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Short-term hypoxic vasodilation *in vivo* is mediated by bioactive nitric oxide metabolites, rather than free nitric oxide derived from haemoglobin-mediated nitrite reduction

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Abstract

Local increases in blood flow - 'hypoxic vasodilation' - confer cellular protection in the face of reduced oxygen delivery. The physiological relevance of this response is well established, yet ongoing controversy surrounds its underlying mechanisms. We sought to confirm that early hypoxic vasodilation is a nitric oxide (NO)-mediated phenomenon and to study putative pathways for increased levels of NO, namely production from NO synthases, intravascular nitrite reduction, release from pre-formed stores, and reduced deactivation by cytochrome c oxidase. Experiments were performed on spontaneously breathing, anaesthetized, male Wistar rats undergoing short-term systemic hypoxaemia, who received pharmacological inhibitors and activators of the various NO pathways. Arterial blood pressure, cardiac output, tissue oxygen tension and the circulating pool of NO metabolites (oxidation, nitrosation and nitrosylation products) were measured in plasma and erythrocytes. Hypoxaemia caused a rapid and sustained vasodilation, which was only partially reversed by non-selective NOS inhibition. This was associated with significantly lower plasma nitrite, and marginally elevated nitrate levels, suggestive of nitrite bioinactivation. Administration of sodium nitrite had little effect in normoxia, but

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produced significant vasodilation and increased nitrosylation during hypoxaemia that could not be reversed by NO scavenging. Methodological issues prevented assessment of the contribution, if any, of reduced deactivation of NO by cytochrome c oxidase. In conclusion, acute hypoxic vasodilation is an adaptive NO-mediated response that is conferred through bioactive metabolites rather than free NO from haemoglobin-mediated reduction of nitrite.

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Abbreviation list: ABP: arterial blood pressure; CPTIO: 2-(4-carboxyphenyl)-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide; HIF: hypoxia-inducible factor; L-NAME: N(G)-nitro-L-arginine methyl ester; NEM: N-ethylmaleimide; NO-Hb: nitrosyl-haemoglobin; NOS: nitric oxide synthase; POE-Hb: pyridoxylated haemoglobin poly-oxoethylene conjugate; RNNO: N-nitrosamines; RSNO: S-nitrosothiols; RXNO: sum of nitrosation products; SEITU: S-ethyl-isothiourea; sGC: soluble guanylate cyclase; SVR: systemic vascular resistance

Keywords: Hypoxic vasodilation; Nitric oxide; Nitrite; Hypoxaemia.

Key points:

- Hypoxia increases blood flow through vasodilation, an adaptive response that increases oxygen availability to tissues.
- This response is likely mediated by nitric oxide (NO); different mechanisms have been postulated (increased *de novo* synthesis, release from pre-formed stores, reduced inactivation), but precise mechanisms remain controversial.
- In a short-term rodent model, hypoxaemia was associated with hypotension and lower plasma nitrite levels suggestive of nitrite bioinactivation; this was only partially reversed by NO synthase inhibition. Administration of sodium nitrite produced marked vasodilation and increased nitrosylation. Scavenging of nitric oxide had little effect.

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- Early hypoxic vasodilation is mediated by a complex interaction of multiple NO-related species, rather than by nitric oxide from haemoglobin-mediated reduction of nitrite *per se*.
- NO and its metabolites play a pivotal role in the body's initial adaptation to an acute decrease in oxygen supply, likely offering protection against a supply-demand mismatch.

Key point word count: 141

INTRODUCTION

Mammals crucially require oxygen (O_2) as the terminal electron acceptor in the mitochondrial respiratory chain to provide sufficient energy for cell metabolism, organ function and survival (Brand, 2005). Any serious imbalance between O_2 delivery and consumption can lead to a state of energetic crisis (Taylor & Pouyssegur, 2007). This is a critical factor underlying cell death (Brunelle & Chandel, 2002) and organ failure (Rixen & Siegel, 2005).

Several complementary mechanisms work in concert to protect the tissues in the face of reduced O_2 delivery. These include measures to facilitate O_2 availability such as local increases in blood flow - 'hypoxic vasodilation' (Roy & Brown, 1880; Dreyer, 1926; Hackel *et al.*, 1954; Heistad & Wheeler, 1970; Rowell & Blackmon, 1987, 1989), with flow redistribution to 'vital' organs and increased recruitment of perfused microvessels (Tsai *et al.*, 2003). In parallel, additional strategies are employed that include decreasing metabolic requirements to match energy supply and demand, up-regulating glycolytic

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generation of ATP, and modifying substrate availability and utilization, for example through the hormonal response to acute stress (Semenza, 2007).

Nitric oxide (NO) is a key determinant of myocardial function and vascular tone. It is essential for both global regulation and regional distribution of blood flow and pressure (Welch & Loscalzo, 1994). NO also plays a crucial role in energy regulation and metabolism through its modulatory effects on mitochondrial activity, and on protein function through nitrosylation, nitrosation or nitration (Clementi *et al.*, 1999; Jobgen *et al.*, 2006; Cooper & Giulivi, 2007). NO levels in blood or tissues represent a balance between production/release and metabolism/bioinactivation.

NO likely plays a key role in the body's early adaptation to an acute energy supply-demand mismatch. However, considerable controversy persists with respect to its sources and mechanisms of action (Umbrello *et al.*, 2013). Using a short-term rodent model of systemic hypoxaemia, we sought to confirm that acute hypoxic vasodilation is a NO-mediated phenomenon, and to study the contribution of putative pathways known or suspected to enhance NO bioactivity, namely increased production from NO synthases, increased reduction of nitrite by red blood cells, increased release from pre-formed storage forms of NO, and reduced deactivation by mitochondrial cytochrome c oxidase.

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METHODS

Male Wistar rats of approximately 300 g body weight were used in all experiments. Animals were purchased from Charles River (Margate, Kent, UK) and housed in cages of four on an alternating 12 h light–dark cycle, with free access to chow (2018 rodent diet, Harlan Teklad, Madison, WI, USA) and tap water. Prior to instrumentation, animals were allowed to acclimatize to the local environment for at least one week. All experiments were performed according to Home Office (UK) guidelines under the 1986 Animal (Scientific Procedures) Act with University College London Ethics Committee approval.

Instrumentation and monitoring

Spontaneously breathing animals were placed into a transparent plastic container and anaesthetised by 5% isoflurane (Baxter Healthcare, Thetford, UK) in room air. Following abolition of the righting reflex, animals were placed in the supine position onto a heated mat to maintain rectal temperature at 37 °C. Under 2-2.5% isoflurane, the left common carotid artery and right internal jugular vein were located, isolated and cannulated using 0.96 mm outside diameter PVC tubing catheter (Biocorp Ltd, Huntingdale, Australia). The arterial line was connected to a pressure transducer (Powerlab, AD Instruments, Chalgrove, UK) for continuous monitoring of arterial blood pressure (ABP). The venous line was used for subsequent administration of fluids and drugs. A tracheostomy was sited using 2.08 mm external diameter polyethylene tubing (Portex Ltd, Hythe, UK) to secure and suction the airway. This was connected to a T-piece to maintain anaesthesia and to vary the fraction of inspired oxygen.

