, which changed either RBC count alone or both RBC count and oxyhaemoglobin. Leg blood flow (LBF), cardiac output (Q) and vascular conductance did not change with either anaemia or polycythaemia alone. However, LBF increased with anaemia + PVX (28 ± 4%) and anaemia + PVX + hypoxia (46 ± 6%) and decreased with polycythaemia + hyperoxia (18 ± 5%). LBF and Q with anaemia + PVX + hypoxia (8.0 ± 0.5 and 15.8 ± 0.7 l min^{-1}, respectively) equalled those during maximal knee-extensor exercise. Collectively, LBF and vascular conductance were intimately related to leg arterial–venous (a–v) O_2 difference ($r^2 = 0.89$–0.93; $P < 0.001$), suggesting a pivotal role of blood O_2 gradients in muscle microcirculatory control. The systemic circulation accommodated to the changes in muscle perfusion. Our results indicate that, when coping with severe haematological challenges, local regulation of skeletal muscle blood flow and O_2 delivery primarily senses alterations in the oxygenation state of haemoglobin and, to a lesser extent, alterations in the number of RBCs and haemoglobin molecules.

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Blood flow to dynamically contracting myocytes is regulated to match O_2 delivery to metabolic demand. This complex regulatory process is classically thought to integrate metabolic, myogenic and neural signals that are either released into the vascular lumen or accumulate in the muscle interstitial space as a result of the enhanced myocyte metabolism (Laughlin *et al.* 1996). Recently, the idea has been advanced that signals released from the circulating erythrocytes in association with the offloading of O_2 from the haemoglobin molecule contribute to the local regulation of blood flow and ultimately O_2 delivery in the microcirculation (Ellsworth *et al.* 1995; Stamler *et al.* 1997; González-Alonso *et al.* 2002). Support for this theory is found in both *in vitro* and *in vivo* reports demonstrating that: (1) red blood cells (RBCs) release the vasodilators ATP and S-nitrosohaemoglobin (SNO-Hb) when exposed to hypoxia (Bergfeld & Forrester, 1992; Ellsworth *et al.* 1995; Jia *et al.* 1996; Stamler *et al.* 1997; McMahon *et al.* 2002; (2) perfusion of the isolated arterioles with a buffer or dextran solution, instead of RBCs, does not increase vessel diameter and affluent ATP with exposure to low P_{O_2} (Dietrich *et al.* 2000); (3) alterations in blood O_2 content with hypoxia and hyperoxia in humans are inversely related to changes in exercising muscle blood flow (Rowell *et al.* 1986; Welch *et al.* 1997; Roach *et al.* 1999; González-Alonso *et al.* 2001, 2002); and (4) muscle perfusion, muscle sympathetic nerve activity and plasma ATP in exercising humans exposed to hypoxia, hyperoxia, carbon monoxide inhalation and

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anaemia are more closely related to the amount of O_2 bound to haemoglobin than the amount of O_2 dissolved in plasma (i.e. P_{O_2}) (González-Alonso *et al.* 2001, 2002; Jagger *et al.* 2001; Hanada *et al.* 2003). In this construct, the RBC itself functions as an O_2 sensor and controller of muscle O_2 delivery by releasing the vasodilators ATP and SNO-Hb. Whether changes in blood O_2 content via manipulation of RBC number are sensed and signalled through ATP release from the erythrocyte have not been investigated.

Elucidating the role of RBC number on the control of blood flow could provide new insights into the regulation of vascular tone in patients suffering from anaemia and polycythaemia, patients receiving volume therapy or erythropoietin, athletes undergoing blood doping and healthy blood donors. Indeed, the capacity of the circulatory system to respond to changes in RBC number appears to be enormous. In a group of chronic anaemic patients, Roy *et al.* (1963) showed that the remarkably high resting cardiac output (Q) of $\sim$15 l min^{-1} was reduced to $\sim$7 l min^{-1} when arterial O_2 content increased from 43 to 143 ml l^{-1} by blood transfusion. In exercising healthy humans, acute anaemia evoked by withdrawal of 1–1.5 l of blood and an equal volume replacement increases systemic and muscle blood flow and vascular conductance (Koskolou *et al.* 1997). Normovolaemic haemodilution also increases Q in pigs (Krantz *et al.* 2005), raising the possibility that the increase in exercising muscle blood flow seen with anaemia in humans is related principally to the haemodilution caused by volume replacement (Koskolou *et al.* 1997). Conversely, normovolaemic polycythaemia at rest and during exercise in dogs reduces Q in a linear fashion as haematocrit increases to 65% (Lindendeld *et al.* 1985). Whereas the importance of the erythrocyte on blood flow control is now recognized (Ellsworth, 2004; Singel & Stamler, 2005), the independent effects of RBC number and the interactions between RBC count and the oxygenation state of haemoglobin on human skeletal muscle blood flow and O_2 delivery remain unexplored.

This study tested the hypotheses that both RBC number and erythrocyte O_2 availability are involved in the local regulation of skeletal muscle blood flow and O_2 delivery and that plasma ATP reflects the changes in blood oxygenation. To accomplish this, we manipulated RBC number in isolation and in combination with acute changes in the oxygenation state of Hb in healthy human subjects and measured local and systemic haemodynamics and plasma ATP at rest and during exercise.

Methods

Ten healthy recreationally active male subjects participated in this study. They had a mean (± S.D.) age of 26 ± 3 years, body weight of 83 ± 11 kg, height of 189 ± 8 cm, quadriceps muscle mass of 2.9 ± 0.3 kg, maximal heart rate of 193 ± 8 beats min^{-1}, maximal cardiac output of 26.1 ± 1.3 l min^{-1} and a $\dot{V}_{O_2,max}$ of 4.8 ± 0.4 l min^{-1}. The subjects were fully informed of any risks and discomforts associated with the experiments before giving their informed, written consent to participate. The studies conformed to the code of Ethics of the World Medical Association (Declaration of Helsinki) and were approved by the Ethics Committee of Copenhagen and Frederiksberg communities (KF 11-047/04).

