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Erythrocyte Dependent Regulation of Human Skeletal Muscle Blood Flow: Role of Varied Oxyhemoglobin and Exercise on Nitrite, S-Nitrosohemoglobin and ATP

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ABSTRACT

The erythrocyte is proposed to play a key role in the control of local tissue perfusion via three O_2-dependent signaling mechanisms: 1) reduction of circulating nitrite to vasoactive NO, 2), S-nitrosohemoglobin (SNO-Hb)-dependent vasodilatation and 3) release of the vasodilator and sympatholytic ATP; however, their relative roles *in vivo* remain unclear. Here we evaluated each mechanism to gain insight into their roles in the regulation of human skeletal muscle blood flow during hypoxia and hyperoxia at rest and during exercise. Arterial and femoral venous hemoglobin O_2 saturation (O_2Hb), plasma and erythrocyte NO and ATP metabolites and leg and systemic hemodynamics were measured in 10 healthy males exposed to graded hypoxia, normoxia and graded hyperoxia both at rest and during submaximal one-legged knee-extensor exercise. At rest, leg blood flow and NO and ATP metabolites in plasma and erythrocytes remained unchanged despite large alterations in O_2Hb. During exercise, however, leg and systemic perfusion and vascular conductance increased in direct proportion to decreases in arterial and venous O_2Hb (r^2=0.86-0.98; P=0.01), decreases in venous plasma nitrite (r^2=0.93; P<0.01), increases in venous erythrocyte nitroso species (XNO) (r^2=074; P<0.05) and to lesser extent increases in erythrocyte SNO (r^2=059; P=0.07). No relationship was observed with plasma ATP (r^2=0.01; P=0.99) or its degradation compounds. These *in vivo* data indicate that during low intensity exercise and hypoxic stress, but not hypoxic stress alone, plasma nitrite consumption and formation of erythrocyte nitroso species is associated with limb vasodilatation and increased blood flow in the human skeletal muscle vasculature.

Key Words: Blood flow control, Erythrocyte signaling, Exercise

Introduction

The erythrocyte has recently been identified as a key player in the regulation of the local processes matching O_2 delivery to tissue aerobic energy demand in vessel preparations *in vitro* (14, 58) and in animals (56, 57) and humans *in vivo* (25, 26). Evidence supports that the erythrocyte functions as an O_2 sensor and controller of local tissue blood flow and O_2 delivery by releasing vasoactive signals in proportion to erythrocyte deoxygenation (14). However, the signaling mechanisms linking erythrocyte deoxygenation to an increased skeletal muscle perfusion are not well understood. The current hypotheses of erythrocyte control of local tissue blood flow including skeletal muscle propose three O_2-dependent signaling pathways: 1) the signal transduction pathway proposed to stimulate ATP release from the erythrocyte (14), 2) the reduction of nitrite to NO by deoxyhemoglobin (8), and 3) the formation of S-nitrosohemoglobin, SNO-Hb (45).

On the one hand, local blood flow might be controlled by deoxyhemoglobin dependent reduction of nitrite to NO (19) and/or erythrocyte O_2-dependent modulation of S-nitrosohemoglobin (SNO-Hb) bioavailability (40, 52). With respect to nitrite, recent studies suggest that T-state deoxyhemoglobin reduces nitrite to NO in a process that is 10 fold faster than R-state oxyhemoglobin, such that maximum NO production occurs at hemoglobin O_2 saturation close to 50%. NO production through this pathway is accelerated at lower pH and may involve intermediate reactive nitrogen species (2, 8, 10). With respect to SNO-Hb, formation of this species is proposed to occur in the lung circulation (52) where a nitroso group is bound to the highly conserved cysteine residue on the β-chain (β93) of Hb. As the erythrocyte deoxygenates in the microcirculation, undergoing allosteric conformational transition from the R-state to the T-state, the ensuing changes in SNO group reactivity is proposed to facilitate the transduction of NO-dependent vasodilatation signals out of the red

cell via transnitrosation reactions with other thiols (13, 45). On the other hand, alongside the modulation of NO bioavailability, erythrocyte deoxygenation is also thought to induce the release of ATP into the lumen (55) which then activates P2-purinergic receptors located on endothelial cells, rapidly triggering a conducted upstream vasodilator response (14). Intravascular ATP is an attractive mediator signal for skeletal muscle blood flow control because it not only can act as a potent vasodilator but also has sympatholytic properties, which are required to maintain or increase perfusion in conditions of augmented sympathetic nerve activity such as hypoxia and exercise (28, 33, 49, 50, 66).

To date the functional relevance of these three individual erythrocyte-dependent signaling pathways and their possible interaction (4) in the regulation of limb muscle blood flow in humans is unknown, particularly in conditions of graded hypoxia, hyperoxia and exercise. Therefore, we performed simultaneous measurements of erythrocyte nitroso species including S-nitrosothiols (SNO), Hg-resistant nitroso species (XNO), nitrite and ATP to gain insight into their relative roles in the regulation of basal and exercising blood flow at different erythrocyte O_2 saturations. We hypothesized that leg and systemic perfusion is closely linked to the interaction between changes in erythrocyte oxygenation and changes in erythrocyte dependent NO and ATP vasodilator activity both during hypoxia and exercise.

Methods

Subjects

Ten healthy recreationally active male subjects with a mean ± S.D. age of 24 ± 2 yr, body weight of 80 ± 2 kg and height of 179 ± 2 cm participated in this study. 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 Brunel University Research Ethics Committee.

Experimental design

The subjects visited the laboratory on 5 different occasions within a two week period to perform preliminary testing and the main experiment. During the first 3 visits, the subjects became familiarized with the one-legged knee-extensor ergometer and were exposed to graded levels of normobaric hypoxia and hyperoxia. The fourth visit consisted of a maximal knee-extensor incremental test to determine the subjects' individual peak power. On the experimental day, the subjects completed six trials comprising 10-min of rest followed by 6-min of submaximal one-legged knee-extensor exercise in the semi-supine position at 22 ± 1 W and ~60 r.p.m. Each trial was performed under the following conditions: (a) severe hypoxia (inspiratory oxygen fraction; F_i,O_2, 0.10); (b) moderate hypoxia (F_i,O_2, 0.13); (c) mild hypoxia (F_i,O_2, 0.16); (d) normoxia (F_i,O_2, 0.21); (e) moderate hyperoxia (F_i,O_2, 0.48); (f) severe hyperoxia (F_i,O_2, 1.00). The order of the six experimental trials was randomized across subjects and a recovery period of ~30 min in normoxia was allowed between trials.

Instrumentation of subjects

Subjects reported to the laboratory at 7:30 a.m., following the ingestion of a light breakfast. Upon arrival to the laboratory, the subjects rested in a supine position while two catheters were inserted using the Seldinger technique under local anesthesia (1% lidocaine), one into the radial artery of the right arm and the other into the femoral vein of the left leg (experimental leg). The femoral vein catheter was positioned ~2 cm distal from the inguinal ligament. Then, the subjects walked to the experimental room were electrodes to measure the electrocardiogram were positioned on their chest. Following ~15 min of semi-supine rest on the knee-extensor ergometer, the subjects were exposed to the first experimental condition for

10-min of rest followed by a 6-min exercise bout while continuously breathing the given FiO_2. During all trials, the subjects breathed through a three-way valve (Hans Rudolph T shape, Cranlea, UK) connected to a 150 L Douglas bag in which the composition of the inspired gas was manually adjusted using gas regulators connected to tanks of the corresponding O_2 concentration (BOC gases, Guildford, UK). All subjects tolerated well the different levels of hypoxia and hyperoxia, showing minimal signs of discomfort. The experiment was performed in a close to thermoneutral and isobaric environment.