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Continuous monitoring of muscle tissue PO_2 (tPO_2) levels was obtained by the use of Large Area Surface (LAS™) oxygen sensors (0.7 mm diameter) connected to the Oxylite™ tissue monitoring system (Oxford Optronix, Abingdon, Oxon, UK), as described in detail elsewhere (Dyson *et al.*, 2007). Briefly, the sensor sends short pulses of light (475 nm) along a fibreoptic cable to a platinum-complex fluorophore situated at the probe tip. This provides an 8 mm^2 surface area in contact with the tissue for tPO_2 measurement. Upon interaction with oxygen, the fluorophore emits light (600 nm) back to the detection unit, the lifetime of which is inversely proportional to the local PO_2 concentration within the tissue of interest. Unlike polarographic techniques, oxygen is not consumed during the measurement process; moreover, as the fluorescence decay is longer at lower values of PO_2, highly accurate measurements can be made in a hypoxic environment. A small incision was made at mid-thigh level, and the sensor inserted into the vastus lateralis muscle to a depth of 10 mm using an 18-gauge cannula for guidance (Venflon™, Becton Dickenson, Franklin Lakes, NJ, USA). The sensor was then withdrawn by 2 mm to prevent erroneous measurements resulting from local haematoma. This was verified by seeing a rapid response in tPO_2 following a change in inspired O_2. Arterial blood pressure (ABP) and muscle tPO_2 measurements were continuously monitored and recorded onto a computer using a Powerlab system and Chart 5.0 acquisition software (AD Instruments, Chalgrove, UK).

Isoflurane anaesthesia was reduced to 1.2% for the remainder of the experiment. Post-instrumentation, intravascular volume optimization was achieved by repeated 0.5 ml intravenous challenges of 0.9% saline given over 10 s every 5 min until ABP failed to increase >10%. A continuous infusion of 0.9% saline was then administered at a rate of

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10 ml/kg/h for the duration of the experiment to ensure adequate filling conditions throughout. All animals were allowed to stabilize for at least 30 min to achieve constant baseline physiological variables before the beginning of the experiment.

At pre-defined time points, arterial blood samples (0.2 ml) were collected into heparinized capillary tubes for blood gas analysis (ABL-625 analyzer, Radiometer, Copenhagen, Denmark). Blood (0.5 ml) was also collected into tubes containing N-ethylmaleimide (NEM; 10 mM) and EDTA (2.5 mM) to prevent artificial thiol nitrosation and to minimize transition metal-catalyzed RSNO degradation and trans-nitrosation reactions (Marley *et al.*, 2000). Plasma and erythrocytes were then obtained by centrifugation at 800g at 4°C for 10 min. Both plasma and erythrocytes were immediately frozen in liquid nitrogen and stored at -80 °C for subsequent analysis.

Transthoracic echocardiography was performed using a Vivid 7 Dimension™ machine (GE Healthcare, Bedford, UK) with a 14 MHz probe recording at a depth of 0-2 cm. Aortic blood flow velocities were determined immediately before the right carotid artery bifurcation using pulsed-wave Doppler; the direction of blood flow was confirmed by colour Doppler imaging. Stroke volume (SV) was determined as the product of the velocity time integral (VTI) and vessel cross-sectional area ($\prod$ x [0.5 x diameter]^2). Rats of this size have an aortic diameter of 0.26 cm (Slama *et al.*, 2003), thus a cross-sectional area of $(0.13)^2$ x $\prod$ was assumed for all animals studied. Heart rate (HR) was determined by measuring the time between six consecutive cycles from the start of each Doppler trace to account for variation with respiration. Cardiac output (CO) was determined as the product of SV and HR. Systemic vascular resistance was calculated as mean blood

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pressure divided by CO. The O_2 content in the arterial blood and global O_2 delivery were calculated using standard formulae.

Experimental protocols

Modulation of the various NO pathways was achieved through separate studies (Figure 1). For each experimental condition, after recording of baseline haemodynamic variables and blood sampling for NO metabolite determination (BL), animals were randomized to either breathing room air (N) or a hypoxic mixture (H) at an inspired oxygen concentration (FiO_2) of 0.125. They were then allowed to stabilize for 10 minutes. Animals (six hypoxic and six normoxic per group) were then randomized into one of the following groups:

i) sham, which only received the background infusion of 0.9% saline;
ii) systemic vasoconstriction, using an intravenous infusion of norepinephrine at a rate of 1.5 µg/kg/min
iii) NOS inhibition, where animals received a 1.5 mg/kg/min infusion of the potent, non-selective NOS inhibitor S-ethyl-isothiourea (SEITU) (Southan *et al.*, 1995);
iv) nitrite (NO_2^-) administration, where animals received a 250 µg/kg/min infusion of sodium nitrite, $NaNO_2$ (Badejo *et al.*, 2010);
v) a combination of NOS inhibition with SEITU, and $NaNO_2$ administration (doses as above);
vi) non-haem NO scavenging, using an infusion of the scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide (C-PTIO) (Yoshida *et al.*, 1994) running at 0.17 mg/kg/min;

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vii) haem-based NO scavenging, where animals received a continuous infusion (50 mg/kg/h) of pyridoxylated haemoglobin poly-oxoethylene (POE-Hb) (Kida *et al.*, 1995).

To avoid any possible confounding effect, drug concentrations were calculated so that all animals received at total of 10 ml/kg/h of intravenous fluid. At the end of each experiment (30'), another aliquot of blood was drawn for the determination of NO metabolites. All drugs used were purchased from Sigma-Aldrich (Gillingham, Dorset, UK) with the exception of norepinephrine (Abbott Labs, Maidenhead, UK). POE-Hb was kindly donated by Apex Bioscience (Chapel Hill, NC, USA), and dissolved in 0.9% saline.

Drug doses were selected after extensive exploratory dose-response experiments (Supplementary Figure S1). We chose a dose of SEITU for subsequent study that gave an approximate 40 mmHg rise in ABP in normoxic rats. Notably, no further increases in blood pressure were observed above this dose in either normoxic or hypoxic animals. We also attempted to modulate NO metabolism by altering the redox state of cytochrome c oxidase through administration of either hydrogen sulphide (given as NaHS) or sodium azide (both 10^{-8} to 10^{-2} g/kg/min). Unfortunately, major side-effects (severe haemodynamic instability leading to premature death) precluded *in vivo* study of this pathway.

Figure 1b depicts the experimental schedule. Blood pressure and muscle tissue oxygen tension were monitored continuously. Echocardiography and blood gas analysis was performed at baseline, 10 and 30 minutes with sampling for plasma and erythrocyte levels of NO-related compounds (see below) at baseline and 30 minutes. In selected

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experiments, additional echocardiography and blood gas analysis was performed at 20 minutes before administration of sodium nitrite.

After analysis of the results, we performed two sets of additional experiments to better understand the mechanisms underlying our findings. The hypotheses we set out to test were: i) whether the vasodilation seen under hypoxia was caused by increased levels of NO *per se* or if a NO-related compound could play a role and ii) similarly, whether the vasodilating effect of nitrite in hypoxia was caused by increased levels of NO *per se* or if a NO-related compound could play a role. We thus treated two groups of hypoxic animals (n=6 each) with a combination of i) NOS inhibition followed by a continuous infusion of POE-Hb and ii) NOS inhibition and $NaNO_2$ administration followed by a continuous infusion of POE-Hb; all drugs were used at the same doses as previously described. To avoid POE-Hb oxidation, the latter experiment required separate venous access for the administration of both drugs: in this group, SEITU and $NaNO_2$ were administered via the jugular line. The left femoral vein was also cannulated for administration of POE-Hb.

