Mechanisms Underlying Erythrocyte and Endothelial Nitrite Reduction to Nitric Oxide in Hypoxia
Role for Xanthine Oxidoreductase and Endothelial Nitric Oxide Synthase

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Abstract—Reduction of nitrite (NO$_2^-$) provides a major source of nitric oxide (NO) in the circulation, especially in hypoxic conditions. Our previous studies suggest that xanthine oxidoreductase (XOR) is an important nitrite reductase in the heart and kidney. Herein, we have demonstrated that conversion of nitrite to NO by blood vessels and RBCs was enhanced in the presence of the XOR substrate xanthine (10 µmol/L) and attenuated by the XOR inhibitor allopurinol (100 µmol/L) in acidic and hypoxic conditions only. Whereas endothelial nitric oxide synthase (eNOS) inhibition had no effect on vascular nitrite reductase activity, in RBCs L-NAME, L-NMMA, and L-arginine inhibited nitrite-derived NO production by >50% (P<0.01) at pH 7.4 and 6.8 under hypoxic conditions. Western blot and immunohistochemical analysis of RBC membranes confirmed the presence of eNOS and abundant XOR on whole RBCs. Thus, XOR and eNOS are ideally situated on the membranes of RBCs and blood vessels to generate intravascular vasodilator NO from nitrite during ischemic episodes. In addition to the proposed role of deoxyhemoglobin, our findings suggest that the nitrite reductase activity within the circulation, under hypoxic conditions (at physiological pH), is mediated by eNOS; however, as acidosis develops, a substantial role for XOR becomes evident. (Circ Res. 2008;103:957-964.)

Key Words: blood vessels ■ cardiovascular research ■ hypoxia ■ nitric oxide

Until recently, nitrite (NO$_2^-$) was considered to be merely an inactive metabolite of the pleiotropic molecule nitric oxide (NO). However, recent studies have demonstrated that this view is incorrect, and, indeed, nitrite is now believed to be an important functional vascular mediator. This functionality is thought to lie in its role as an important storage form of NO that is released particularly in situations where conventional NO synthesis, via the L-arginine–NO synthase (NOS) pathway, has been compromised. This reduction to NO has been implicated as underlying nitrite-induced protection against ischemia/reperfusion (I/R) and hypoxic injury in the myocardial, hepatic, renal, pulmonary, and cerebral vasculature. More recently, the functional remit of nitrite has been extended further with the proposal that it is active in physiological conditions. Indeed, nitrite causes dose-dependent vasodilatation in the brachial artery of normal volunteers, and we have recently demonstrated that dietary nitrate, via its bioconversion to nitrite, causes a marked decrease in blood pressure, inhibition of platelet aggregation, and the prevention of endothelial dysfunction following an I/R insult in the human forearm. Such findings support the thesis that nitrite may have an important role in maintaining vascular homeostasis, in addition to its protective effects against cardiovascular disease.

A number of distinct endogenous pathways have been identified in facilitating reduction of nitrite to NO in the circulation. Over and above that achieved by simple chemical acidification, in particular, a role in RBCs for deoxyhemoglobin (deoxyHb) has been proposed. However, this hypothesis is controversial; the known scavenging capacity of oxyHb for NO makes it difficult to envisage how nitrite-derived NO might escape the RBC, although there is recent evidence that this may be achieved via the intermediate N$_2$O$_3$. Indeed, free oxyHb in blood greatly lowers the NO concentration at the endothelial layer. A related globin, deoxymyoglobin, has recently been shown to reduce nitrite and generate NO at a faster rate than deoxyhemoglobin. However, blood vessels lack myoglobin, indicating that,

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although likely important for cardiomyocytes, other pathways are likely to be more important within the circulation.