Leg and systemic hemodynamics and oxygenation

Heart rate was obtained from the ECG signal whereas intravascular pressures were measured in the radial artery and the femoral vein with transducers positioned at heart level (Edwards Life Science, Saint Prex, Switzerland) and connected to a blood pressure amplifier and a data acquisition system (Power Laboratory 16/30, ADInstruments, Cahlgrove, UK). Cardiac output ($\dot{Q}$) was calculated by multiplying stroke volume by heart rate, using the modelflow method to determine stroke volume from directly measured arterial pressure recordings (BeatScope 1.1a; Finapres Medical Systems BV, Amsterdam, the Netherlands) (3, 23). Systemic vascular conductance was calculated as the ratio between $\dot{Q}$ and mean arterial pressure (MAP). Anterograde, retrograde and net leg blood flow (LBF) were measured for ~1 min by ultrasound Doppler (Vivid 7, GE Medical Systems, Bedford, UK) (46) after 8 min of rest and after 4 min of exercise. Each LBF value represents the mean of 4 measurements performed over 12-s windows of continuous anterograde, retrograde and net blood velocity and arterial diameter measurements ($LBF_{net} = LBF_{anterograde} - LBF_{retrograde}$). Leg vascular conductance was established as the ratio between LBF and leg perfusion pressure. Leg perfusion pressure was the difference between MAP and femoral vein pressure. Anterograde, retrograde and net shear rate in the femoral artery were calculated using Poiseuille's equation: shear rate (s^{-1}) = (4 × mean blood velocity)/r, where r represents the radius of the

artery lumen (44, 61). Leg VO_2 was calculated by multiplying the net LBF by the difference in O_2 content between the radial artery and the femoral vein ($a–vO_2$) (23-26).

Blood collection and analyses

Immediately after resting and exercising LBF measurements, paired radial artery and femoral venous blood samples were collected simultaneously. All samples were processed for either immediate analysis or storage.

Blood gases

Blood gas variables, hemoglobin, glucose and lactate concentrations were measured in arterial and venous samples (1 ml) using an automated analyzer (ABL 825 M Flex, Radiometer, Denmark).

Blood nitric oxide metabolites

For the measurement of plasma nitrite and erythrocyte SNO and Hg-resistant nitroso species (XNO), blood (4 ml) was collected and immediately drawn into a first stabilizing solution (i.e., 400 µl, SS1 containing sodium citrate (1.5% w/v), N-ethylmaleimide (NEM, 5 mmol/L), and DTPA (100 µmol/L) in PBS; all concentrations refer to final). After gentle mixing, all blood samples were centrifuged at 3000 g for 180 s at 22 °C. Plasma aliquots (1.5 ml) were then pipetted into vials containing 200µl of a second stabilising solution (SS2 comprising NEM (1 mmol/L) and DTPA (100 µmol/L) in PBS), and erythrocytes aliquots (1.6 ml) were pipetted into vials containing 200 µl of a third stabilizing solution (SS3 comprising NEM (20 mmol/L), DTPA (100 µmol/L), potassium ferricyanide (10 mmol/L) and IGEPAL CA-630 (1% v/v)). Plasma and erythrocyte vials were mixed and left at room temperature for 30 to 60-s before freezing in liquid nitrogen. For NO-metabolite measurements, samples were thawed on ice in the dark and measured by reductive (I_3^-) chemiluminescence as previously described with a limit of detection of 10 nmol/L (65). We did not specifically separate high

and low Mwt components for these measurements, and hence refer to erythrocyte SNO to indicate total SNO.

Blood adenine nucleotides and adenosine and blood soluble nucleotide converting activities

In a subgroup of 7 subjects, plasma and whole-blood nucleotides (AMP, ADP, ATP) and whole-blood adenosine concentrations were determined from 2 ml arterial and venous blood samples. 1 ml was immediately frozen in liquid nitrogen for later determination of whole blood nucleotides concentrations. The remaining 1 ml was immediately pipetted into plasma separation tubes and centrifuged for 3 min at 4,000 g. Plasma was then pipetted into 0.5 ml vials and immediately frozen in liquid nitrogen. All samples were analyzed using high performance liquid chromatography with UV detection as described previously (53, 54). Briefly, blood samples were extracted using PCA on ice (whole-blood: 1.3 mol/L PCA, ratio 1:1; plasma: 2.4 mol/l PCA, ratio 1:0.25) and then centrifuged at 13,000 rpm for 3 min at 4 °C. Supernatant was collected and neutralized using 2 mol/L KOH (whole blood samples) or 3 mol/L K_3PO_4 (plasma samples) before being centrifuged again at 13,000 rpm for 3 min at 4 °C. Then, the samples were left on ice for 30 min before analysis. This method gives good recovery (>95%) and reproducibility (CV<10%), with values well above the detection limits of the method (>10 for ATP) (53, 54). Erythrocyte nucleotides concentrations were calculated as follows: [erythrocyte] = ([whole-blood]-[plasma])/hematocrit. In addition, plasma ATP was also determined with the luciferin–luciferase technique (37), using a luminometer with three automatic injectors (Orion Microplate Luminometer, Berthold Detection System GmbH, Pforzheim, Germany). In seven subjects, 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 14000 r.p.m

(18000 g) (4˚C; Sigma, 1–15 K) before measurement, 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. In three additional subjects, blood samples (1 ml) were drawn into a stop solution (1.7 ml) containing S-(4-nitrobenzyl)-6-thioinosine (NBTI; 5 nmol/l), 3-isobutyl-1-1methylxantine (IBMX; 100 μmol/l), forskolin (10 μmol/l), EDTA (4.15 mmol/l), NaCl (118 mmol/l), KCl (5 mmol/l) and Tricine buffer (40 mmol/l) (27). After centrifugation and separation, plasma ATP was measured with the luciferin–luciferase technique as described above. As an indicator of hemolysis, plasma Hb was measured spectrophotometrically (11) and samples with Hb > 1 mg/dl were excluded.

Statistical analysis

Two-way analysis of variance with repeated measures (ANOVA) was performed to test significant differences between treatments. Following a significant *F* test, pair-wise differences were identified using Tukey's *post hoc* test. Correlations between variables were established using Pearson product moment correlation and step-wise forward multiple regression analysis. The latter was used to test the strength of the association between LBF and $\dot{Q}$ as dependent variables and blood NO metabolites as independent variables. The significant level was set at $P<0.05$ and $P<0.10$ was considered as a tendency. Data are presented as mean±S.E.M. unless otherwise stated.

RESULTS

Blood oxygenation

Both at rest and during exercise, graded hypoxia progressively reduced normoxic arterial O_2 content by -5±1%, -10±2% and -22±2%, in mild, moderate and severe hypoxia, respectively, independently of any changes in Hb concentration (Table 1). This was largely the result of a gradual reduction in arterial hemoglobin O_2 saturation (-4±1%, -9±1% and -21±2%). On the other hand, moderate and severe hyperoxia gradually increased arterial O_2 content by +4±1% and +9±1%, respectively, both at rest and during exercise (Table 1) due solely to a ~1.6- and 4.8-fold elevation in PO_2, respectively. Exercise further decreased arterial O_2 content by -3±2% and -6±2% in moderate and severe hypoxia, respectively, mainly via reduction of arterial hemoglobin O_2 saturation. Similar directional changes were observed for venous O_2 content indicating that progressive changes in arterial and venous erythrocyte O_2 saturation were experimentally obtained between the two extreme conditions of severe hypoxia and severe hyperoxia both at rest and during exercise. As a direct consequence of changes in blood O_2 content, leg a-v O_2 difference was generally maintained with exposure to graded hypoxia and hyperoxia, except for severe hypoxia (31±7% and 23±2% lower than in normoxia at rest and during exercise respectively). No or minimal alterations were observed in arterial and venous MetHb and COHb (Table 1).