To address the main aim of the study, the subjects were tested at rest and during 3 min of submaximal one-legged knee-extensor exercise (52–53 W (± 5 W); peak power 101 ± 4 W) under conditions that changed RBC count alone (points 2, 3 and 5 below) or both RBC count and O_2 content (points 4, 6 and 7 below): (1) normocythaemia control; (2) anaemia (withdrawal of 20 ± 3% blood volume); (3) anaemia combined with plasma volume expansion (anaemia + PVX; infusion of 1.3 ± 0.2 l Voluven (Fresenius Kabi AB, Sweden); (4) anaemia + PVX combined with hypoxia (F_{IO_2} = 10%); (5) polycythaemia (reinfusion of 0.8 ± 0.1 l packed RBCs); (6) polycythaemia + hyperoxia (F_{IO_2} = 100%); and (7) polycythaemia + hypoxia. The control and anaemic conditions (1–4; part A – see below) and polycythaemic conditions (5–7; part B) were performed on two different days, separated by $\sim$4 weeks. During part A, the subjects performed the control condition followed by anaemia, anaemia + PVX and anaemia + PVX + hypoxia. The trials were separated by 30–90 min of rest necessary to implement the experimental condition and to recover from previous exercise. During part B, the subjects performed the polycythaemia condition followed by polycythaemia + hyperoxia and polycythaemia + hypoxia. These trials were separated by 30 min of rest. Prior to the control condition in part A, the subjects underwent incremental one-legged knee-extensor exercise (26 ± 3, 52 ± 6, 79 ± 9 and 95 ± 11 W).

In both part A and B, the subjects reported to the laboratory at 8 a.m., following the ingestion of a normal breakfast. Upon arrival, they rested in the supine position while catheters were placed under local anaesthesia into the femoral artery and vein of the exercising thigh and the brachial artery and an antecubital vein, with the latter catheter being advanced to the right atrium. The femoral artery and vein catheters were positioned 1–2 cm proximal or distal from the inguinal ligament. A thermistor to measure venous blood temperature was inserted through the femoral venous catheter orientated in the anterograde direction for the determination of femoral venous blood flow. The subjects then walked to the experimental room and sat on the knee-extensor ergometer while instrumentation was attached. Following $\sim$10 min of seated rest, baseline leg and systemic haemodynamics and blood samples were obtained.

In part A, during each stage of incremental knee-extensor exercise, arterial and femoral venous blood samples were withdrawn after 40 s followed by the measurement of leg blood flow (LBF) after 60 s and repeated after 10 min of recovery. During the submaximal 3 min exercise bouts, blood samples were withdrawn after 1.5 min whereas LBF was measured after 1 and 2.5 min. Acute anaemia was induced by withdrawal of ∼20% of the subjects blood volume (i.e. 1.3 ± 0.2 l; range 0.9–1.5 l). Following appropriate separation of blood, packed RBCs from each subject were stored at the hospital blood bank for reinfusion 4 weeks later. To restore plasma volume in the anaemic state, 1.3 ± 0.2 l of a plasma volume expander (hydroxyl-ethyl-starch; Voluven, 60 mg ml^{-1}) was infused intravenously. To superimpose systemic hypoxia onto anaemia and PVX, the subjects breathed from a bag containing 10% O_2 in N_2 before and during exercise and recovery.

In part B, packed RBCs were reinfused (0.84 ± 0.16 l; range 0.66–1.02 l) 2 days before the invasive experiment. On the experimental day, the subjects underwent incremental knee-extensor exercise before the submaximal exercise protocols. To superimpose hypoxia and hyperoxia onto polycythaemia, the subjects breathed from a bag containing either 10% or 99% O_2, respectively.

Throughout the studies, pulmonary $\dot{V}_{O_2}$ was measured online (Cosmed Quark b^2, Italy), heart rate was obtained from an electrocardiogram while arterial and central venous pressures were monitored with transducers positioned at heart level (Pressure Monitoring Kit, Baxter). The LBF was measured by the constant-infusion thermodilution method (Andersen & Saltin, 1985; González-Alonso *et al.* 2000), while Q was calculated by multiplying stroke volume by heart rate, using the Modelflow method, incorporating age, gender, height and weight to determine stroke volume (BeatScope version 1.1; Finapress Medical Systems BV, Amsterdam, the Netherlands) (Bogert & van Lieshout, 2005). This method computes an aortic flow waveform from peripheral arterial pressure by simulating a non-linear three-element model of aortic input impedance (Wesseling *et al.* 1993), detecting the arterial pressure upstroke and the dicrotic notch at the end of left ventricular ejection. This flow pulsation was integrated over the period between the beginning of the upstroke and the notch to yield stroke volume. To report absolute values, resting values were calibrated against the Fick method $Q = \dot{V}_{O_2}$/a−v O_2 difference), assuming negligible differences in blood oxygenation between the right atrium and the pulmonary artery (Barratt-Boyes & Wood, 1956). Systemic and leg vascular conductance were calculated as the ratio between Q or LBF and their perfusion pressures, respectively. Leg and systemic plasma flows were calculated by multiplying Q or LBF by haematocrit, whereas the RBC flows were calculated as the difference between LBF or Q and the corresponding plasma flow. Perfusion pressure was the difference between mean arterial (MAP) and central venous pressures. For systemic O_2 delivery, Q was multiplied by the arterial O_2 content whereas systemic O_2 extraction was the ratio between the systemic a–v O_2 difference and the arterial O_2 content. Blood gases and haemoglobin concentration were measured using an ABL700 analyser (Radiometer, Copenhagen, Denmark). Erythrocyte and reticulocyte parameters were analysed using an automatic haematology system, which performs flow cytometric measurements (ADVIA 120, Haematology System; Bayer Diagnostics, Tarrytown, NY, USA). Quadriceps femoris muscle mass was estimated using the antroprometric method (Andersen & Saltin, 1985), while blood volume was measured using the CO inhalation method (Burge & Skinner, 1995).

ATP in plasma was determined with the luciferin–luciferase technique (Lundin, 2000), using a luminometer with two automatic injectors (Orion Microplate Luminometer, Berthold Detection System GmbH, Pforzheim, Germany). Blood samples (2.7 ml) for determination of plasma ATP were obtained using syringes containing EDTA (S-monovette, 2.7 ml KE; Sarstedt, Nümbrecht, Germany) and were centrifuged immediately for 30 s at 14 000 r.p.m (18000 g) (4°C; Sigma, 1–15 K). Plasma was then pipetted into pre-chilled tubes, frozen in dry ice and stored for later analysis. Plasma ATP was measured in duplicates at room temperature (∼20°C) using an ATP kit (ATP Kit SL 144-041; BioTherma AB, Dalarö, Sweden) with an internal ATP standard procedure. Plasma haemoglobin was also analysed spectrophotometrically to determine if haemolysis had occurred during the handling of the samples (Cripps, 1968). Plasma noradrenaline and adrenaline concentration were determined with high performance liquid chromatography with electrochemical detection.