Biochemical analyses

Frozen sample aliquots were thawed just before use. Plasma was used undiluted while the erythrocyte pellet was subjected to hypotonic lysis in cold aqueous NEM/EDTA (10/2.5 mM; 1:4 v/v) immediately before analysis. The concentration of nitroso compounds was assessed using group-specific reductive denitrosation by iodine-iodide in glacial acetic acid, with subsequent detection of liberated NO into the gas phase by its chemiluminescent reaction with ozone (Feelisch *et al.*, 2002). 'RSNO' signifies mercury-labile S-nitrosated species (including nitrosothiols), whereas 'RNNO' signifies mercury-

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resistant nitroso adducts and may include N-nitrosamines and metal nitrosyls other than NO-haem species. 'RXNO' represents the total pool of nitrosated species (i.e. the sum of RSNOs and RNNOs). NO-haem species in erythrocytes (nitrosylhaemoglobin) were determined by parallel injection of replicate aliquots of blood homogenates into a solution of 0.05 M ferricyanide in PBS at pH 7.5 and 37˚C (Bryan *et al.*, 2004). This method employs one-electron oxidation rather than reduction to achieve denitrosation, with the liberated NO being quantified by gas-phase chemiluminescence (CLD 77 AM, Eco Physics, Ann Arbor, MI, USA). Nitrate and nitrite were quantified by ion chromatography with on-line reduction of nitrate to nitrite and post-column Griess diazotization (ENO20 Analyzer; Eicom, Kyoto, Japan) (Rassaf *et al.*, 2002) .

Statistical Analysis

All data are presented as means ± SEM (n=6 per group) unless otherwise specified. Statistics were performed on raw data using a repeated measures two-way ANOVA followed by Tukey's post-hoc test (SigmaStat 11.0, Systat Software Inc, San Jose, CA) to compare multiple groups at multiple time points or unpaired Student's t test to compare two groups. For all comparisons, a p value <0.05 was considered statistically significant.

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RESULTS

Model characterisation and effects of hypoxia

Hypoxia (12.5% O_2) significantly reduced arterial PO_2 and haemoglobin saturation (Table 1). Muscle tPO_2 levels halved. Despite an unchanged respiratory rate, hypoxia was associated with reduced $PaCO_2$, but similar arterial pH. Though tidal volumes were not formally measured, there was an obvious increase in chest wall excursion.

Blood pressure fell significantly during hypoxia, and this was maintained over time despite no significant change in either stroke volume or heart rate (Figure 2, Suppl Fig. S1). As a consequence, systemic vascular resistance (SVR) was significantly lower in the hypoxic group. Global oxygen delivery was reduced by half, and blood lactate levels increased significantly (Table 1).

Norepinephrine, titrated to increase mean ABP by 40 mmHg above normoxic levels, failed to reach this target under hypoxia (Fig. 2 and Table 1). Whereas this agent increased SVR in the normoxic group, it had little effect during hypoxia. HR was significantly increased by norepinephrine in both groups to the same extent. Oxygen delivery increased in both groups, though failed to reach baseline levels in the hypoxia group. Lactate levels were lower than during hypoxia alone, and similar to that measured in normoxic sham animals.

Haemodynamic effects of the modulation of the NO system (Figure 2)

Non-selective NOS inhibition with SEITU increased ABP to a greater extent in normoxic compared to hypoxic animals. A similar pattern was observed with the structurally distinct NOS inhibitor, L-NAME (Supplementary Figure S2). However, while SEITU

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significantly reduced cardiac output, mainly through its effect on stroke volume, hypoxic animals showed a lesser fall. Oxygen delivery was substantially reduced but did not differ between the two groups. Lactate levels increased to a greater extent in hypoxic animals treated with SEITU compared to that seen during hypoxia alone.

Although nitrite administration had only a mild hypotensive effect in either normoxia or hypoxia, stroke volume increased in the hypoxia group and vascular resistance fell significantly, indicating further vasodilatation. This resulted in an overall oxygen delivery that was similar to the normoxic group treated with nitrite, although lactataemia was significantly greater. When nitrite was given after non-selective NOS inhibition with SEITU, the hypotensive effect was significantly more pronounced under hypoxic conditions.

NO scavenging with C-PTIO had a greater effect on blood pressure in the normoxic group. Cardiac output also significantly increased with C-PTIO in normoxia, mainly through an increase in HR. On the other hand, the haem-based NO scavenger POE-Hb produced similar changes in blood pressure in both groups, though ABP values did not reach the levels obtained with NOS blockade or norepinephrine.

When an NO scavenger was added after NOS inhibition in the hypoxic group, ABP failed to increase further (mean change +10.7 mmHg, p=0.799), with no changes seen in either cardiac output (mean increase 11.1 ml/min, p=0.478) nor SVR (mean increase 0.60 mmHg/(ml/min), p=0.247). Similarly, when an NO scavenger was added after NOS inhibition and nitrite administration in the hypoxic group, ABP failed to increase (mean change +14.3 mmHg, p=0.154), with no changes in either cardiac output (mean increase

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11.7 ml/min, p=0.424) or SVR (mean increase 0.09 mmHg/(ml/min), p=0.982) (Supplementary Figure S3).

Plasma NO metabolites (Figure 3, Table 2)

Despite similar concentrations of circulating total NO oxidation products, 30 minutes of hypoxia significantly reduced plasma nitrite levels. Nitrate levels did not change significantly at this time point. Administration of norepinephrine resulted in lower plasma levels of nitrite and nitrate compared to the sham-treated groups, with no differences between normoxia and hypoxia. On the other hand, NOS inhibition reduced plasma nitrite levels during normoxia, but without any further reduction during hypoxia, whereas the concentration of total nitrosation products (RXNO) increased. S-nitrosothiols (RSNOs) accounted typically for less than half of the RXNO signal, and that this ratio didn't change much under any of the experimental conditions applied.

Administration of nitrite increased plasma nitrite by approximately 20-fold, with a near-doubling of plasma nitrate and an increase in plasma total nitrosation products. However, no differences were seen between hypoxic and normoxic groups. Addition of NOS inhibition to nitrite did not change the plasma NO metabolite profile.

Scavenging NO with C-PTIO reduced plasma nitrite levels to virtually zero, whereas nitrate levels were conserved. Plasma nitrosation products were also reduced, though no differences were seen between hypoxia and normoxia. Utilization of POE-Hb as the NO scavenger was associated with a similar profile of plasma oxidation products. However, in stark contrast to C-PTIO, plasma nitrosation products increased compared to sham in both normoxic and hypoxic animals. Table 2 shows the relative contribution of nitrite to total oxidation products, and that of RSNO to total nitrosation products.

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Erythrocyte NO metabolites (Figure 4, Table 2) and nitrosyl-Hb (Figure 5)

In sham animals, erythrocyte nitrite and nitrate levels were conserved during hypoxia, with no differences seen in nitrosation products. Norepinephrine reduced intracellular nitrite levels in normoxic animals but RSNO and RNNO remained largely unchanged. NOS inhibition by SEITU had no impact upon erythrocyte NO oxidation and nitrosation products, and no differences were seen between hypoxia and normoxia states.