Endothelial (e)NOS has been localized to the internal surface of the RBC membrane, and both recombinant bovine and murine endothelial cell eNOS have been shown to reduce nitrite to NO under anaerobic conditions in vitro. However, the presence of functional NOS in RBCs remains controversial, and whether it has potential for nitrite reductase activity in this location has not been tested. Our own work has implicated xanthine oxidoreductase (XOR) in facilitating nitrite reduction in the rat and human heart and rat kidney, confirming the results of previous studies using purified XOR. However, whether RBCs contain and use XOR to reduce nitrite is unknown.

Although it is likely that the RBC is an important source of nitrite-derived NO within the circulation, other cellular sites within the blood vessel are also likely to contribute to the nitrite reductase capacity of the circulation. Of particular interest is the possibility that a significant component of this activity resides within the blood vessel wall. Our previous findings in the isolated Langendorff rat heart demonstrated that nitrite-derived NO generation was abolished following removal of the endothelium, highlighting the likely importance of the endothelium and the blood vessel wall in circulatory nitrite reduction. Moreover, because XOR and eNOS are present on the endothelium, there is a clear rationale for proposing it as a site of NO production from nitrite.

Herein, we demonstrate the nitrite reductase activities of both XOR and eNOS in suspensions of washed RBCs prepared from blood taken from healthy volunteers and XOR activity in supernatants of human blood vessels.

Materials and Methods

Blood and Tissue Collection

Animal Studies

Experiments were conducted in accordance with the Animals (Scientific Procedures) Act, UK 1986. Male Wistar rats (250 to 350 g; Charles River) were euthanized by cervical dislocation, and aortae and vena cavae were removed. Tissues were washed in saline and snap-frozen in liquid N2. At a later date, supernatants were prepared as previously described.

Human Studies

All studies involving human subjects were conducted with the approval of the local ethics research committee (NELCHA REC: P2/04/045 and 04/Q0603/118). As above, supernatants were prepared from samples of excess left internal mammary artery obtained from patients undergoing coronary artery bypass grafting.

RBCs were purified from blood (15 mL) collected from healthy volunteers (see the expanded Materials and Methods section in the online data supplement, available at http://circres.ahajournals.org).

Measurement of NO Production

NO production from tissue supernatants or washed RBCs (10 µL) in the presence of sodium nitrite (10 to 100 µmol/L) at pH 7.4 (physiological levels), pH 6.8 (representing clinically severe acidosis in the blood), and pH 5.5 (representing tissue ischemia) was determined using ozone chemiluminescence, as previously described.

Mechanisms of Nitrite Reduction

To investigate the role of XOR in tissue-dependent nitrite reduction samples were incubated with the selective XOR inhibitor allopurinol (100 µmol/L) or diphenyleneiodonium (DPI) (10 µmol/L) or the XOR substrates xanthine (10 µmol/L) or NADH (1 mmol/L). To investigate the role of NOS, supernatants were treated with the NOS inhibitors Nω-methyl-L-arginine (L-NNAME) (300 µmol/L) or Nω-methyl-L-arginine (L-NMMA) (300 µmol/L), the NOS substrate L-arginine (300 µmol/L), or inactive isomer D-arginine (300 µmol/L). All drug pretreatments were for 30 minutes before nitrite incubation.

Fluorometric XOR Assay

Conventional XOR activity was determined in rat aorta and vena cava and human plasma using a specific fluorometric assay (see supplemental Materials and Methods).

XOR Immunohistochemistry

RBC pellets were obtained and prepared for immunohistochemistry and sections (4 µm) incubated with a rabbit polyclonal antibody against bovine buttermilk XOR (1:50; Chemicon) for visualization of XOR expression (see supplemental Materials and Methods).

Western Blot Analysis for eNOS

RBC membranes were prepared as previously described. Western blotting for eNOS was conducted as previously described using an eNOS (C-20) rabbit polyclonal antibody (1:2000 dilution; Santa Cruz Biotechnology). Bands were detected by enhanced chemiluminescence (ECL, Amersham) and autoradiographic film.