Statistical analysis

A one-way repeated measures analysis of variance (ANOVA) was performed to test significance between treatments. Following a significant F test, pair-wise differences were identified using Tukey's honestly significant difference (HSD) *post hoc* procedure. When appropriate, significant differences were also identified using Student's paired t tests. The significance level was set at $P < 0.05$. Data are presented as means ± S.E.M.

Results

Erythrocyte count and blood oxygenation

Compared with baseline values (control 1), the withdrawal of 1.3 ± 0.2 l of blood and the subsequent infusion of the same volume of a plasma volume expander reduced

Table 1. Haematological responses after blood withdrawal and reinfusion

Variables	Control 1	Anaemia	Anaemia + PVX	Control 2	Polycythaemia Acute	Polycythaemia Day 1	Polycythaemia Day 2
RBC count ($\times 10^6$ cells ml^{-1})	4.84 ± 0.08	4.29 ± 0.13*	3.39 ± 0.09*	4.64 ± 0.07	5.04 ± 0.10†	5.23 ± 0.07*†	5.03 ± 0.03†
Hct (%)	42.9 ± 0.6	38.0 ± 0.9*	30.1 ± 1*	41.9 ± 0.5	45.4 ± 0.8†	47.3 ± 0.5*†	45.4 ± 0.8†
Hb (g l^{-1})	147 ± 2	131 ± 3*	103 ± 3*	143 ± 2	153 ± 2†	160 ± 2*†	154 ± 3†
MCHC (g l^{-1})	343 ± 1	344 ± 1	346 ± 2	340 ± 3	338 ± 3	338 ± 2	341 ± 2
MCH (pg)	30.5 ± 0.3	30.5 ± 0.3	30.7 ± 0.3	30.7 ± 0.4	30.5 ± 0.3	30.6 ± 0.4	30.7 ± 0.4
MCV (fl)	88.7 ± 1.0	88.8 ± 1.0	89.0 ± 1.0	90.3 ± 0.9*	90.2 ± 0.9*	90.3 ± 1.0*	89.8 ± 0.9*
Reticulocytes count ($\times 10^9$ cells ml^{-1})	53 ± 3	50 ± 3	40 ± 3	59 ± 4	63 ± 4	59 ± 4	54 ± 3
Reticulocytes (%)	1.1 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	1.3 ± 0.1	1.3 ± 0.1	1.1 ± 0.1	1.1 ± 0.1

Values are means ± S.E.M. for 10 subjects. PVX, plasma volume expansion; RBC, red blood cell; Hct, haemotocrit, Hb, total haemoglobin concentration; MCHC, mean corpuscular haemoglobin concentration; MCH, mean cell haemoglobin; MCV, mean cell volume.% reticulocyte, percentage of total RBC count. Control 2, before packed RBC reinfusion 4 weeks after control 1. *Significantly different from control 1, $P < 0.05$. †Significantly different from control 2, $P < 0.05$.

RBC count by 11% and 30%, respectively (Table 1). Four weeks after the anaemic studies (control 2), RBC count was restored to baseline values ($P = 0.19$). Compared with control 2, the reinfusion of 0.84 ± 0.16 l of packed RBCs increased RBC count by 9, 13 and 8% at day 0, 1 and 2, respectively. Based on the measured blood volume, RBC volume increased from 2.94 ± 0.11 l at baseline to 3.36 ± 0.19 l after RBC reinfusion, whereas plasma volume increased from 3.45 ± 0.14 to 3.67 ± 0.20 l (both $P < 0.05$). In all experimental conditions, mean corpuscular haemoglobin concentration (MCHC) and mean cell haemoglobin (MCH) were similar, indicating that the changes in total Hb among treatments reflected only changes in RBC count. Due to independent changes in total Hb, or combined changes in Hb, with oxyhaemoglobin (O_2Hb) and P_{O_2}, arterial O_2 content declined by 10, 27 and 41% with anaemia, anaemia + PVX and anaemia + PVX + hypoxia, but increased by 7 to 11% with polycythaemia and polycythaemia + hyperoxia, respectively (Table 2).

Leg and systemic haemodynamics

Neither anaemia nor polycythaemia altered LBF or Q at rest or during exercise (Figs 1 and 2). However, the combination of anaemia + PVX + hypoxia increased LBF and Q to levels observed during maximal knee-extensor exercise (i.e. ∼8 and ∼16 l min^{-1} for LBF and Q, respectively). In contrast, the combination of polycythaemia + hyperoxia tended to reduce LBF and Q ($P = 0.07$–0.13). MAP and perfusion pressure remained stable in all conditions or changed only slightly indicating that increases in LBF were due to vasodilatation and the reductions to vasoconstriction. At rest and during exercise, LBF and vascular conductance were closely related to changes in leg a–v O_2 difference and arterial O_2 content ($r^2 = 0.89$–0.93; $P < 0.001$) and to a lesser extent to arterial [Hb], arterial RBC count, femoral venous O_2 content and femoral venous P_{O_2} (Figs 1 and 3). Q was also tightly correlated to systemic a–v O_2 difference and arterial O_2 content ($r^2 = 0.86$–0.96; $P < 0.001$). At rest and during exercise, leg and systemic RBC flow varied within a small range in all conditions despite the large changes in LBF and Q (Fig. 4). This reflected the maintenance of O_2 delivery to the exercising quadriceps muscles and the intimate relationship between LBF and Q *versus* arterial O_2 content. Leg and systemic $\dot{V}_{O_2}$ remained unchanged at rest and during exercise in all experimental conditions (Figs 1 and 2).

During exercise, heart rate was 99 ± 3 beats min^{-1} in the control, anaemia and anaemia + PVX, increased to 128 ± 2 beats min^{-1} with anaemia + PVX + hypoxia, but was attenuated to 93 ± 3 and 87 ± 2 beats min^{-1} with polycythaemia and polycythaemia + hyperoxia (Fig. 2). In contrast, stroke volume was higher than control only during anaemia + PVX (124 ± 5 *versus* 139 ± 6 ml beat^{-1}, respectively; $P < 0.05$). Changes in Q were therefore largely due to changes in heart rate. Central venous pressure increased from 6 ± 2 mmHg in control to 11 ± 1 mmHg during anaemia + PVX and anaemia + PVX + hypoxia, decreasing to ∼2 mmHg during all polycythaemia trials (Fig. 2). Stroke volume and central venous pressure were unrelated ($r^2 = 0.21$; $P = 0.300$). Plasma noradrenaline was elevated with anaemia and anaemia + PVX + hypoxia whereas plasma ATP remained unchanged (Table 2).