Notably, nitrite administration did not modify intraerythrocytic nitrite levels. However, nitrate concentrations were higher than in sham animals, particularly under hypoxic conditions. Nitrosyl haemoglobin levels were also higher during hypoxia. Similar results were seen when nitrite was co-administered with the NOS inhibitor, SEITU. NO scavenging was associated with lower intracellular nitrite levels, yet most other NO metabolites were unchanged. Of note, the NO metabolite profile observed in the presence of CPTIO was different from that of a typical NO-scavenger; besides the expected reductions in blood nitrite concentrations under normoxic and hypoxic conditions due to oxygen-independent conversion of NO to NO_2, hypoxia was associated with increases in nitrosation products other than RSNOs and increases in nitrosylhaemoglobin. This suggests preferential formation of N-nitrosated species in red blood cells that act as potential NO-donors with this particular compound. Thus, care should be taken when CPTIO is used in *in vivo* studies, emphasizing the need to compare mechanistically distinct NO scavengers before reaching conclusions about the possible involvement of NO in a given physiological process.

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DISCUSSION

Several complementary mechanisms protect the body's tissues in the face of a reduced O_2 delivery. These include redistribution of blood flow to 'vital' organs, and increased recruitment of perfused microvessels to facilitate O_2 availability (Tsai *et al.*, 2003). At the systemic level, the most evident adaptation to hypoxaemia involves an increase in blood flow - 'hypoxic vasodilation' (Roy & Brown, 1880; Dreyer, 1926; Hackel *et al.*, 1954; Heistad & Wheeler, 1970; Rowell & Blackmon, 1987, 1989). This aims to compensate for the reduction in arterial O_2 content by attempting to maintain an acceptable O_2 supply-demand balance. The sympathetic nervous system is activated with increased catecholamine levels, albeit with a reduced response to exogenous vasopressors (vascular hyporeactivity).

Although the physiological relevance of hypoxic vasodilation is well established, ongoing controversy surrounds its underlying mechanisms. An increase in nitric oxide (NO) is strongly implicated. This key signalling and effector molecule is a major determinant of vascular tone, an important modulator of myocardial function, and essential for both global regulation and regional distribution of blood flow and pressure (Welch & Loscalzo, 1994).

NO levels in blood and tissues represent a balance between production/release and metabolism/bioinactivation. Its best-recognized biosynthetic route is the L-arginine-NO pathway (Moncada & Higgs, 1993; Boucher *et al.*, 1999). However,

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alternative pathways are proposed, involving (i) nitrite and nitrate, oxidative end-products traditionally considered to be inert (Modin *et al.*, 2001), and (ii) S-nitrosothiols (RSNO), the reaction products between reactive nitrogen oxide species and thiol residues (Diesen *et al.*, 2008).

Particularly under conditions of hypoxia, proteins from the haem-globin family (Gladwin & Kim-Shapiro, 2008) or from pteryn-based molybdenum enzymes (Li *et al.*, 2008) may catalyze the reduction of nitrite to NO, beyond their conventional physiological functions. Release of NO from RSNO storage pools may also be enhanced under low oxygen conditions (Jia *et al.*, 1996). These pathways may provide important alternative sources of NO where increased blood flow may be beneficial, such as during hypoxia, particularly recognizing the critical requirement for O_2 as a cofactor for NOS. However, to date, many of these mechanisms have only been demonstrated in non-physiological conditions, either *in vitro* or *ex vivo*, or indirectly implicated by utilization of pharmacological tools. Thus, an open question still remains as to their *in vivo* (patho)-physiological relevance.

The *in vivo* fate of NO is also highly complex; several catabolic pathways exist with varying relevance in different body compartments. The reaction with haemoglobin to form NO_3^- constitutes the major catabolic pathway. However, in plasma in the presence of O_2, the principal reaction is formation of NO_2^- (Ignarro *et al.*, 1993) occurring through oxidation by mitochondrial cytochrome c oxidase (Shiva *et al.*, 2001; Pearce *et al.*, 2002). An alternative theory for the hypoxia-induced increase in NO is reduced elimination. Using an *in vitro* model, Moncada and colleagues demonstrated that cytochrome c oxidase in its oxidized state constantly inactivates NO, contributing to its intracellular regulation (Palacios-Callender *et al.*, 2007; Unitt *et al.*, 2010). However, in the reduced

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state typical of hypoxia, an impaired inactivation would account for the witnessed increase in NO.

NO acts at multiple sites within the body (Martinez-Ruiz *et al.*, 2011). It promotes vasorelaxation either via activation of soluble guanylate cyclase (sGC) and subsequent generation of cyclic GMP (cGMP), or through S-nitrosation of critical thiols sited on ion channels. NO also inhibits the mitochondrial electron transport chain through direct competition with oxygen at Complex IV (cytochrome c oxidase), and via nitrosation/nitration of other respiratory chain complexes (Frost *et al.*, 2005), ultimately causing a decrease in O_2 consumption.

As the vasodilating (Movahed *et al.*, 2003) and metabolic (Frost *et al.*, 2005) effects of NO are both enhanced during hypoxia, it likely plays a major role in re-equilibrating any O_2 supply-demand mismatch (Umbrello *et al.*, 2013). Hypoxia-induced vasorelaxation may be reversed by scavenging NO (Pohl & Busse, 1989), or inhibiting sGC with methylene blue (Iwamoto *et al.*, 1992). Whereas increased NO synthesis by NOS may be a potential cause of hypoxic vasodilation (Edmunds & Marshall, 2001; Hunter *et al.*, 2003), others report an ineffectiveness of NOS inhibition on decreased hind limb resistance (Vallet *et al.*, 1994) and dilation of small coronary arteries (Liu & Flavahan, 1997) induced by hypoxia.

We set out to study mechanisms underlying hypoxic vasodilation *in vivo* using a well-established short-term rodent model of systemic hypoxia (Dyson *et al.*, 2007, 2009). We chose an FiO_2 of 0.125 as this concentration was associated with the largest fall in PaO_2

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that did not carry short-term lethality (Dyson *et al.*, 2007). Our findings support the role of NO as a key mediator of hypoxic vasodilation.

Hypoxaemia caused an immediate and sustained hypotension associated with muscle tissue hypoxia and increased glycolytic flux. Vasopressor hyporesponsiveness was confirmed by the limited increase in ABP with norepinephrine. Notably, hypoxia reduced ABP and SVR but had little effect on cardiac output: this may be explained by the balanced effect of an increased perfusion due to vasodilation and some degree of hypoxic myocardial depression, possibly NO-mediated (Brady *et al.*, 1993; Mohan *et al.*, 1996).