Human Endothelial Cell Experiments

Human umbilical vein endothelial cells (HUVECs) treated with the calcium ionophore A23187 (10 µmol/L; 10 minutes before nitrite) to activate eNOS were then exposed to nitrite (0.1 to 1000 µmol/L; 30 minutes) before collection of cells for measurement of cGMP as an index of NO generation under normoxic or hypoxic conditions (see supplemental Materials and Methods).

Data Analysis and Statistics

Data processing was performed with GraphPad Prism software (version 4). All data are shown as means±SEM. Statistical significance was tested using the paired t test and 1-way ANOVA with Bonferroni’s correction was used for multiple group comparisons. P<0.05 was considered significant.

Results

Blood Vessel Nitrite Reductase Activity

Under anaerobic conditions, rat aortic supernatants facilitated NO generation from nitrite: an effect greatly enhanced with increasing acidity from pH 7.4 to pH 5 (Figure 1A) and greater than that achieved by chemical acidification alone (ie, in the absence of tissue) or the addition of tissue alone (ie, absence of nitrite; see the expanded Results section and Figure I in the online data supplement). NO generation was also evident in the presence of O2, although this was substantially reduced compared to anaerobic conditions (Figure 1B). Vena cava had very similar activity to aorta under anaerobic or oxygenated conditions (Figure 1C). For all further experimentation, nitrite reduction was assessed under anaerobic and, predominantly, acidic pH conditions to simulate the ischemic environment.

Denaturation (by boiling the aorta supernatant) abolished tissue facilitated reduction of nitrite under anaerobic and pH 5.5 conditions (Figure 2A). At this pH, allopurinol suppressed (P<0.01) NO production by ~20% (Figure 2B), whereas...
L-NAME and L-NMMA had no significant effect (Figure 2C). Xanthine (n=6) and NADH (n=5) both significantly (P<0.05) elevated nitrite-derived NO production by 57±18% and 37±8%, respectively (data not shown). DPI had no significant effect on NO production (Figure 2D). Similarly to rat aorta, homogenates of human LIMA expressed significant nitrite reductase activity that increased with decreasing pH (rate of NO production=74.6±83.9 pmol/g per second (n=6) at pH 6.8, 103.0±18.6 pmol/g per second (n=3) at pH 6.0, and 367.0±75.0 pmol/g per second (n=8) at pH 5.5 under anaerobic conditions. At pH 5.5, the effect of nitrite was significantly attenuated by allopurinol (n=5) and enhanced by xanthine (n=5; Figure 2E).

Fluorometric XOR Assay
Whereas the XO activity of rat aorta and vena cava supernatants were similar (Figure 3A), the XDH activity of vena cava was double that of aorta supernatants (Figure 3B), resulting in significant differences between the total XOR activity between the 2 vessel types (Figure 3C).

RBC Nitrite Reductase Activity
The addition of RBCs to nitrite resulted in a significant increase in NO production above that caused by simple chemical disproportionation of nitrite in the absence of RBCs (Figure 4). The rate of NO production was greater with deoxygenated, rather than oxygenated, RBCs and was further enhanced by increasing nitrite concentration and decreasing pH (Figure 4).

At pH 7.4, neither xanthine nor allopurinol affected nitrite-derived NO production (Figure 4C). However, at pH 6.8, allopurinol resulted in ≈65% inhibition of erythrocyte-related NO production, whereas xanthine increased NO production by ≈43% (Figure 4D). L-NAME inhibited NO production by ≈60% and 75% at pH 7.4 and pH 6.8, respectively (Figure 4E and 4F). L-Arginine also inhibited NO production by ≈50% and ≈60% at pH 7.4 and pH 6.8, respectively, whereas the inactive isomer, D-arginine had no significant effect.

Specific staining for XOR on RBCs demonstrated the presence of XOR, predominantly within the membrane, on RBCs collected from healthy volunteers (Figure 5C and 5D). In addition, expression of eNOS protein was confirmed on RBC membranes collected from blood of healthy volunteers (Figure 5C and 5D). Wherever XOR, predominantly within the membrane, on RBCs collected from healthy volunteers (Figure 5C and 5D). In addition, expression of eNOS protein was confirmed on RBC membranes collected from blood of healthy volunteers (Figure 5C and 5D).