Discussion

This study manipulated RBC number in isolation and in combination with acute changes in the oxygenation state of Hb to gain insight into the role of the erythrocyte in the regulation of skeletal muscle and systemic circulations. The major observations are: (1) LBF or Q did not change with small changes in RBC count evoked by either anaemia or

Table 2. Blood parameters during submaximal knee-extensor exercise with control, anaemia, anaemia combined with plasma volume expansion, anaemia superimposed with plasma volume expansion and hypoxia and polycythaemia combined with either hypoxia or hyperoxia

Variables	Control	Anaemia	Anaemia + PVX	Anaemia + PVX + hypoxia	Polycythaemia	Polycythaemia + hyperoxia	Polycythaemia + hypoxia
Haemoglobin (g l^{-1})							
a	153 ± 5	137 ± 3	109 ± 4*	119 ± 3*	161 ± 4	158 ± 4	160 ± 4
v	153 ± 4	140 ± 5	115 ± 3*	117 ± 5*	164 ± 3	162 ± 3	169 ± 3
O_2 saturation (%)							
a	98.4 ± 0.2	98.5 ± 0.1	98.9 ± 0.2	75.6 ± 2.7*	98.4 ± 0.1	100.0 ± 0.0	73.4 ± 1.7*
v	30.9 ± 2.0	25.9 ± 2.3	29.7 ± 4.1	20.7 ± 3.0*	30.2 ± 1.7	36.3 ± 2.2	19.3 ± 1.1*
P_{O_2} (mmHg)							
a	107 ± 2	108 ± 2	116 ± 3	40 ± 2*	104 ± 1	507 ± 19*	38 ± 1*
v	22 ± 1	20 ± 1	22 ± 1	17 ± 1*	21 ± 1	24 ± 1	16 ± 1*
O_2 content (ml l^{-1})							
a	204 ± 7	184 ± 4	148 ± 6*	121 ± 5*	216 ± 5	227 ± 6*	158 ± 6*
v	64 ± 5	49 ± 5*	47 ± 7*	33 ± 5*	67 ± 4	79 ± 4*	44 ± 3*
P_{CO_2} (mmHg)							
a	38 ± 1	39 ± 1	39 ± 1	32 ± 1*	39 ± 1	40 ± 1	35 ± 1*
v	56 ± 1	61 ± 1*	58 ± 2	48 ± 3*	58 ± 1	60 ± 1	52 ± 2
pH							
a	7.39 ± 0.01	7.38 ± 0.01	7.37 ± 0.01	7.44 ± 0.01*	7.38 ± 0.01	7.39 ± 0.01	7.43 ± 0.01*
v	7.31 ± 0.01	7.28 ± 0.02	7.27 ± 0.02*	7.32 ± 0.02	7.30 ± 0.01	7.29 ± 0.01	7.33 ± 0.01
Temperature (°C)							
v	37.2 ± 0.1	37.2 ± 0.1	37.0 ± 0.1	37.1 ± 0.1	37.1 ± 0.1	37.1 ± 0.1	37.0 ± 0.1
Noradrenaline (nmol l^{-1})							
a	1.6 ± 0.1	2.5 ± 0.4*	1.5 ± 0.1	2.7 ± 0.3*	1.1 ± 0.2	0.9 ± 0.1	1.3 ± 0.1
v	1.7 ± 0.2	2.6 ± 0.3	1.7 ± 0.1	2.7 ± 0.3	1.2 ± 0.2	1.0 ± 0.1	1.8 ± 0.1
Adrenaline (nmol l^{-1})							
a	0.7 ± 0.1	0.8 ± 0.1	0.6 ± 0.1	1.0 ± 0.2	0.5 ± 0.1	0.4 ± 0.1*	0.8 ± 0.2
v	0.6 ± 0.1	0.7 ± 0.1	0.5 ± 0.1	0.8 ± 0.1	0.4 ± 0.1	0.3 ± 0.1*	0.5 ± 0.2
ATP (nmol l^{-1})							
a	545 ± 66	496 ± 53	425 ± 53	557 ± 45	553 ± 95	518 ± 78	654 ± 40
v	551 ± 81	585 ± 95	534 ± 135	475 ± 94	547 ± 156	514 ± 69	538 ± 86

Values are means ± S.E.M. for 10 subjects. a, femoral artery; v, femoral vein; PVX, plasma volume expansion.*Significantly different from control, $P < 0.05$.

polycythaemia alone, but increased with a large reduction in RBC count evoked by the combination of anaemia + PVX; (2) LBF and Q increased with anaemia + PVX + hypoxia to values observed during maximal exercise, despite exercise intensity eliciting only 50% of peak power; (3) LBF, Q and vascular conductance were tightly correlated to a–v O_2 difference, suggesting an important role of blood O_2 gradients in muscle and systemic circulatory control. Collectively, these results suggest that, when coping with severe haematological challenges, local regulation of human skeletal muscle blood flow and O_2 delivery primarily senses alterations in the oxygenation state of Hb and, to a lesser extent, changes in the total number of RBCs and Hb molecules.

An unexpected finding was that anaemia and polycythaemia alone did not change LBF or Q either at rest or during exercise. Adjustments in O_2 extraction, rather than in blood flow, afforded the maintenance of leg and systemic $\dot{V}_{O_2}$. Studies in anaemic and polycythaemic patients as in animals show that Q is drastically altered with large changes in RBC count and thus total [Hb] (Richardson & Guyton, 1959; Roy *et al.* 1963; Ekblom *et al.* 1972; Gustafsson *et al.* 1980; Lindendeld *et al.* 1985; Krantz *et al.* 2005). For instance, an extremely high resting Q of ∼15 l min^{-1} was found in 10 chronic anaemic patients with a critically low [Hb] of 35 g l^{-1}. Remarkably, Q fell to ∼7 l min^{-1} when [Hb] increased to 108 g l^{-1} after blood transfusion (Roy *et al.* 1963). On the other hand, normovolaemic polycythaemia, at rest and during exercise in dogs, reduces Q in a linear fashion as haematocrit increases to 65% (Richardson & Guyton, 1959; Lindendeld *et al.* 1985). In the present study, acute anaemia was induced by withdrawal

of 20% of the subjects' blood volume, which resulted in a smaller decrease in RBC count (∼11%) due to the ensuing plasma volume expansion. Similarly, the autologous reinfusion of 0.84 l of packed RBC, expected to evoke a ∼20% increase in RBC count, only increased RBC count by ∼9% as plasma volume was also augmented. These modest changes in RBC count were inadequate stimuli to change local and systemic blood flow either at rest or during exercise. In contrast, the 30% drop in RBC count with combined anaemia + PVX was associated