Plasma nucleotides and NO metabolites

Plasma Hb did not change across all interventions, indicating that no significant hemolysis occurred during the processing of the samples (mean plasma Hb = 0.69±0.03 mg/dl). Gradual hypoxia and hyperoxia did not significantly alter arterial or venous plasma ATP (either measured by HPLC or by luminometry without (n=7) or with the stop solution, n=3), ADP, AMP, SNO or nitrite at rest or during exercise (Table 2). Similarly, whole blood adenosine

concentration remained unchanged at rest and during exercise. However, in an analysis of arterial and venous samples drawn under all conditions, significant a-v gradients in plasma nitrite were present across the leg at rest and during exercise ($P<0.05$, Table 2) but not in plasma ATP or plasma SNO.

Erythrocyte nucleotides and NO metabolites

Arterial erythrocyte SNO and XNO did not change with graded hypoxia or graded hyperoxia. Similar observations also apply to arterial erythrocyte nucleotides with the exception of arterial ADP which decreased with severe hypoxia (Table 3). During exercise, venous erythrocyte SNO, XNO and SNO+XNO were significantly or tended to be elevated with severe hypoxia compared to normoxia (+160±77%, $P=0.091$; +39±19%, $P<0.05$; +54±21%, $P<0.01$). Severe hypoxic exercise increased venous SNO (+230±102%, $P<0.05$), XNO (+70±24%, $P<0.001$) and SNO+XNO (+72±19%, $P<0.001$) above resting values. No changes in arterial SNO or XNO were observed. In contrast, severe hyperoxia tended to lower venous erythrocyte XNO compared to normoxia (-26±11%, $P=0.086$). In an analysis of arterial and venous samples drawn under all conditions, significant or close to significant negative a-v gradients were observed during exercise for XNO ($P=0.06$), SNO+XNO ($P=0.03$) and to a lower extent for SNO ($P=0.11$) (Table 3). Venous erythrocyte nucleotides did not change with graded hypoxia or graded hyperoxia.

Leg and Systemic Hemodynamic Responses

At rest, anterograde, retrograde and net LBF and shear rates as well as perfusion pressure, vascular conductance and $\dot{V}O_2$ did not change significantly with graded hypoxia or graded hyperoxia (Fig. 1 and Fig. 2). However, $\dot{Q}$ and systemic vascular conductance increased with moderate (+18±4% and +24±6%) and severe hypoxia (+37±5% and +41±6%) compared to

normoxic control, suggestive of a slight but significant increases in systemic blood flow (+0.9-1.6 l min^{-1}) via vasodilatation in territories other than the experimental leg such as the trunk or the brain. During exercise, net LBF and $\dot{Q}$ increased above normoxic control with severe hypoxia (+23±5% and +28±3%), but remained unaffected with graded hyperoxia when O_2Hb did not change significantly (Fig. 1). The change in net LBF with severe hypoxia was a consequence of a significant increase in anterograde LBF (+26±5%) with no effect on retrograde LBF. As a result, net shear rate increased with severe hypoxia (+22±4%), secondary to the increase in anterograde shear rate (+25±5%) while retrograde shear rate remained unchanged (Fig. 2). Arterial and leg perfusion pressure did not change with moderate and severe hypoxia indicating that increases in blood flow were associated with increases in leg and systemic vascular conductance suggestive of enhanced leg and systemic vasodilatation. Increases in $\dot{Q}$ with hypoxia were only associated with increases in heart rate (r^2 = 0.95: P<0.01) as stroke volume remained unchanged.

Relationships between leg perfusion, erythrocyte de-oxygenation and NO /ATP metabolites

Venous plasma nitrite decreased progressively with venous hemoglobin O_2 saturation during exercise (r^2=0.88, P<0.01; Fig. 3) but not at rest (P=0.4-0.5) whereas venous erythrocyte XNO increased with decreasing venous O_2 saturation both at rest (r^2=0.70, P<0.05) and during exercise (r^2=0.79, P<0.01). In contrast, venous and arterial plasma ATP and erythrocyte SNO levels were unrelated to alterations in hemoglobin O_2 saturation either at rest or during exercise.

During exercise, leg vascular conductance increased with decreasing venous plasma nitrite (r^2=0.93, P<0.01) and increasing venous erythrocyte XNO (r^2=0.74, P<0.05), erythrocyte SNO+XNO (r^2=0.96, P<0.001) and to a lesser extent SNO (r^2=0.59, P=0.07; Fig.4). These changes in leg vascular conductance accompanied similar changes in LBF and $\dot{Q}$, as flows

increased with decreasing venous hemoglobin O_2 saturation (r^2=0.98, P<0.001 and r^2=0.86, P<0.01) and plasma nitrite (r^2=0.93 and r^2=0.88, both P<0.01) but with increasing erythrocyte XNO (r^2=0.79, P<0.05 and r^2=0.62, P=0.06), and erythrocyte SNO+XNO (r^2= 0.96 and r^2=0.94, both P<0.001) and to a lower extent with increasing venous erythrocyte SNO (r^2=0.53, P=0.10 and r^2=0.70, P<0.05). Multiple forward regression analysis performed with pooled resting and exercising data indicated that venous O_2Hb, plasma nitrite and erythrocyte XNO are significant predictors of LBF (Fig. 4), while the same parameters and erythrocyte SNO are significant predictors of $\dot{Q}$.

DISCUSSION

This investigation explored the possible relationships between erythrocyte-dependent NO and ATP signaling pathways and limb muscle and systemic blood flow in resting and exercising humans exposed to graded hypoxia and graded hyperoxia. The observation that net LBF and $\dot{Q}$ were linearly related (r^2=0.75-0.98, P<0.05) to arterial, venous and arterio-venous difference in O_2Hb extends data from previous studies underscoring the modulatory role exerted by the oxygenation state of hemoglobin in erythrocyte-dependent control of blood flow and emphasizes the importance of exercise stress in revealing this function. Importantly, mean arterial and leg perfusion pressures together with retrograde LBF were unchanged while anterograde and net LBF and vascular conductance increased, indicating that local vasodilatation overrode the enhanced vasoconstrictor activity associated with exercise (51) and hypoxia (28, 66). The mechanisms coupling erythrocyte deoxygenation with improved vascular conductance are thought to proceed via stimulation of NO-dependent vasodilatation (5, 7). A salient novelty of this work was the concomitant measurement of parameters related to the three known pathways (ATP (14), SNO (59) and nitrite (18)) of erythrocyte O_2-dependent control of vascular NO-signaling at graded erythrocyte O_2 saturations from severe

hypoxia through to severe hyperoxia, in the absence and presence of exercise. The strongest associations were found between plasma nitrite consumption / erythrocyte XNO formation and net LBF and O_2Hb (see below). Interestingly, in contrast to the observations made during exercise, no significant relationship was observed at rest between O_2Hb, net LBF or $\dot{Q}$ and plasma nitrite, erythrocyte SNO, plasma ATP or erythrocyte XNO, suggesting that resting blood flow in the leg results from the integration of physiological signals that might not involve erythrocyte deoxygenation as a primary sensor. We limit the discussion of this matter and focus below on the possible roles for different NO-dependent pathways in controlling exercise induced hyperemia in the human leg microvasculature and on the plausible explanations for the lack of change in plasma ATP and its degradation compounds.

Nitrite Pathways

Significant arterio-venous nitrite gradients in plasma were observed across the exercising limb, in line with previous data across the human forearm (8, 20, 38) or across the systemic circulation (41). Lower venous vs. arterial plasma nitrite concentrations is in agreement with the maximal nitrite reductase activity of deoxyhemoglobin, estimated at 50% O_2Hb saturation (17), a level never encountered in our arterial blood samples but of increasing prevalence in venous blood with exercise and graded hypoxia (range in venous O_2Hb saturation during exercise = 21-45%). Recent studies have shown that hypoxic tissues including erythrocytes, metabolize nitrite faster compared to normoxic tissues (31). We expected therefore that plasma nitrite levels would decrease with decreasing erythrocyte oxygenation, independently of rest or exercise. However, changes in arterial and venous plasma nitrite were small at rest and during exercise despite profound alterations in erythrocyte oxygenation (range in venous plasma nitrite = 4-5 pmol/mg prot vs. range in venous O_2Hb saturation = 24-81%). Hypoxia is known to stimulate nitrite formation from eNOS (6) which may in turn explain the failure

to observe decreased nitrite within arterial or venous circulations at rest with graded hypoxia. Additional considerations include that we did not control for dietary nitrate consumption which in turn can influence circulating nitrite levels (36). Despite this limitation, significant associations between venous plasma nitrite, venous erythrocyte deoxygenation and leg and systemic blood flow were observed during exercise, consistent with the hypothesis that nitrite reduction by deoxyhemoglobin may play a role in controlling local and systemic hemodynamics.