Nitrite Reductase Activity of HUVECs
Basal levels of cGMP in HUVECs in the absence of ionophore under normoxic and hypoxic conditions were 12.2±1.7 fmol per well (n=5) and 4.4±2.7 fmol per well (n=3), respectively. A23187 appeared to raise basal cGMP levels in HUVECs under both normoxic (17.1±1.2 fmol, n=7) and hypoxic (10.3±2.8 fmol, n=6) conditions; however, neither of these reached significance. Nitrite induced concentration-dependent elevation of cGMP in hypoxic conditions only (EC50=2.0±0.6 μmol/L; maximum=37.0±4.0 fmol; n=4 to 7), an effect peaking at 10 μmol/L and decreasing with further increasing nitrite concentration (100 μmol/L, 1000 μmol/L; Figure 6B). NOS inhibition abolished nitrite-induced (10 μmol/L) elevations of cGMP under hypoxic conditions.
conditions (n=6, P<0.001). Under normoxic conditions only, 1000 μmol/L nitrite was able to cause elevation of cGMP levels (31.1±4.3, n=5, P<0.01); however, this response was abolished by treatment with L-NMMA (7.5±0.7, n=4, P<0.001).

Discussion
We have demonstrated that XOR plays a major role in vascular nitrite reductase activity under conditions of hypoxemia and acidosis. We show that XOR is localized to at least 2 distinct sites within the circulation: the blood vessel wall (likely on the endothelial cell because previous studies in the isolated heart preparation demonstrate abolition of nitrite reduction following endothelium denudation)3; and the RBC. Furthermore, we have confirmed the presence of eNOS on RBCs and provided the first demonstration, ex vivo tissue, of the nitrite reductase function of eNOS. As such, we propose that there are 2 enzymes with nitrite reductase activity ideally located within the circulation (Figure 7) to facilitate the production of NO in ischemic conditions in addition to, and likely complementing, the nitrite reductase activity of intracellular deoxyHb.12

Nitrite Reductase Activity in the Circulation
The nitrite reductase activities of human LIMA and rat aorta/vena cava were similar to that demonstrated previously with human right atrium and rat heart (ie, activity increased with hypoxia and with decreasing pH).3 The rate of NO production in these tissues under ischemic conditions (=0.5 nmol/g per second) was similar to the levels produced by
Nitrite Reductase Activity of XOR and eNOS

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Figure 5. Evidence for XOR on RBC membrane. A, Hematoxylin/eosin stain of washed RBCs (×60 magnification). B through D, Immunohistochemistry for XOR on RBCs. B, Negative control (×60 magnification). C, XOR antibody (×60 magnification). D, XOR antibody (×100 magnification).

conventional NO synthesis pathways (ie, NOS) under oxygenated conditions, indicating the likely functional relevance of the levels of NO synthesis in our study. In addition, comparison between the aorta and the vena cava revealed almost identical nitrite reductase activity demonstrating no venseselectivity in NO-generating capacity, unlike the organic nitrates. The degree of nitrite reductase activity was dependent on the prevailing conditions of anaerobiosis and acidosis, rather than the vessel type, consistent with recent demonstrations of hypoxia-dependent dilatation of vessels to nitrite. Interestingly, at physiological pH, very little nitrite reductase activity was evidenced, suggesting that within the blood vessel wall there is little capacity for reduction of nitrite under physiological conditions. These findings imply that the blood vessel is unlikely to play a role in the nitrite reductase activity that has been demonstrated under physiological conditions.