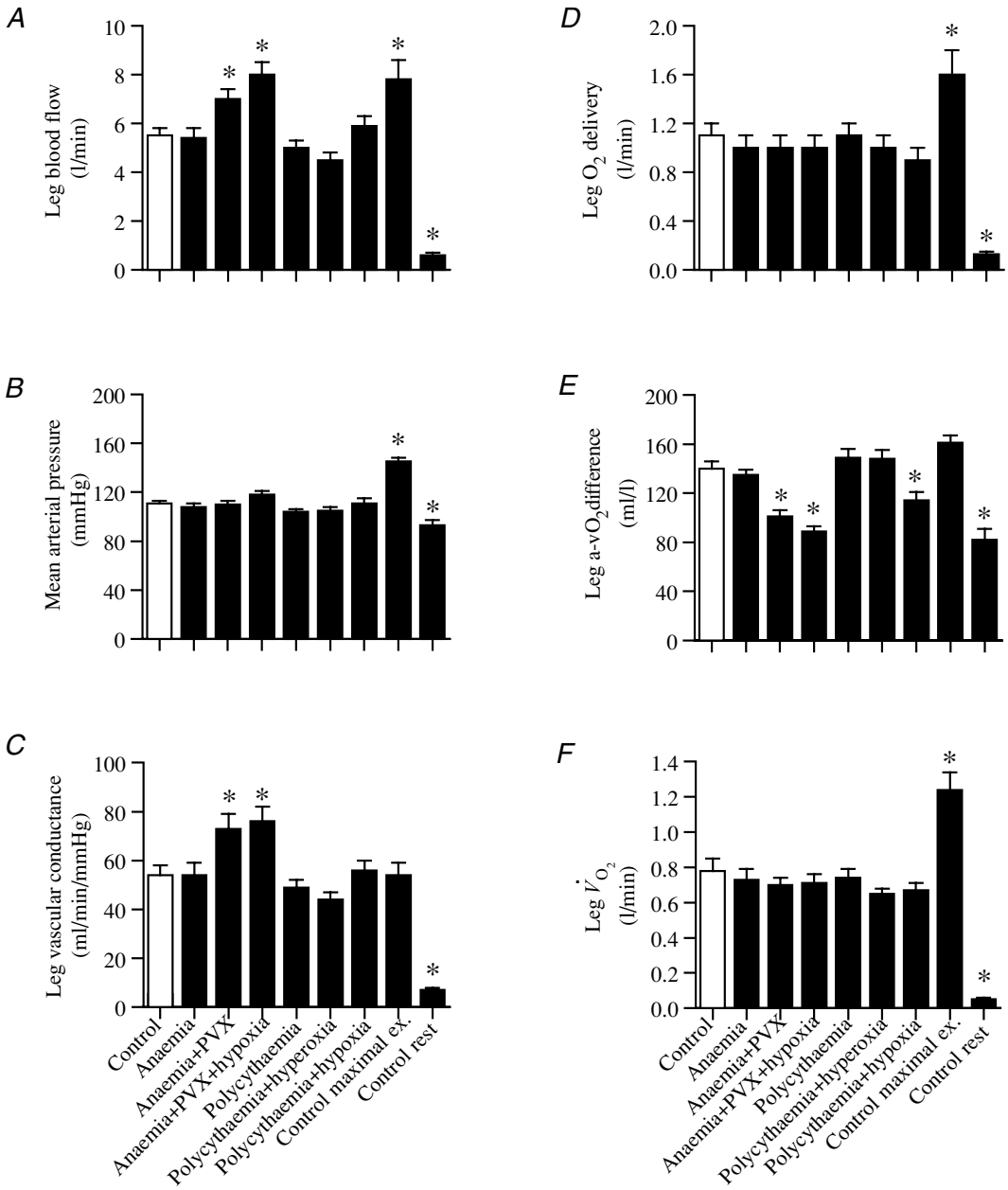

Figure 1. Leg haemodynamics with alterations in RBC count and blood oxygenation
Leg haemodynamics and oxygenation during submaximal one-legged knee-extensor exercise (52–53 W (± 5 W)) in normocythaemia (control), anaemia, anaemia combined with plasma volume expansion (anaemia + PVX), anaemia combined 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. *A*, leg blood flow; *B*, mean arterial pressure; *C*, leg vascular conductance; *D*, leg O_2 delivery; *E*, leg a–v O_2 difference; *F*, leg $\dot{V}_{O_2}$. Data are means ± S.E.M. for 9 subjects. *Significantly different from control, $P < 0.05$.