Interestingly, opposite correlations of plasma nitrite and erythrocyte XNO as a function of venous O_2Hb saturation (r^2=0.88 and 0.79, respectively, P<0.01) were observed whereas the relationship between erythrocyte SNO and venous O_2Hb saturation failed to reach significance (r^2=0.48, *P*=0.12). Given the variability in the individual responses, we also ran correlation analyses using all individual values. Although the relationships were weaker, we still observed that venous plasma nitrite and erythrocyte XNO were significantly related to venous O_2Hb saturation (r =0.27, P=0.04 and r= -0.33, P<0.01, respectively). Therefore, plasma nitrite consumption might be primarily associated with XNO and, to a lower extent, SNO formation within the erythrocyte. The exact identity of XNO is not known but these species likely represent nitroso adducts (C-, N- or O-) and, together with SNO formation, indicate increased formation of nitrosative species (16, 42). We acknowledge that our measurements of XNO may be derived in part from HbNO since the latter is a product of deoxyhemoglobin-nitrite reactions. Due to restrictions in the amount of RBC that could be collected, HbNO measurements were not possible and thus lack of these data is a limitation of the study. Irrespective of whether XNO measurements comprised HbNO, however, the present results are consistent with findings suggesting that nitrite reactions with deoxygenated Hb proceeds through formation of nitrosating species, including dinitrogen

trioxide (N_2O_3) (2), and also support previous data demonstrating an association between plasma nitrite and erythrocyte SNO-Hb in models of endotoxemia (9) and cardiovascular disease (62). In this context, these nitroso species represent a marker of nitrite reactivity with the erythrocyte, although functional roles cannot be excluded at this time. Therefore, a major finding of this study is that plasma nitrite consumption and erythrocyte XNO/SNO formation occur simultaneously in the vasculature of exercising humans *in vivo* and emphasizes the functional relevance of the nitrite/NO pathway in the control of mild exercise hyperemia. Collectively, these data are consistent with previous reports demonstrating i) a potent vasodilator action of plasma nitrite in the physiological range (8, 12, 35, 38); ii) nitrite conversion to erythrocytic NO adducts during arterial-venous transit in humans (8, 48) and iii) that plasma nitrite is related to aerobic exercise capacity in healthy populations (47) or in patients suffering from peripheral artery disease (1).

Blood oxygenation and erythrocyte SNO

Plasma and erythrocyte SNO did not change significantly in either arterial or venous blood (severe hypoxia vs. severe hyperoxia, P=0.18). Consequently, neither plasma nor erythrocyte SNO was related to O_2Hb saturation and minor negative arterio-venous gradients in plasma or erythrocyte SNO were observed across the leg. As such, these results extend previous findings obtained in the human forearm (8, 20, 21) but do not fit with previous reports in rodents (32) nor in humans (40) exposed to various levels of ambient O_2 availability or with previous data showing reciprocal changes in plasma SNO and erythrocyte SNO upon erythrocyte deoxygenation (13, 43). Previous studies have suggested that these differences stem from problems associated with the use of triiodide to measure SNO-Hb and specifically underestimation of SNO-Hb (29). However, data from studies, which spike blood or red cell samples with SNO-Hb standards and then process these samples according to methods used

in this study show that SNO-Hb is stable and completely recoverable with SNO-Hb concentrations being similar to those measured by non-triiodide based methods (e.g. CuCl/Cys) (34, 65). Moreover, our finding that erythrocyte SNO is higher in venous compared to arterial samples specifically under severe hypoxia (+159%; P=0.023), is consistent with increasing nitrosative species formation during arterial-venous transit and suggest that a local mechanism of SNO formation might operate in addition to the traditional view of central SNO formation in the pulmonary circulation where the erythrocyte oxygenates (40, 60).

Blood oxygenation and plasma/erythrocyte ATP and ATP metabolites

As a third tested pathway of erythrocyte O_2-dependent vasodilatation, we found that plasma and erythrocyte ATP were not significantly altered by graded hypoxia, graded hyperoxia or low intensity knee-extensor exercise. Therefore, no relationship appeared between plasma or erythrocyte ATP levels and O_2Hb saturation. This is at odds with our previous findings showing increased venous plasma ATP levels with exercise of increasing intensity to near maximal power output (25, 49, 68) and other studies documenting erythrocyte deoxygenation dependent release of ATP and subsequent stimulation of *in-vitro* vasodilatation (14, 30). However, they are in agreement with our previous results comparing venous plasma ATP at rest and during low-to-moderate intensity knee-extensor exercise in the presence and absence of severe hypoxia (24) or with anemia and polycythemia in isolation and in combination with hypoxia (23). One possibility explaining the discrepancy is that the increased plasma ATP levels with moderate and intense exercise might be related to mechanical deformation in addition to erythrocyte deoxygenation "*per se*" (15, 64). In our setting, mechanical power output (22±1 W) and therefore mechanical deformation imposed on the red cells was kept constant across all exercise interventions, allowing us to investigate the isolated effect of

altered O_2Hb levels on plasma ATP. This contrasts with the expected large changes in red cell deformability and other metabolic factors occurring during incremental exercise, which could provide a greater stimulus for erythrocyte ATP release. This view is supported by the moderate levels of exercising blood flow and shear rate reported in the present study (~2 L/min and 200 s^{-1} in normoxia) which are quite low compared to values observed during near maximal knee-extensor exercise in active subjects (typically >4 L/min and >300 s^{-1} in normoxia) (22, 25, 63), suggesting that the plasma ATP response might be magnified at higher exercise intensity. Another possibility is that the expected low nanomolar changes in erythrocyte ATP release with hypoxic and hyperoxic low intensity exercise might be difficult to detect in a large vein such as the common femoral vein. In support of this, plasma ATP has been shown to remain unaltered or to be slightly decreased in this vessel during intrafemoral artery ATP infusion despite the up to ~20-fold increase in leg blood flow (25, 49, 50). It should be kept in mind that the level of plasma ATP represents a net balance between ATP release from the erythrocyte and other sources and ATP inactivation by vascular (39) and serum soluble nucleotide-hydrolysing enzymes (67). It is therefore still possible that erythrocyte ATP release is altered at the level of the microcirculation even though plasma ATP values were unchanged in the large femoral vessels. Hence, despite combined measures of plasma and erythrocyte ATP levels and ATP breakdown products, the O_2-dependent release of ATP from the human erythrocyte remains difficult to ascertain during mild exercise *in vivo*.

In summary, this study provides new evidence that demonstrates : 1) increases leg and systemic perfusion with progressive hypoxia during low-intensity exercise are closely related to reductions in erythrocyte oxygenation, associated declines in venous plasma nitrite, increases in venous erythrocyte XNO and to a lesser extent erythrocyte SNO; 2) plasma

nitrite and erythrocyte XNO/erythrocyte XNO+SNO display opposite relationships to venous O_2Hb saturation, especially during exercise, consistent with the reduction of circulating nitrite to nitroso species (SNO+XNO) by the deoxygenated erythrocyte; 3) at rest, however, neither leg perfusion nor NO and ATP metabolites were altered. Together, these findings in humans point to plasma nitrite consumption / erythrocyte XNO formation across the vasculature as the dominant erythrocyte-dependent signaling pathway during low-intensity exercise hyperemia.