RBCs displayed a similar profile of nitrite reductase activity to blood vessels (ie, increased generation of NO under acidic and hypoxic conditions). The maximal measurable rate of synthesis was ~10-fold less than that evidenced in the vascular tissue. It is possible that this difference reflects a lesser role for the RBC in provision of nitrite-derived NO production in an ischemic environment, although clearly the magnitude of the role for RBC will depend on the number present within a specific volume at any 1 time. In clinical practice, the lowest pH encountered on blood gas analysis in profoundly acidotic patients is ~6.8 to 6.9 and, therefore, we tested the effect of pH 6.8 on RBC nitrite reductase activity. Interestingly, in contrast to blood vessels, RBCs displayed significant nitrite reductase activity during hypoxia at pH 7.4, indicating that acidosis is not essential for this function. Furthermore, this raises the possibility that nitrite reduction may occur under physiological conditions, as has been demonstrated in other recent studies, although, under oxygenated conditions, we failed to measure any significant nitrite reduction at pH 7.4 by RBCs or blood vessel supernatants (data not shown).

Blood Vessel XOR-Dependent Reduction of Nitrite

The significant inhibition of nitrite reductase activity of rat aorta and vena cava and human LIMA by allopurinol suggests a contribution by XOR. Indeed, XOR is likely to be a key nitrite reductase in blood vessels because vascular smooth muscle cells lack expression of the other key proteins (globins) implicated in nitrite reduction, particularly myoglobin. Substantial evidence supports the concept that nitrite is reduced directly to NO at the molybdenum site of XOR. However, there is some evidence suggesting that the bioactivity of nitrite relates to the formation of S-nitrosothiol intermediates. Interestingly, purified XOR has been shown to facilitate S-nitrosothiol decomposition to NO in a DPI-sensitive manner, implicating the flavin adenine dinucleotide site of the enzyme in this activity. In the present study, DPI had no effect on nitrite-derived NO production, suggesting that S-nitrosothiol formation is unlikely to underlie this process in blood vessels under hypoxic conditions. Interestingly, the inhibitory activity of allopurinol was substantially greater in LIMA than in rat aorta (ie, 75% compared to 25%), suggesting that although XOR is the predominant enzyme in human blood vessels, other, as of yet unknown, mechanisms contributed to the activity in rat blood vessels. The marked increases in nitrite reductase activity following incubation with xanthine further implicate the involvement of XOR and suggest the importance of adequate concentrations of substrate, which may be lost during processing of the samples. The concentration of xanthine (10 μmol/L) used for the present study was chosen because peak increases in the nitrite reductase activity of XOR have
been demonstrated with 10 to 20 μmol/L, whereas concentrations >20 μmol/L result in XOR inhibition, thought to be attributable to competition between xanthine and nitrite at the molybdenum site. NADH is a less potent substrate for XOR in terms of its nitrite reductase activity, acting at the flavin adenine dinucleotide site; however, provision of NADH also increased activity by ~35% at a concentration of 1 mmol/L. This upregulation of activity by 2 distinct XOR substrates provides further confirmation of an important role for XOR in vascular nitrite reductase activity. Conventional XOR activity was confirmed in the tissues using a validated fluorometric method. Whereas the XO activity was similar between aorta and vena cava, XDH activity was significantly greater in vena cava compared to aorta, suggesting that the nitrite reductase activity, which was similar between the tissues, is likely dependent on XO rather than XDH activity. This echoes the activity of the 2 forms with respect to other facets of XOR enzymology, in particular, superoxide production where XO reduces O₂ 4 times faster than XDH. Our evidence would suggest that the nitrite reductase activity of XOR lies with the XO form of the enzyme. Exactly why this might be the case is uncertain at present and warrants further investigation.

RBC XOR-Dependent Reduction of Nitrite

Another major site for nitrite reduction in the circulation is at the level of the RBC, although this has been predominantly attributed to the activity of deoxyHb. However, we tested the hypothesis that XOR might also contribute to the nitrite reductase activity of RBCs. Indeed, the inhibitory effect of allopurinol at pH 6.8 suggested that XOR activity accounted for ~2/3 of all nitrite-derived NO production. Although xanthine showed a trend for an effect at pH 7.4, allopurinol had no effect, consistent with the thesis that the nitrite reductase activity of XOR is upregulated, in concert with its conventional activity, under pathological conditions, with increasing acidosis and during hypoxia, but is likely less active under normal physiological conditions and has little or no role to play at physiological pH.