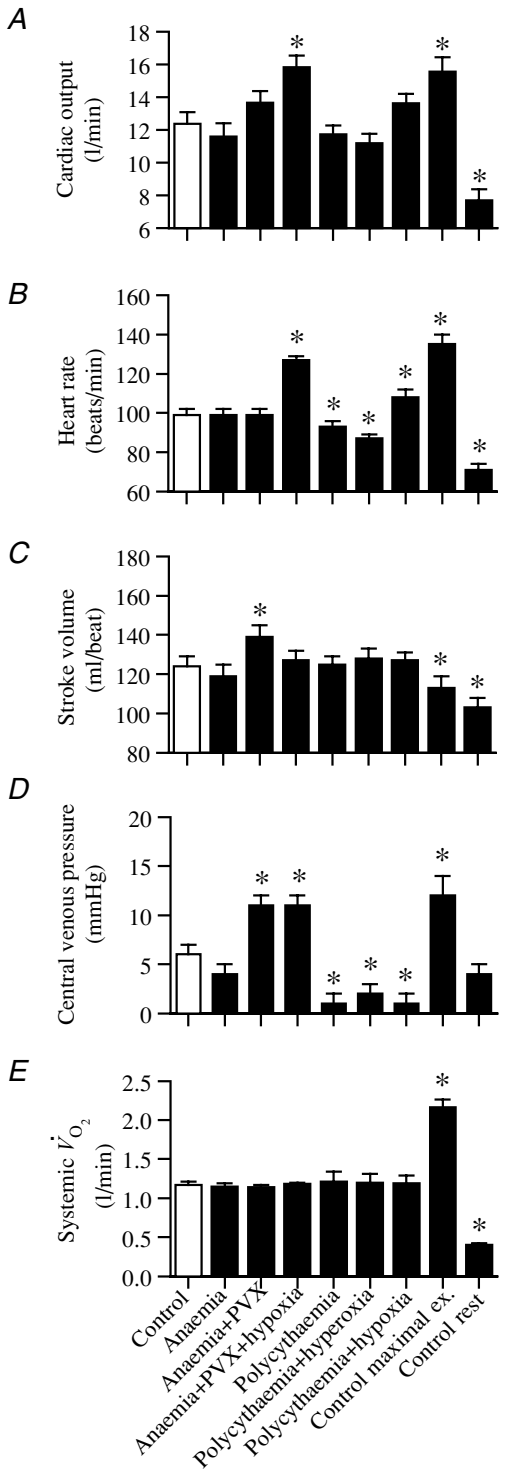

Figure 2. Systemic haemodynamics with alterations in RBC count and blood oxygenation
Systemic haemodynamics and $\dot{V}_{O_2}$ during submaximal one-legged knee-extensor exercise (52–53 W (± 5 W)) in normocythaemia (control), anaemia, anaemia + PVX, anaemia + PVX + hypoxia, polycythaemia, polycythaemia + hyperoxia and 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. *A*, cardiac output; *B*, heart rate; *C*, stroke volume; *D*, central venous pressure; *E*, systemic $\dot{V}_{O_2}$. Data are means ± S.E.M. for 8–10 subjects. *Significantly different from control, $P < 0.05$.

with a 27% (i.e. 1.5 l min^{-1}) elevation in LBF and a corresponding increase in *Q*, allowing the maintenance of O_2 supply (Koskolou *et al.* 1997). Therefore, the enhanced exercising muscle blood flow reported in humans with 'isovolaemic' anaemia is owing to the haemodilution and concomitant reduction in blood O_2 content caused by volume replacement, rather than the acute reduction in RBC count (Koskolou *et al.* 1997). A threshold for the circulatory response to changes in erythrocyte content appears to exist, whereby only alterations in RBC count larger than ∼10% are compensated for by a change in blood flow.

Compelling evidence in both humans and animals demonstrates that pronounced alterations in blood O_2 content with hypoxia and hyperoxia are associated with inverse changes in limb muscle blood flow and *Q*, thereby maintaining a constant systemic and contracting muscle O_2 supply (Rowell *et al.* 1986; Welch *et al.* 1997; Roach *et al.* 1999; González-Alonso *et al.* 2001, 2002). In agreement with reports using knee-extensor exercise, we found that the superimposition of hypoxia (F_{IO_2} 10%) onto anaemia or polycythaemia increased LBF by ∼20% (i.e. 1 l min^{-1}), whereas the superimposition of hyperoxia onto polycythaemia reduced LBF by ∼10% (Rowell *et al.* 1986; Richardson *et al.* 1995; Roach *et al.* 1999; González-Alonso *et al.* 2001, 2002). The *Q* response mirrored the haemodynamic response of the exercising muscles. Hence, the effects of altered blood oxygenation on exercising muscle blood flow are similar when examined independently or combined with mild changes in RBC count. Importantly, regression analysis of the haemodynamic data from the present seven experimental conditions revealed an intimate correlation between LBF and *Q* *versus* both arterial O_2 content and a–v O_2 difference ($r^2 = 0.91$–0.93), reflecting the unchanged O_2 delivery and $\dot{V}_{O_2}$ across all conditions. Compatible with a favoured O_2 delivery control, leg RBC flow only varied from 2.1 to 2.9 l min^{-1}, despite LBF ranging from 4.5 to 8.0 l min^{-1} (Fig. 4), with most of the difference in leg RBC flux explained by the different O_2Hb. This indicates that O_2 availability in the erythrocyte and large changes in RBC count are sensed and that O_2 delivery, rather than blood flow, is the controlled variable producing exercise hyperaemia.

RBC and particularly Hb have been postulated to function as O_2 sensors and controllers of local blood flow and O_2 delivery, by releasing ATP and SNO-Hb in proportion to the offloading of O_2 from Hb (Ellsworth *et al.* 1995; Stamler *et al.* 1997; González-Alonso *et al.*

2002). ATP and SNO-Hb will then act on the vascular endothelium and initiate a conducted vasodilator response upstream (Ellsworth, 2004; Singel & Stamler, 2005). Indeed, the Hb molecule is the main locus of vascular O_2 sensing in the light of the observation that it is O_2Hb rather than P_{O_2} that determines muscle blood flow (see Fig. 3*F*; Roach *et al.* 1999; González-Alonso *et al.* 2001, 2002; Hanada *et al.* 2003). The present design affords the possibility to independently separate the haemodynamic effects of total number of Hb molecules (and/or RBC number) from those of the oxygenation state of Hb. It is worth noting that the variations in total [Hb] seen here only reflect changes in the total number of erythrocytes, as mean cell haemoglobin remained constant at ~30.5 pg in all interventions. The similar exercising LBF when RBC count ranged from 4.48×10^6 cells ml^{-1} with anaemia to 5.32×10^6 cells ml^{-1} with polycythaemia, and the markedly elevated LBF when RBC count was 3.59×10^6 cells ml^{-1} with anaemia + PVX, suggest that the regulation of the muscle circulation is sensitive only