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Table 1. Hematological responses to graded systemic hypoxia and hyperoxia in resting and exercising humans

	Severe Hypoxia	Moderate Hypoxia	Mild Hypoxia	Normoxia	Moderate Hyperoxia	Severe Hyperoxia
$[Hb]_a$ (g/l)						
rest	151±3	150±3	150±2	150±3	150±2	150±3
exercise	155±3*†	153±3†	153±3†	152±3†	153±3†	152±3†
$[Hb]_v$ (g /l)						
rest	152±3	150±3	149±2	149±2	150±2	150±3
exercise	156±3*†	153±3†	154±3†	153±3†	153±3†	153±3†
Sa,O_2 (%)						
rest	76.8±1.9*	88.6±1.4*	93.6±0.6*	97.8±0.1	99.5±0.1	99.8±0.1
exercise	70.1±2.0*†	84.2±1.1*†	93.2±0.7*	97.7±0.7	99.5±0.1	99.7±0.1
Sv,O_2 (%)						
rest	54.4±2.4*	60.9±3.3	61.3±3.6	64.7±3.7	70.4±3.1*	77.1±4.0*
exercise	23.7±2.4*†	29.1±2.5*†	35.4±1.7†	36.6±1.8†	38.7±2.3†	42.6±2.4†
$MetHb_a$ (%)						
rest	0.7±0.1	0.7±0.0	0.7±0.0	0.7±0.1	0.7±0.0	0.7±0.1
exercise	0.7±0.0	0.7±0.0	0.7±0.0	0.7±0.1	0.7±0.0	0.7±0.0
$MetHb_v$ (%)						
rest	0.7±0.0	0.7±0.0	0.7±0.0	0.7±0.1	0.6±0.0	0.7±0.0
exercise	0.7±0.0	0.6±0.0	0.7±0.0	0.7±0.0	0.6±0.0	0.7±0.0
$COHb_a$ (%)						
rest	0.5±0.1*	0.3±0.1	0.4±0.1	0.2±0.1	0.1±0.1	0.0±0.1
exercise	0.5±0.1*	0.4±0.1*	0.3±0.1	0.2±0.1	0.1±0.1	0.0±0.1
$COHb_v$ (%)						
rest	0.4±0.1	0.4±0.0	0.3±0.1	0.3±0.1	0.3±0.1	0.3±0.1
exercise	0.2±0.1†	0.2±0.0†	0.2±0.0†	0.2±0.0†	0.3±0.0†	0.2±0.0†
Pa,O_2 (mmHg)						
rest	40±2*	55±3*	69±2*	102±2	258±4*	582±2*
exercise	35±1*	48±2*	67±2*	101±1	262±3*	589±9*
Pv,O_2 (mmHg)						
rest	29±1*	33±1*	34±2*	38±2	40±2	58±10 *
exercise	18±1*†	21±1*†	24±1†	25±1†	26±1§†	27±1*†
Ca,O_2 (ml/L)						
rest	161±6*	184±5*	195±3*	205±3	213±3*	224±4*
exercise	150±5*†	178±4*†	198±4*	208±3	216±4*	226±4*
Cv,O_2 (ml/L)						
rest	115±6*	126±7	127±7	134±7	146±6*	160±9*
exercise	52±6*†	62±6 *†	76±4†	78±4†	82±5†	90±5*†
$Lactate_a$ (mmol/L)						
rest	0.9±0.1	1.0±0.1	0.9±0.1	1.1±0.2	0.9±0.1	0.9±0.1
exercise	1.8±0.2	1.4±0.2	1.2±0.2	1.4±0.2	1.1±0.2	1.1±0.1
$Lactate_v$ (mmol/L)						
rest	1.0±0.1	1.0±0.1	0.9±0.1	1.2±0.2	0.9±0.1	1.0±0.1
exercise	2.9±0.5	1.9±0.3	1.7±0.4	1.9±0.3	1.4±0.3	1.4±0.3
$Glucose_a$ (mmol/L)						
rest	5.7±0.2	6.1±0.2	6.1±0.3	6.4±0.4	6.3±0.2	6.3±0.3
exercise	5.7±0.2	6.0±0.2	5.7±0.2	6.0±0.3	6.1±0.2	6.0±0.3
$Glucose_v$ (mmol/L)						
rest	5.4±0.2	5.7±0.2	5.6±0.2	5.8±0.3	5.8±0.2	5.7±0.2
exercise	5.6±0.2	5.8±0.1	5.6±0.1	5.9±0.2	5.9±0.2	5.8±0.2

Data represent mean ± SEM for 10 subjects. $_a$ and $_v$ indicate radial arterial and femoral venous samples; [Hb] hemoglobin concentration; SO_2, oxygen saturation, $MetHb_v$, methemoglobin; COHb, carboxyhemoglobin; PO_2, partial pressure for O_2; CO_2, O_2 content. *, $P<0.05$ vs. normoxic control; §, $P<0.1$ vs normoxic control; †, $P<0.05$ vs rest.

Table 2. Plasma nucleotides and nitric oxide metabolites during graded systemic hypoxia and hyperoxia in resting and exercising healthy humans

	Severe Hypoxia	Moderate Hypoxia	Mild Hypoxia	Normoxia	Moderate Hyperoxia	Severe Hyperoxia
$[ATP]_a$ (nmol/L)						
rest	485±50	475±40	424±35	436±39	540±84	412±50
exercise	560±80	544±90	543±70	438±27	475±51	525±43
$[ATP]_v$ (nmol/L)						
rest	436±52	467±32	381±52	457±43	490±43	472±23
exercise	518±57	453±42	474±85	487±67	454±33	554±60
$[ADP]_a$ (nmol/L)						
rest	134±19	134±22	119±18	121±29	135±24	112±18
exercise	169±43	160±46	171±35	116±21	138±28	144±17
$[ADP]_v$ (nmol/L)						
rest	138±24	139±22	112±19	143±31	140±30	136±24
exercise	152±30	135±22	129±28	148±25	121±16	163±37
$[AMP]_a$ (nmol/L)						
rest	89±17	83±18	95±21	88±17	105±23	110±24
exercise	107±21	107±34	96±22	90±29	106±23	99±20
$[AMP]_v$ (nmol/L)						
rest	89±17	92±17	110±20	90±14	107±24	99±17
exercise	116±32	97±17	98±23	89±16	113±35	99±15
$[Adenosine]_a$ (nmol/L) *						
rest	46±14	39±10	43±12	41±9	46±11	46±9
exercise	45±12	38±9	40±8	48±14	40±9	47±11
$[Adenosine]_v$ (nmol/L) *						
rest	46±11	41±9	49±13	57±14	57±13	46±11
exercise	50±12	53±16	44±10	62±17	56±15	48±12
$[SNO]_a$ (pmol/g prot)						
rest	50±30	35±26	98±95	26±21	37±25	56±37
exercise	13±24	63±34	96±78	17±20	42±25	72±41
$[SNO]_v$ (pmol/g prot)						
rest	17±17	23±22	30±21	36±23	38±22	20±25
exercise	21±17	3±20	16±18	9±24	34±25	24±24
$[Nitrite]_a$ (pmol/mg prot)						
rest	4.83±0.34	5.39±0.34	5.43±0.43	5.47±0.42	5.06±0.55	5.84±0.56
exercise	5.04±0.38	4.42±0.35	4.74±0.53	4.78±0.32	5.09±0.38	5.96±0.46
$[Nitrite]_v$ (pmol/mg prot)						
rest	4.19±0.49	4.40±0.49	5.04±0.83	4.48±0.53	4.36±0.45	4.20±0.37‡
exercise	3.96±0.45‡	4.11±0.36	4.31±0.39	4.50±0.34	4.43±0.45	4.53±0.51‡
$[Nitrite]_{a-v}$ (pmol/mg prot)						
rest	0.64±0.55	0.99±0.31	0.40±1.02	0.99±0.73	0.70±0.24	1.64±0.45
exercise	1.08±0.41	0.31±0.46	0.43±0.40	0.28±0.15	0.66±0.31	1.44±0.49

Data represent mean ± SEM for 10 subjects except for nucleotides values where n=7. $_a$ and $_v$ indicate radial arterial and femoral venous samples. * Adenosine concentrations were measured in whole blood samples. $[Nitrite]_{a-v}$, arteriovenous difference in plasma nitrite. No significant differences were observed with graded hypoxia, graded hyperoxia or exercise. ‡, $P<0.05$ vs. corresponding arterial value.