Non-selective NOS inhibition with SEITU reversed hypoxic vasodilation but failed to achieve ABP values obtained from normoxic, SEITU-treated animals; we observed a similar pattern using the non-selective NOS inhibitor L-NAME and infer the difference in blood pressure between normoxic and hypoxic rats is due to NO not generated by NOS but derived from other pathways (nitrite reduction or release from pre-formed nitroso-pools). Hypoxia was associated with significantly lower plasma levels of nitrite, supporting the concept of increased NO production originating from nitrite reduction. When NOS was blocked by SEITU, plasma nitrite was lowered even under normoxic conditions. Most likely, this change is unrelated to nitrite reduction but a direct consequence of reduced NO production from NOS. Administration of nitrite caused little hypotensive effect in normoxia, and only small reductions of ABP under hypoxia. However, stroke volume and cardiac output, and thus SVR, were significantly higher in the hypoxic group, suggesting a stronger vasodilating effect of nitrite administration under hypoxia. The failure of the blood pressure to fall any further may be explained by

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being already low due to hypoxia alone. However, nitrite administration significantly increased nitrosyl haemoglobin generation during hypoxia, consistent with higher levels of NO production. When given after non-selective NOS-blockade with SEITU, nitrite caused a significantly greater hypotensive effect under conditions of hypoxia, accompanied by a further increase in nitrosyl haemoglobin levels.

Experiments with NO scavengers could distinguish whether the vasodilating factor acting in hypoxia was due to intravascular generation of NO *per se* or one or more NO-related compound(s). Both C-PTIO and POE-Hb are specific extracellular NO scavengers and both increased ABP irrespective of the presence of hypoxia. A possible explanation is that NO produced by endothelial cells exerts its vasorelaxing activity by a paracrine effect on surrounding vascular smooth cells, with no transit into the bloodstream. However, the changes in the circulating pool of NO oxidation and nitration products during NO scavenging suggest that a portion of the NO generated in vascular tissue does get transferred to the bloodstream. Alternatively, NO does not exert its vasodilatory action directly, but rather through the action of other downstream mediators. A study performed under physiological conditions found that nitrite was not directly reduced to NO but instead modulated several different signalling pathways (Bryan *et al.*, 2005). In addition to sGC activation, post-translational modifications typically associated with NO occur, such as the formation of nitrosated, nitrosylated and nitrated species (Bryan *et al.*, 2005; Perlman *et al.*, 2009). Thus, nitrite may exert its signalling functions directly, without the need for intermediary formation of free NO. Hypoxia markedly potentiates tissue NO production from nitrite in a dose-dependent manner (Feelisch *et al.*, 2008). This occurs particularly in heart, liver and vascular tissue, with multiple haem, iron-sulfur

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cluster and molybdenum-based reductases distributed among distinct subcellular compartments acting in a multifactorial and cooperative manner to catalyze the reaction. Acute hypoxia also reduced nitrite concentrations yet simultaneously enhanced the formation of NO metabolites such as RSNOs and RNNOs in an NO-independent manner, again consistent with a pathway that generates bioactive NO metabolites without the intermediacy of free NO. In this paradigm, conversion of nitrite to NO and the storage of NO bioactivity as RSNOs may both be constituents of a more complex regulatory mechanism of interaction of multiple NO-related species. To further test this hypothesis, and its *in vivo* relevance, we designed experiments whereby NO was scavenged after hypoxia and NOS inhibition ± nitrite administration. Notably, in both cases, blood pressure failed to increase upon NO scavenging, further supporting the hypothesis that actions of NO in hypoxia are mediated by NO metabolite signalling pathways rather than by NO directly.

Our study has several limitations: first, we focused on the acute hypoxic response, which may present different mechanisms from the response to chronic hypoxia. However, ten-fold higher levels of circulating NO products were found in Tibetan plateau residents compared to sea-level dwellers (Erzurum *et al.*, 2007). This was associated with increased resting forearm blood flow, suggesting a NO-mediated adaptive mechanism that offsets the O_2 lack caused by high altitude and which persists over time. Our rats were anaesthetized and spontaneously breathing, and responded to hypoxia with an increase in their ventilatory drive. The subsequent reduction in $PaCO_2$ (compared to group-matched normoxic rats) was, however, limited, and probably more representative of the situation that occurs *in vivo*. The (patho)physiology of vascular adaptation to hypoxia is

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indeed complex. Studies have demonstrated that NO is a major, though not the sole, contributor to this response, (Liu & Flavahan, 1997), or that it acts within a network of synergistic factors (von Beckerath *et al.*, 1991; Park *et al.*, 1992). Indeed, some of our results may be explained by concomitant activation of other signalling pathways. Another limitation of our study is that changes in NO metabolite status have only been assessed in blood and not in the vasculature itself. While a contribution by NO generated in the vascular wall can thus not be entirely excluded, this is unlikely to have occurred under our experimental conditions as the high concentrations of the two NO scavengers used should have created a sink for NO that would be efficient enough to show clear reversal of the effects of nitrite – however, this is not what we observed. Regrettably, we were unable to explore the possibility that NO levels may be increased in hypoxia because of a reduced metabolism/bioinactivation. Although we tried several strategies to modulate the redox state of cytochrome c oxidase (namely azide and hydrogen sulphide administration), unavoidable side-effects prevented the *in vivo* construction of such a model. Due to these methodological constraints we cannot exclude that reduced inactivation of NO by cytochrome c oxidase may be an additional contributory factor.

In conclusion, we demonstrated that short-term hypoxic vasodilation *in vivo* is an adaptive response mediated by NO. The mediator seems to act through bioactive metabolites, in a complex regulatory network of interaction of multiple NO-related species, rather than directly by NO itself.

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Figure 1: Sites of intervention and study protocol

A: Pathways of NO production with identification of sites of action of inhibitors and activators used in the study. For details about the pathways, please refer to the text.

NOS: Nitric Oxide synthase; RSNO: products of the nitrosation of critical thiol residues; sGC: soluble Guanylate Cyclase; $NaNO_2$: sodium nitrite; NO_2^-: nitrite anion; SEITU: non-selective NOS inhibitor S-ethyl-isothiourea; L-NAME: non-selective NOS inhibitor N (G)-nitro-L-arginine methyl ester; C-PTIO: organic NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide; POE-Hb: haem-based NO scavenger pyridoxylated haemoglobin poly-oxoethylene. The bolt denotes increase in the target concentration, the dashed line denotes a decrease, through enzymatic inhibition (SEITU) or conversion/direct scavenging (C-PTIO and POE-Hb).

B: Study design showing doses and timings of pharmacological inhibitors and activators

ABP: arterial blood pressure; BL: baseline measurement; N1/H1: measurement made 10 min after randomisation to either hypoxia or normoxia; N2/H2: measurement made 20 min after the commencement of pharmacological inhibitors or activators. BGA: arterial blood gas analysis; ECHO: echocardiography; tPO_2: tissue (muscle) PO_2. $NaNO_2$: nitrite-donor sodium nitrite; SEITU: non-selective NOS inhibitor S-ethyl-isothiourea; C-PTIO: organic NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide; POE-Hb: haem-based NO scavenger pyridoxylated haemoglobin poly-oxoethylene.