In cultured human endothelial cells XOR has been localized to the outer surfaces of the cell membrane and, indeed, our immunohistochemical studies of human RBCs, for the first time, have identified the abundant presence of XOR particularly on the membrane. The presence of XOR on RBCs was postulated in 1998 and confirmed, e.g., in RBCs in an experimental model of guinea pig otitis media and in patients with lung cancer, atherosclerosis, and autism. RBC XOR is most likely derived from circulating XOR in the plasma. XOR is released into the circulation from the liver, especially following periods of metabolic stress, and carried to vascular endothelium in distant parts of the circulatory tree. Our studies herein suggest that just as circulating XOR binds to the surface of endothelial cells, it may also bind to RBCs.

RBC NOS

Surprisingly, NOS inhibition substantially diminished RBC nitrite reductase activity at pH 7.4 and pH 6.8. The presence of NOS in RBCs was postulated in 1998 and confirmed,
using confocal microscopy, by Kleinbongard et al.\(^2\) and further supported by Western blotting for eNOS in the present study. In 2006 Gautier et al, using recombinant eNOS, identified nitrite reductase activity of the oxygenase domain during anoxia,\(^2\) and then went on to confirm this activity in cultured endothelial cells.\(^2\) Using concentrations of nitrite closer to the physiological range, we now demonstrate that such a mechanism is not limited to endothelial cells but provides RBCs with a further source of nitrite-derived NO at the membrane, free from scavenging by intracellular oxyHb.

In the present study, L-arginine, but not L-arginine, inhibited nitrite-derived NO formation by RBCs. This inhibition may be a reflection of competition for the active site, the heme domain of NOS, between L-arginine and nitrite and may be a reflection of competition for the active site, the heme domain of NOS, between L-arginine and nitrite and clearly warrants further investigation (Figure 7). That L-arginine did not cause as great a degree of inhibition as L-NAME may reflect that it is a substrate for NOS and, therefore, would result itself in the generation of NO. Nonetheless, the ability of both L-NAME and L-arginine to block nitrite reduction confirms that this occurs at the heme site. Gautier et al postulated that the crossover of eNOS activity from arginine to nitrite consumption relates to the ambient O\(_2\) levels.\(^2\) This group demonstrated that the rate of NO production by eNOS from L-arginine was reduced 60-fold in hypoxemic tissues in comparison to normoxic tissues. However, they also showed that under hypoxic conditions nitrite-derived NO production was 6-fold greater than that generated from L-arginine, suggesting that this eNOS-dependent nitrite-derived NO production may provide a compensatory synthetic pathway for endothelial cells to provide NO in acute hypoxemia when conventional L-arginine-derived NO is suppressed.

To further confirm our findings that eNOS contributes to nitrite reduction under hypoxic conditions, we measured cGMP synthesis as a measure of nitrite-derived NO production in a cell type that expresses substantial eNOS protein: HUVECs. Interestingly, in these cells, we found that under normoxic conditions, nitrite did not cause concentration-dependent increases in cGMP synthesis, whereas, in contrast, it was a potent inducer of cGMP under hypoxic conditions, an effect blocked by NOS inhibition. Thus, once conventional NO synthesis is blocked by an absence of O\(_2\), endothelial cells will convert nitrite (at concentrations found in vivo ie, 0.1 to 10 \(\mu\)mol/L) to generate NO. The presence of this pathway in both RBCs and endothelial cells\(^2\) (which lie within a RBC-free zone\(^2\)) would enable local generation of protective NO, under hypoxic conditions, across the blood vessel that has the capacity to repress many of the pathogenic activation events that occur within the cellular components of the circulation (RBCs, platelets, and leukocytes), as well as within the blood vessel wall (via the endothelium). There is substantial support for the theory that within the circulation nitrite reductase activity is, at least in part, dependent on interactions with deoxyHb.\(^1\) In particular, it has been proposed that such interactions underlie the vasodilator effects of exogenously administered nitrite in physiological conditions, such as that evidenced in the human forearm,\(^9\) but also underlie endogenous nitrite reduction associated with exercise or moderate hypoxia-induced vasodilatation in humans and primates.\(^9,10\) Our findings now suggest, therefore, that in addition to the proposed role of deoxyHb,\(^1\) eNOS likely plays a major role in mediating the nitrite reductase activity evident within the circulation under physiological pH conditions.