Table 3. Erythrocyte nucleotides and nitric oxide metabolites during graded systemic hypoxia and hyperoxia in resting and exercising healthy humans

	Severe Hypoxia	Moderate Hypoxia	Mild Hypoxia	Normoxia	Moderate Hyperoxia	Severe Hyperoxia
$[ATP]_a$ (µmol/L)						
rest	1441±100	1433±77	1441±75	1399±55	1417±77	1414±52
exercise	1441±88	1502±81†	1359±59*†	1488±96†	1449±75	1462±64
$[ATP]_v$ (µmol/L)						
rest	1424±77	1405±79	1479±79*	1395±62	1482±86*	1382±105
exercise	1462±94	1431±93	1467±76	1463±96†	1408±93†	1463±67†
$[ADP]_a$ (µmol/L)						
rest	149±7*	158±7	164±7	164±8	169±6*	164±7
exercise	147±9*	157±6	156±8†	162±6	171±8	168±8
$[ADP]_v$ (µmol/L)						
rest	147±7	150±8	165±7*	149±9	168±6*	162±9
exercise	148±11	149±7	149±7†	154±10†	155±8	158±7
$[AMP]_a$ (µmol/L)						
rest	16±2	15±1	16±1	16±1	17±1	19±2
exercise	14±1	15±1	16±1	17±1	15±2	19±2
$[AMP]_v$ (µmol /L)						
rest	15±1	15±2	18±2	16±1	18±2	18±1
exercise	16±4	14±2	17±1	15±1	15±1	19±2
$[SNO]_a$ (µmol/mol heme)						
rest	0.60±0.21	0.83±0.24	0.62±0.13	0.83±0.18	0.80±0.36	0.72±0.20
exercise	1.20±0.33	1.52±0.34	0.69±0.21	0.97±0.35	0.65±0.18	0.95±0.37
$[SNO]_v$ (µmol/mol heme)						
rest	1.57±0.71	0.79±0.22	1.26±0.41	1.58±0.57	0.94±0.26	1.16±0.41
exercise	3.05±1.00§†‡	2.41±0.98†	0.76±0.25	1.13±0.35	1.88±0.62	1.69±0.43
$[SNO]_{a-v}$ (µmol/mol heme)						
rest	-0.97±0.56	0.02±0.37	-0.64±0.52	-0.75±0.68	-0.14±0.51	-0.43±0.44
exercise	-1.85±1.04	-0.89±1.13	-0.07±0.22	-0.16±0.61	-1.24±0.74	-0.74±0.56
$[XNO]_a$ (µmol/mol heme)						
rest	3.70±0.44	2.92±0.33	3.66±0.76	3.34±0.40	2.86±0.33	2.78±0.26
exercise	5.36±0.20	3.28±0.41	3.64±0.70	2.87±0.34	2.73±0.27	3.04±0.45
$[XNO]_v$ (µmol/mol heme)						
rest	3.71±0.51	4.18±0.56	3.31±0.64	3.26±0.37	2.55±0.53	2.69±0.26
exercise	6.09±1.07*†	4.31±0.60	4.72±0.58†	4.48±0.53†‡	3.43±0.52	3.14±0.52§
$[XNO]_{a-v}$ (µmol/mol heme)						
rest	-0.01±0.62	-1.26±0.40	0.35±1.05	0.09±0.25	0.31±0.63	0.08±0.62
exercise	-0.73±1.28	-1.03±0.48	-1.08±0.66	-1.60±0.42	-0.70±0.48	-0.10±1.39

Data represent mean ± SEM for 10 subjects except for nucleotides values where n=7.

$_a$ and $_v$ indicate radial arterial and femoral venous samples. $[SNO]_{a-v}$ and $[XNO]_{a-v}$, arteriovenous difference in erythrocyte SNO and erythrocyte XNO respectively. * $P<0.05$ vs. Control Normoxia; § $P<0.1$ vs. Control Normoxia; †, $P<0.05$ vs rest; ‡, $P<0.05$ vs. corresponding arterial value.

FIGURE LEGENDS

Figure 1: **Net leg and systemic hemodynamics at rest and during exercise**

Note that leg blood flow, $\dot{Q}$, leg and systemic vascular conductance were elevated above control with severe hypoxia. Values are means±SEM of 10 subjects except for $\dot{Q}$ and systemic vascular conductance (n=9). * P<0.05 vs. Normoxic control.

Figure 2: **Anterograde, retrograde and net leg blood flows and shear rates at rest and during exercise**

Note that anterograde but not retrograde flows and shear rates were elevated above control with severe hypoxia. Values are means±SEM of 10 subjects. * P<0.05 vs. Normoxic control.

Figure 3: **Mean and individual data for plasma nitrite, erythrocyte XNO, plasma ATP, and erythrocyte SNO as a function of hemoglobin O_2 saturation during exercise**

Note the tight positive relationship between venous O_2Hb and venous plasma nitrite which mirrors the tight inverse relationship between venous O_2Hb and venous erythrocyte XNO. No significant relationship was observed between venous O_2Hb and venous plasma ATP or venous erythrocyte SNO. N=10 subjects for mean points except for venous plasma ATP (n=7 subjects). N=60 samples for individual data except for individual venous plasma ATP (n=42).

Figure 4: **Leg vascular conductance as a function of hemoglobin O_2 saturation and plasma Nitrite, erythrocyte XNO, erythrocyte SNO and plasma ATP during exercise**

Notice that leg vasodilatation during hypoxic exercise, as reflected by increase in leg vascular conductance, is closely associated with progressive reductions in hemoglobin O_2 saturation and concomitant gradual reductions in plasma nitrite and increases in erythrocyte XNO. This supports that plasma nitrite consumption and formation of erythrocyte nitroso species across the leg muscle vasculature is associated with vasodilatation and increased blood flow in the human leg skeletal muscle vasculature. Values are means±SEM of 10 subjects.