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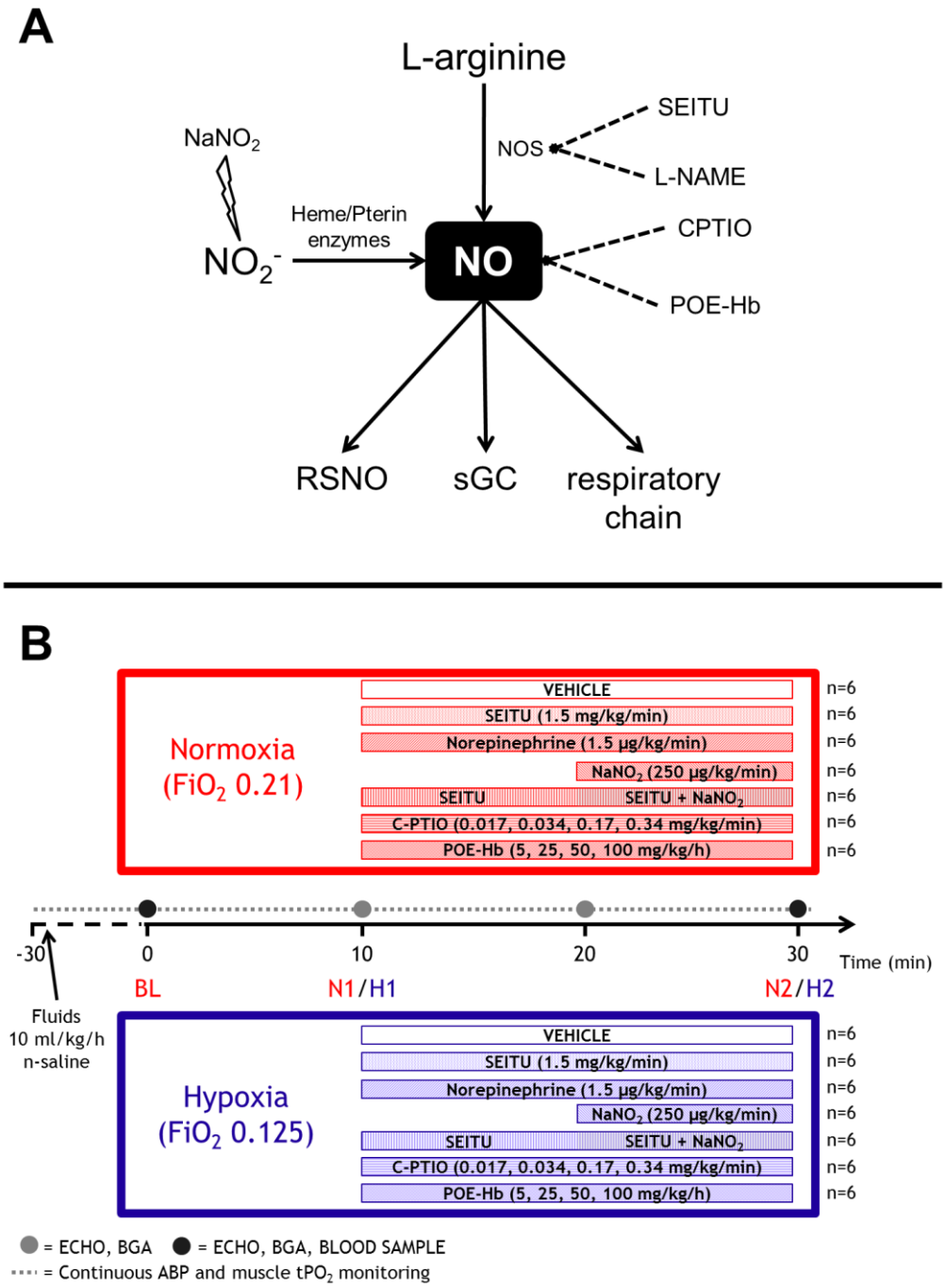

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Figure 2: Haemodynamic effects of modulation of NO bioavailability

NEPI: norepinephrine group; SEITU: non-selective NOS inhibitor S-ethyl-isothiourea group; NaNO2: nitrite-donor sodium nitrite group; C-PTIO: organic NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide group; POE-Hb: haem-based NO scavenger pyridoxylated haemoglobin poly-oxoethylene group.

BL, H1, N1, H2, N2 refer to the timepoints shown in Figure 1

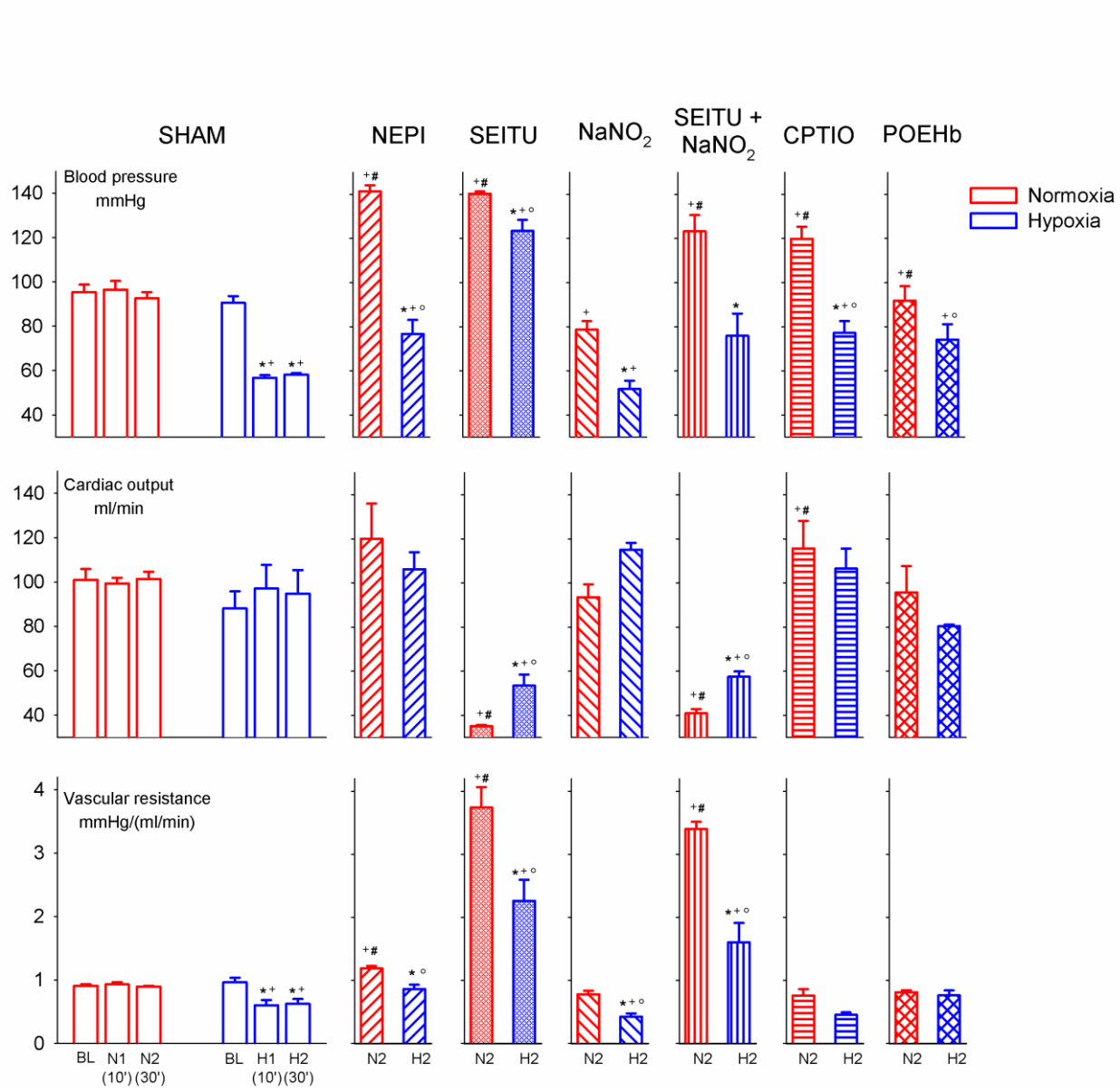

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Figure 3: Plasma metabolites related to oxidation of and nitrosation by NO