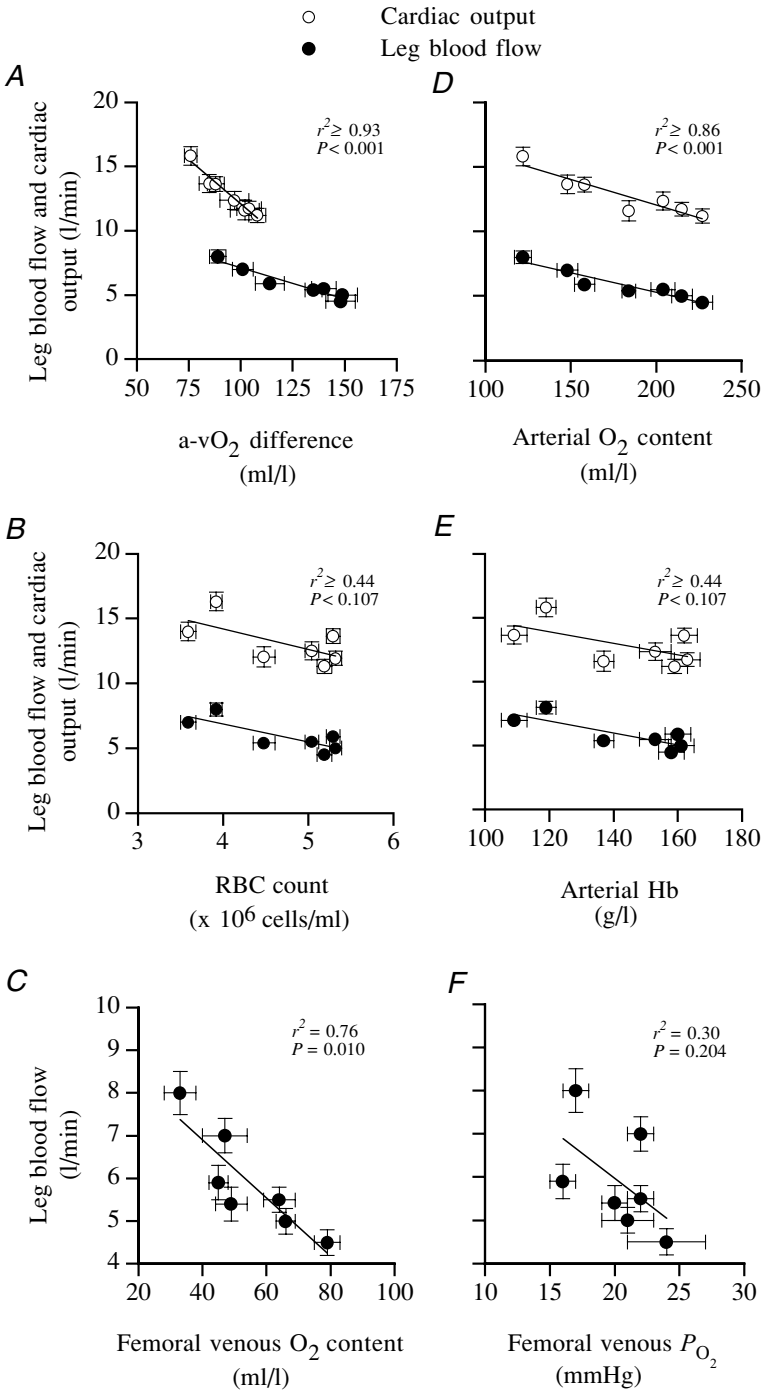

Figure 3. Blood flow and blood O_2
Relationships between leg and systemic blood flow *versus* a–v O_2 difference and arterial O_2 and Hb contents. Note the tight inverse relationships between leg and systemic blood flow *versus* a–vO_2 difference and arterial O_2 content, but the weak relationship with total Hb and RBC. *A*, leg blood flow and cardiac output *versus* a-v O_2 difference; *B*, leg blood flow and cardiac output *versus* RBC count; *C*, leg blood flow *versus* femoral venous O_2 content; *D*, leg blood flow and cardiac output *versus* arterial O_2 content; *E*, leg blood flow and cardiac output *versus* arterial Hb; *F*, leg blood flow *versus* femoral venous P_{O_2}. Data are means ± S.E.M. for 9 subjects. *Significantly different from control, $P < 0.05$.

to large changes in RBC count and/or total number of Hb molecules (Fig. 3). This contrasts with the linear increase in LBF with progressive reductions in O_2Hb with graded carbon monoxide hypoxaemia (González-Alonso *et al.* 2001). These results collectively suggest a greater sensitivity of the skeletal muscle circulatory control system to perturbations in O_2Hb than to total number of RBCs and Hb molecules.

A striking finding was that combined changes in RBC count and blood oxygenation evoked an ~2-fold change in quadriceps muscle blood flow, with muscle blood flow and Q reaching peak exercise levels with exposure to anaemia + PVX + hypoxia, despite the unchanged metabolic energy demand (50% quadriceps $\dot{V}_{O_2,peak}$). Perfusion pressure was also stable suggesting that the increase in blood flow was due to vasodilatation (Fig. 1). Knee-extensor exercise mainly engages the quadriceps muscles, as the momentum inflected in the flywheel allows for a passive movement and thereby a stable metabolism of the knee-flexors muscles (Andersen & Saltin, 1985; González-Alonso *et al.* 2000). Since $\dot{V}_{O_2}$ was the same in all conditions, the differences in LBF and/or leg O_2 extraction observed during exercise were confined to the contracting quadriceps muscles. The quadriceps muscle mass of the subjects was 2.9 kg. If we assumed that one-half of the quadriceps muscle fibres are recruited when working at 50% of the peak