FIGURE 1 R2

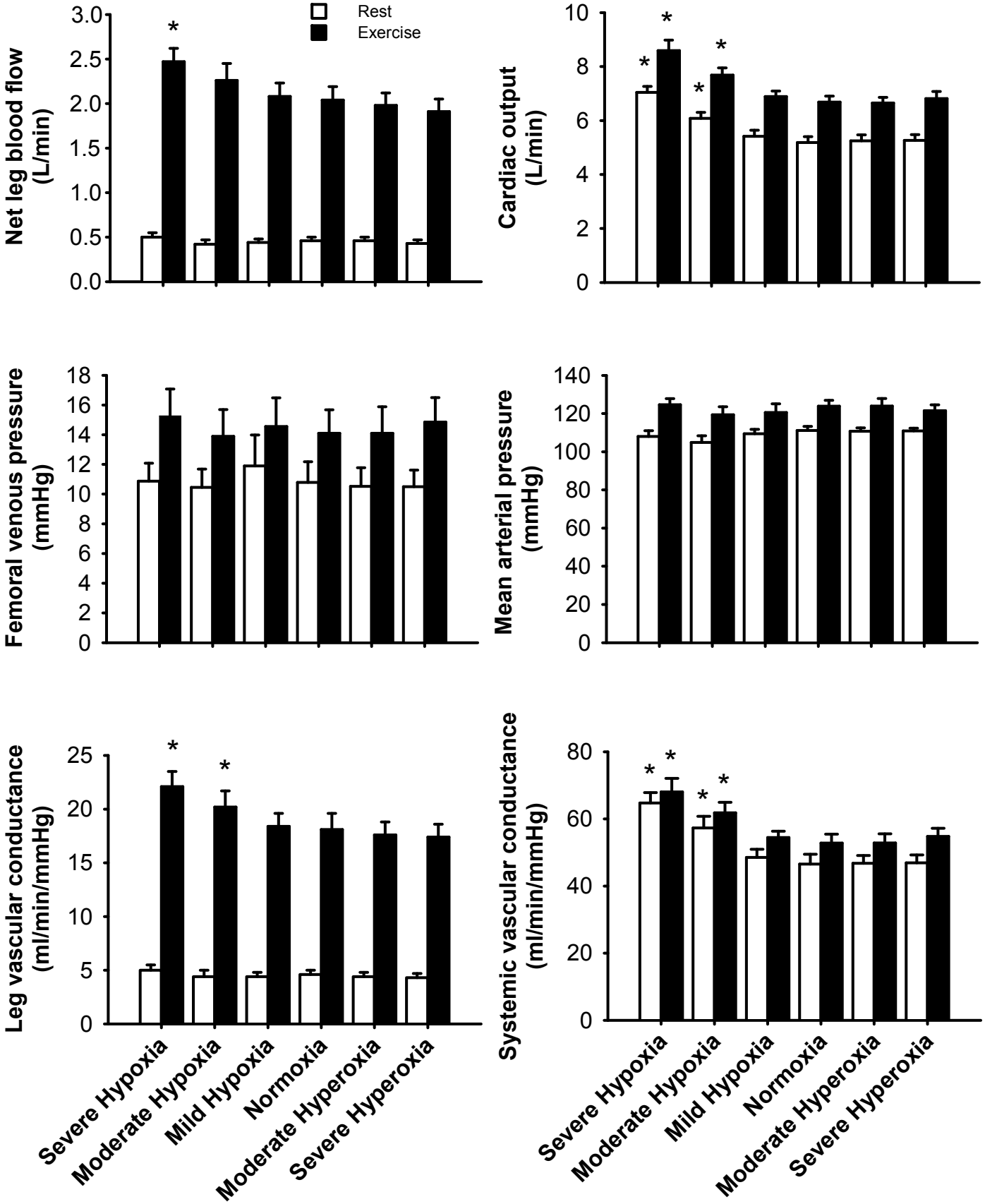

Rest
Exercise
Net leg blood flow (L/min)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Cardiac output (L/min)
10
8
6
4
2
0
Femoral venous pressure (mmHg)
18
16
14
12
10
8
6
4
2
0
Mean arterial pressure (mmHg)
140
120
100
80
60
40
20
0
Leg vascular conductance (ml/min/mmHg)
25
20
15
10
5
0
Systemic vascular conductance (ml/min/mmHg)
80
60
40
20
0
Severe Hypoxia
Moderate Hypoxia
Mild Hypoxia
Normoxia
Moderate Hyperoxia
Severe Hyperoxia

FIGURE 2 R2

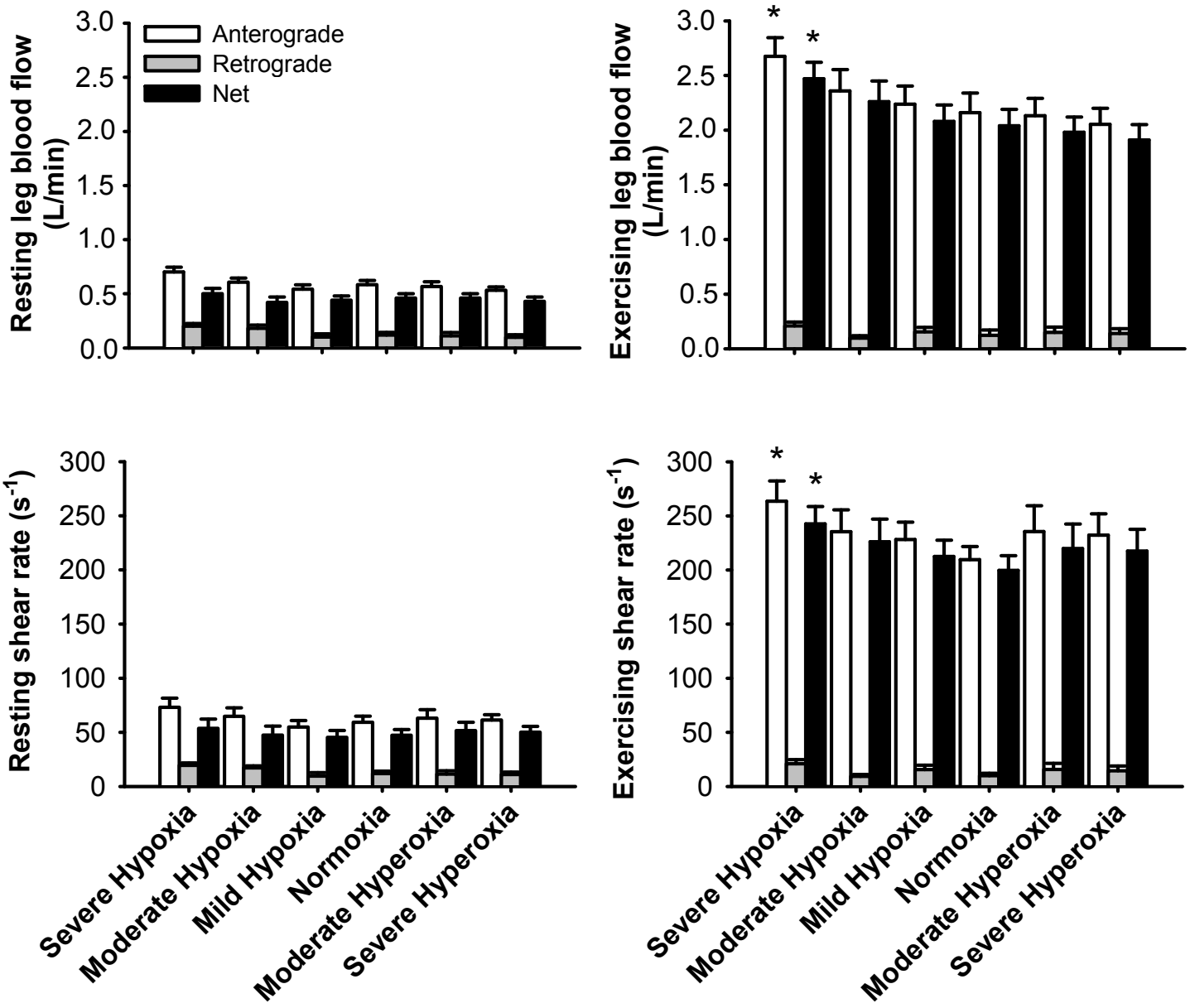

Anterograde
Retrograde
Net
Resting leg blood flow (L/min)
Exercising leg blood flow (L/min)
Resting shear rate (s-1)
Exercising shear rate (s-1)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
300
250
200
150
100
50
0
*
Severe Hypoxia
Moderate Hypoxia
Mild Hypoxia
Normoxia
Moderate Hyperoxia
Severe Hyperoxia