NEPI: norepinephrine group; SEITU: non-selective NOS inhibitor S-ethyl-isothiourea group; $NaNO_2$: nitrite-donor sodium nitrite group; C-PTIO: organic NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide group; POE-Hb: haem-based NO scavenger pyridoxylated haemoglobin poly-oxoethylene group. Norm: normoxia; Hypo: hypoxia. NOx: total oxidation products (sum of nitrite and nitrate); RSNO: S-nitrosothiols; RNNO: N-nitrosamines; RXNO: total nitrosation products (sum of RSNO and RNNO). Dashed bars denote the hypoxic groups. *p<0.05 vs normoxia; #p<0.05 vs sham normoxia; °p<0.05 vs sham hypoxia. N=6 animals per group

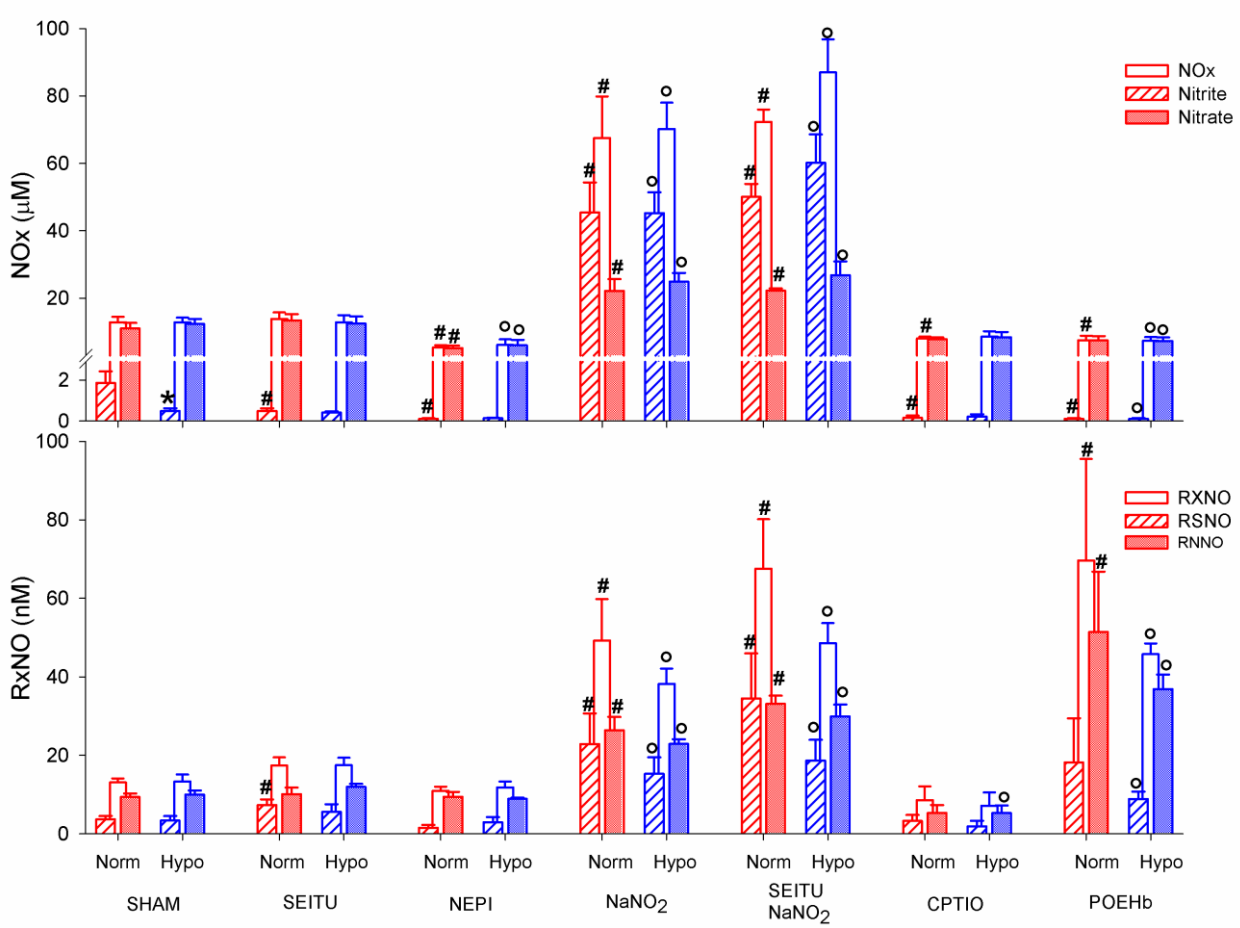

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Figure 4: Erythrocytic metabolites related to oxidation of and nitrosation by NO

NEPI: norepinephrine group; SEITU: non-selective NOS inhibitor S-ethyl-isothiourea group; NaNO2: nitrite-donor sodium nitrite group; C-PTIO: organic NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide group; POE-Hb: haem-based NO scavenger pyridoxylated haemoglobin poly-oxoethylene group. Norm: normoxia; Hypo: hypoxia. NOx: total oxidation products (sum of nitrite and nitrate); RSNO: S-nitrosothiols; RNNO: N-nitrosamines; RXNO: total nitrosation products (sum of RSNO and RNNO). Dashed bars denote the hypoxic groups. *p<0.05 vs normoxia; #p<0.05 vs sham normoxia; °p<0.05 vs sham hypoxia. N= 6 animals per group