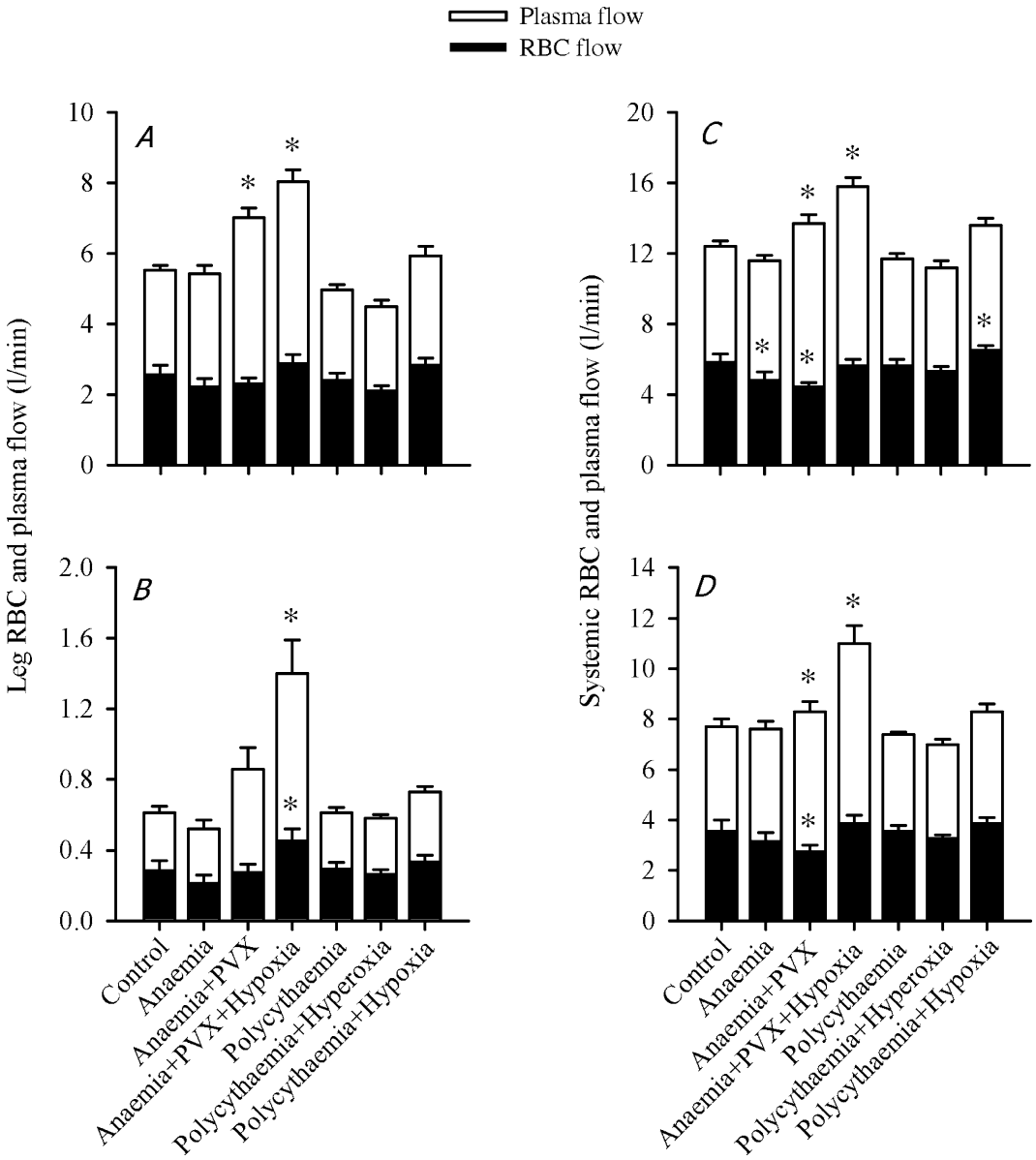

Figure 4. Partition of leg and systemic blood flow into RBC and plasma flows
Leg RBC and plasma flows during exercise (*A*) and at rest (*B*) and systemic RBC and plasma flows during exercise (*C*) and at rest (*D*) in the control, anaemia, anaemia + PVX, anaemia + PVX + hypoxia, polycythaemia + hyperoxia and polycythaemia + hypoxia conditions. Data are means ± S.E.M. for 9 subjects. *Significantly different from control, $P < 0.05$.

$\dot{V}_{O_2}$ (Ray & Dudley, 1998), the 6.9 l min^{-1} difference in LBF between the exercise and resting conditions with exposure to anaemia + PVX + hypoxia, would represent a peak quadriceps muscle perfusion of 4.6 l min^{-1} kg^{-1}, the highest value reported (Andersen & Saltin, 1985; Richardson *et al.* 1993). In sharp contrast, the estimate of quadriceps muscle perfusion during polycythaemia + hyperoxia is 2.6 l min^{-1} kg^{-1}. This difference in muscle perfusion with varied blood O_2 content but clamped muscle metabolic demand supports a pivotal role of the erythrocyte in the local control of muscle perfusion and O_2 delivery.

Skeletal muscle blood flow regulation reflects the interplay of neural sympathetic vasoconstrictor activity, myogenic factors and locally derived vasoactive substances, which ensure the optimal muscle nutritive supply, especially O_2, during the majority of physical activities in humans (Laughlin *et al.* 1996). With interventions such as those used here, the contribution vasodilatation and vasoconstriction to blood flow regulation change (Dinenno & Joyner, 2003; Hanada *et al.* 2003; Rosenmeier *et al.* 2004; Coney *et al.* 2004). The herein parallel changes in LBF and vascular conductance to altered RBC and blood oxygenation during moderate exercise indicate that the increases in LBF were due to net vasodilatation and the reductions to net vasoconstriction. Moreover, the blood noradrenaline data suggest that sympathetic vasoconstrictor activity was augmented with anaemia and anaemia + PVX + hypoxia, but was reduced with polycythaemia and polycythaemia + hyperoxia. Therefore, the unchanged LBF with anaemia and the increased hyperaemia with anaemia + PVX + hypoxia must have resulted from an elevation in vasodilator activity, offsetting or overriding the augmented sympathetic vasoconstrictor activity. We hypothesized that plasma ATP would reflect differences in O_2Hb and vasodilator activity based on the observation that plasma ATP and skeletal and cardiac muscle vascular conductance increase during incremental exercise in humans and dogs (González-Alonso *et al.* 2002; Farias *et al.* 2005). The potent vasodilatory and sympatholytic effects of ATP in the leg make it an attractive candidate to maintain or increase muscle blood flow in conditions that increase sympathetic vasoconstrictor activity such as anaemia and hypoxia (González-Alonso *et al.* 2002; Rosenmeier *et al.* 2004). However, the herein unchanged plasma ATP does not seem to support an involvement of ATP released from RBCs in blood flow regulation.

The level of circulating ATP represents a net balance between ATP release and inactivation by vascular endothelial (Marcus *et al.* 2003) and serum soluble nucleotide-hydrolysing enzymes (Yegutkin *et al.* 2003), rendering the measure of plasma [ATP] in femoral vein as a rough index of ATP release from RBCs in the active quadriceps microcirculation. In support of this, we have recently observed that plasma ATP and serum nucleoside triphosphate-diphosphohydrolase (ATP/ADP-hydrolysing enzyme) remain unchanged under conditions of pharmacological ATP-induced vasodilatation in resting subjects, but increase during strenuous cycling exercise (G.G. Yegutkin, S.S. Samburki, S. P. Mortensen, S. Jalkanen & J. González-Alonso, unpublished observation). The unchanged plasma ATP in the femoral vein might then represent the prevalence of nucleotide-inactivating mechanisms over nucleotide release. Studies directly measuring plasma ATP turnover in the muscle microcirculation are therefore required to elucidate whether ATP released from the erythrocyte is indeed a mediator of O_2 sensing transduction between the erythrocyte and the vascular endothelium.

In conclusion, this study provides evidence that mild anaemia and polycythaemia do not alter skeletal muscle or systemic blood flow either at rest or during exercise in healthy humans. However, when combined with volume replacement and altered blood oxygenation, anaemia and polycythaemia drastically change resting and exercising muscle blood flow and cardiac output. When encompassing all the submaximal exercise conditions, muscle perfusion and vascular conductance are tightly related to a–v O_2 difference, suggesting an important role of blood O_2 gradients in muscle microcirculatory control. Our results may have implications for the understanding of the blood flow regulation of patients and normal people exposed to haemorrhage and dehydration *versus* transfusion therapy and rehydration.

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