FIGURE 3 R2

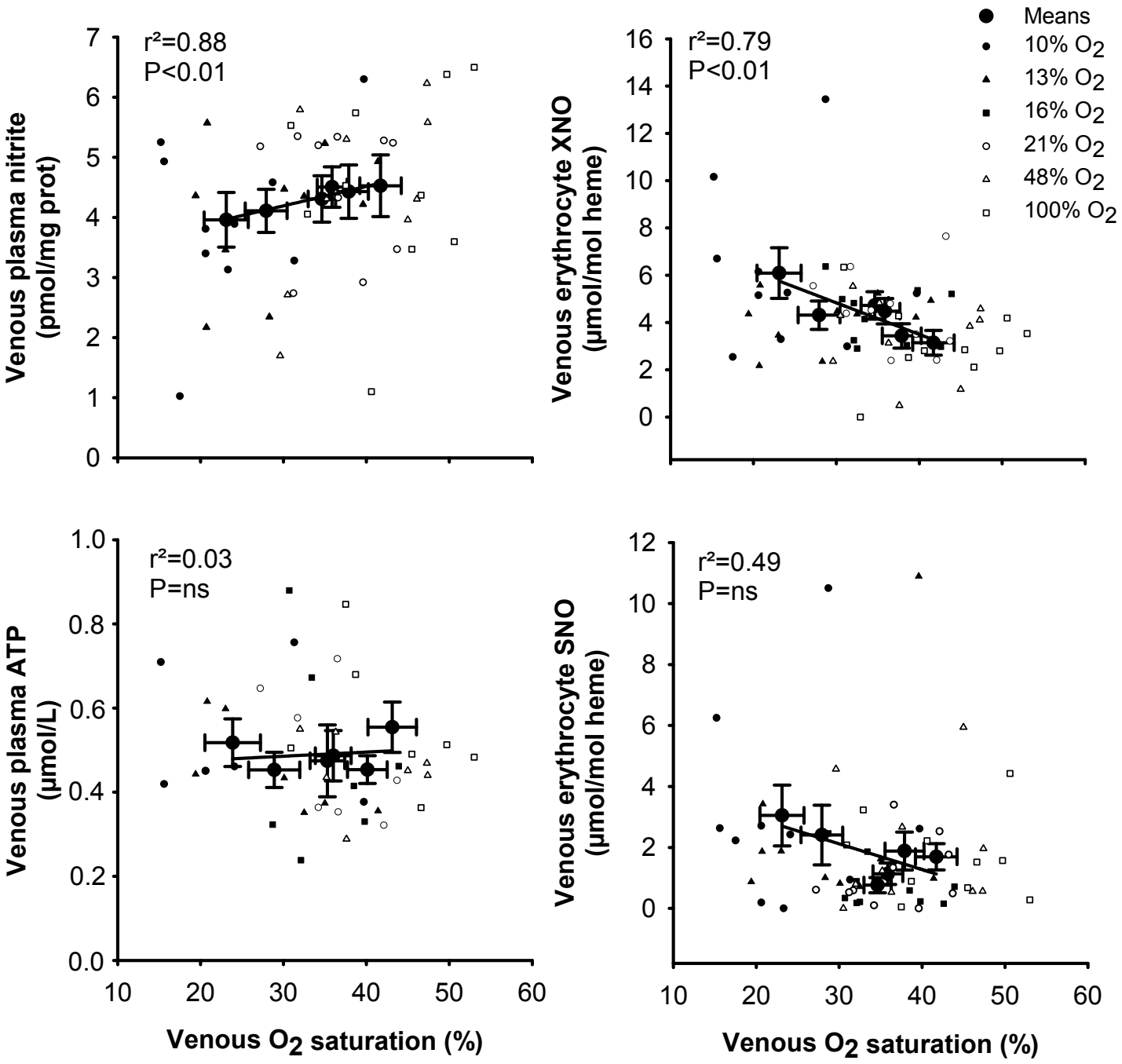
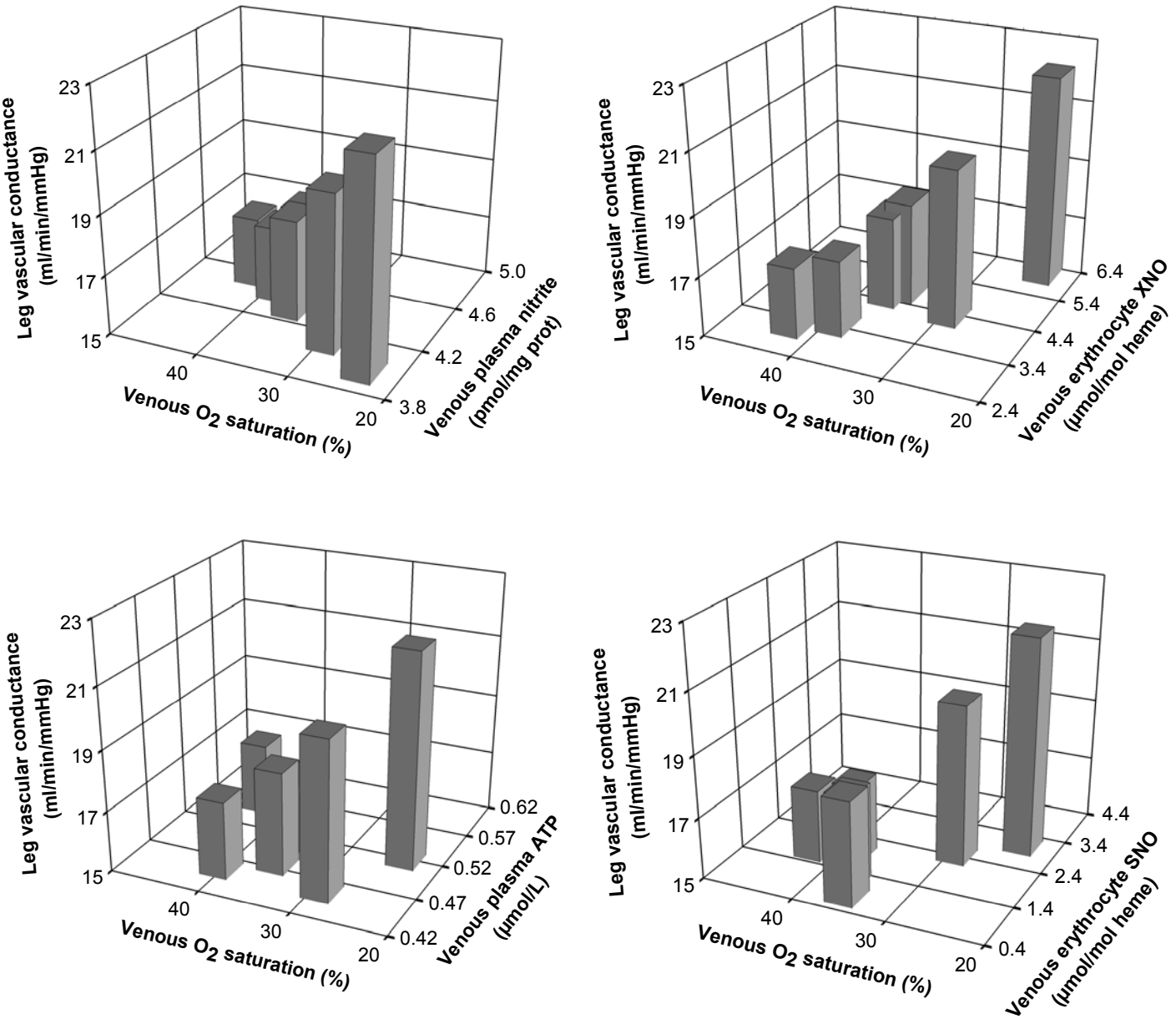

r²=0.88
P<0.01
Venous plasma nitrite (pmol/mg prot)
r²=0.79
P<0.01
Venous erythrocyte XNO (µmol/mol heme)
Means
10% O2
13% O2
16% O2
21% O2
48% O2
100% O2
r²=0.03
P=ns
Venous plasma ATP (µmol/L)
r²=0.49
P=ns
Venous erythrocyte SNO (µmol/mol heme)
Venous O2 saturation (%)
Venous O2 saturation (%)

FIGURE 4.R2


Leg vascular conductance (ml/min/mmHg)
23
21
19
17
15
40
30
20
Venous O2 saturation (%)
5.0
4.6
4.2
3.8
Venous plasma nitrite (pmol/mg prot)
6.4
5.4
4.4
3.4
2.4
Venous erythrocyte XNO (µmol/mol heme)
0.62
0.57
0.52
0.47
0.42
Venous plasma ATP (µmol/L)
4.4
3.4
2.4
1.4
0.4
Venous erythrocyte SNO (µmol/mol heme)