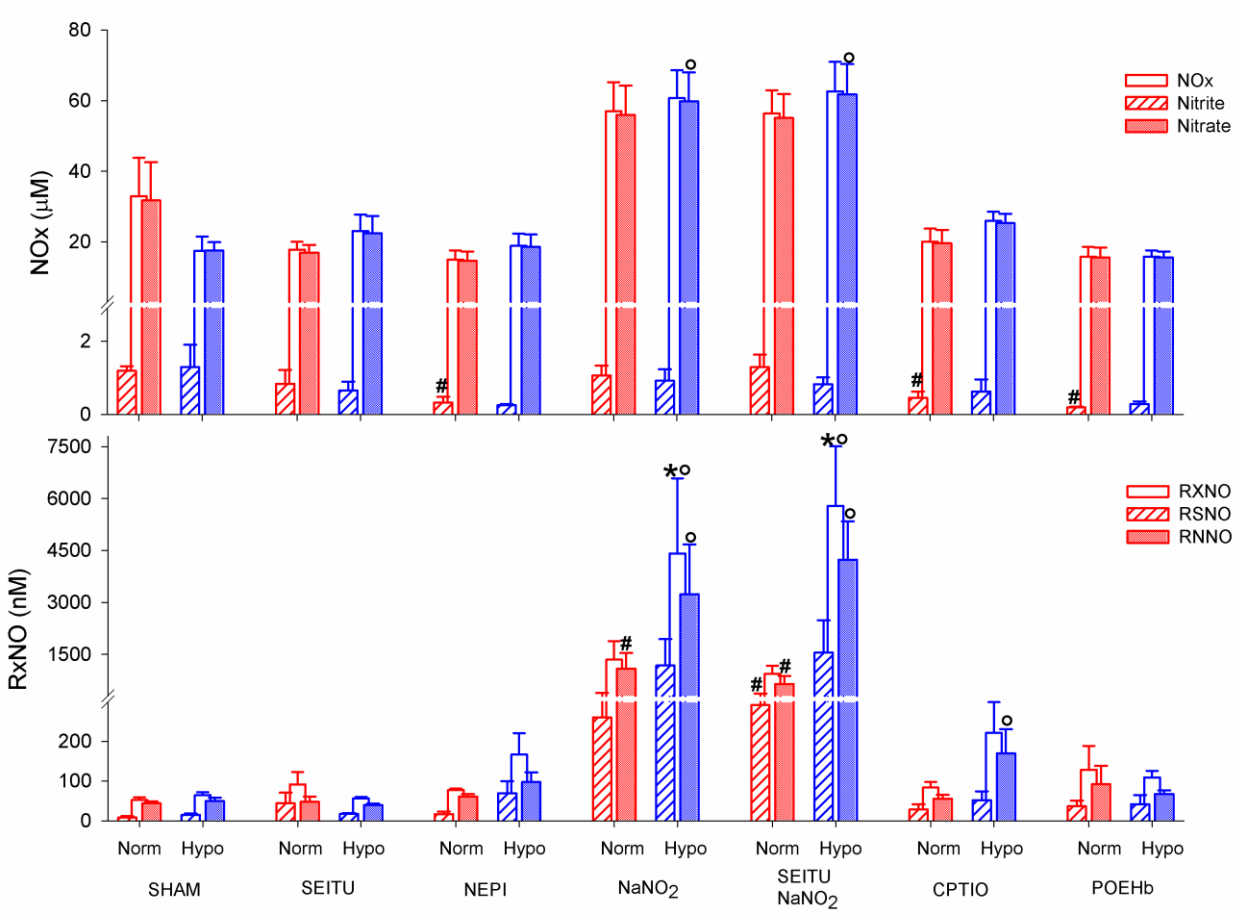

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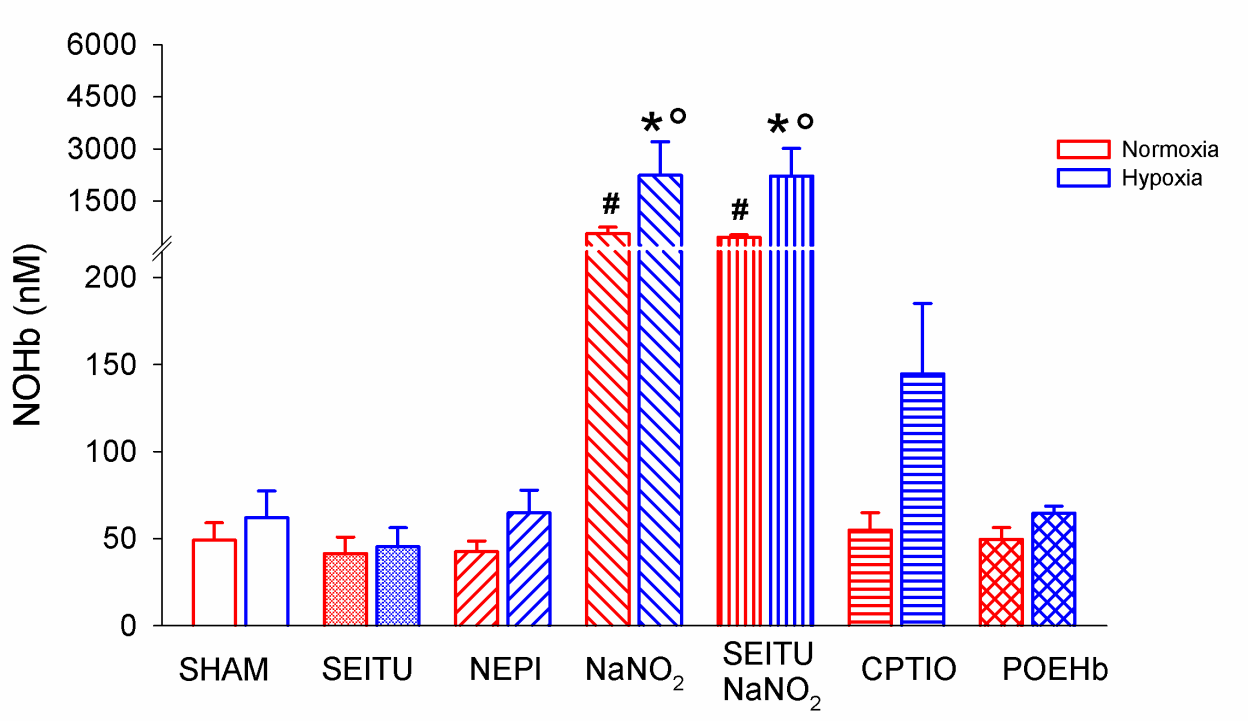
Figure 5: Haem nitrosylation as marker of NO availability in erythrocytes

NEPI: norepinephrine group; SEITU: non-selective NOS inhibitor S-ethyl-isothiourea group; NaNO2: nitrite-donor sodium nitrite group; C-PTIO: organic NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide group; POE-Hb: haem-based NO scavenger pyridoxylated haemoglobin poly-oxoethylene group. NOHb: nitrosyl-haemoglobin. *p<0.05 vs normoxia; #p<0.05 vs sham normoxia; °p<0.05 vs sham hypoxia. N=6 animals per group



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Supplementary figure S1: Dose-response curves

BL: baseline measurement; H/N: measurement after randomisation to hypoxia or normoxia. Open symbols represent normoxic rats, closed symbols are hypoxic rats. SHAM: normoxic and hypoxic control groups; SEITU: non-selective NOS inhibitor S-ethyl-isothiourea group; NEPI: norepinephrine group; POE-Hb: haem-based NO scavenger pyridoxylated haemoglobin poly-oxoethylene group; C-PTIO: organic NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide group; $NaNO_2$: sodium nitrite group. N=4 animals per group.

Supplementary figure S2: Dose-response to L-NAME

BL: baseline measurement; H/N: measurement after randomisation to hypoxia or normoxia. Open symbols represent normoxic rats, closed symbols are hypoxic rats. L-NAME: N (G)-nitro-L-arginine methyl ester. N=3 animals per group.

Supplementary figure S3: Haemodynamic effects of NO scavenging after hypoxia and NOS inhibition and after hypoxia, NOS inhibition and nitrite administration

BL: baseline measurement; SEITU: non-selective NOS inhibitor S-ethyl-isothiourea; NaNO2: sodium nitrite; POE-Hb: haem-based NO scavenger pyridoxylated haemoglobin poly-oxoethylene. *$p<0.05$ vs baseline; #$p<0.05$ vs previous step. N=6 animals per group.

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