In summary, we have demonstrated that both blood vessels and RBCs reduce nitrite to NO through the enzymatic actions of XOR and eNOS. In addition, our demonstration of the presence of both enzymes within the RBC membrane suggest a potential advantage over intracellular nitrite reductases, because they are ideally located to facilitate NO release for functional effects in the vasculature while simultaneously avoiding the problems of Hb scavenging.

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References
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SUPPLEMENTAL INFORMATION

Mechanisms underlying erythrocyte and endothelial nitrite reduction to NO: role for xanthine oxidoreductase and eNOS


Running Title: Nitrite reductase activity of XOR and eNOS
MATERIALS AND METHODS

Preparation of RBC and RBC membranes

The blood was centrifuged at 1600 rpm for 5 min at 25°C. The plasma and buffy layer were removed. The RBCs were washed in phosphate buffer solution (PBS) and the centrifuge/wash process repeated twice.

For RBC membranes 4.33 ml of washed RBC was added to 60.67 ml of phosphate buffer solution (PBS; 5 mmol), kept on ice for 30 min and then centrifuged at 15000 rpm (100,000 g) for 15 min (Beckman Ultracentrifuge). The RBC membrane pellet was washed. The wash step was repeated a further two times until the RBC membrane pellet was white and the supernatant had lost its pinkish tinge. To dissolve the membrane, 1 ml of 1% Igepal was added to the membrane and incubated for 48 hours. The supernatant was then removed and aliquoted into eppendorfs in preparation for the Bradford Assay.

Determination of RBC protein concentration

RBC protein levels are difficult to ascertain due to the interference of hemoglobin with standard colorimetric assessment of protein concentration. Due to this difficulty we estimated hemoglobin concentration in each sample and expressed all NO production relative to this. Haemoglobin concentration was measured using the Beckman Coulter LH750 Haematology analyser. A lysed sample solution is added to the Haemoglobin cuvette for measurement. Briefly light (wavelength of 525nm) transmission was assessed and levels of hemoglobin in samples determined relative to a standard curve. The mean Hb concentration of the blood samples collected in these studies was 29.4±0.8 g/dl (n=?) with no differences between groups.

Human umbilical vein endothelial cell studies

Human umbilical vein endothelial cells (HUVECs at ~300,000 cells per well: cultured in EGM-2 media, Lonza) were treated with potassium nitrite (0.1-1000µM; 30 min) prior to collection of cells for measurement of cGMP (GE Healthcare; RPN226) as a functional index of NO generation. Cells were exposed to normoxic (21% O₂, 5% CO₂) or hypoxic
conditions (5% O₂, 5% CO₂; Coy hypoxic glove box, Coy laboratory products Ltd) during nitrite treatment; with and without 500uM L-NMMA. All HUVECs were treated with the phosphodiesterase inhibitor, IMBX (100uM; 20min prior to nitrite) and the calcium ionophore A23187 (10µM; 10min prior to nitrite) to activate eNOS.

**Fluorometric XOR assay**

This assay was based on the method of Beckman *et al.,* (1989) measuring the rate of production of isoxanthopterin from pterin¹ using an Hitachi F-4500 Fluorescence Spectrophotometer: in our hands the greatest sensitivity was found with an excitation wavelength of 340 nm and an emission wavelength of 400 nm and this was used for all studies. The increase in fluorescence following the addition of a known concentration of isoxanthopterin (0.01 µM) at the end of each experiment served as an internal standard, against which the rates of XO and XDH activity could be calculated.

**XOR immunohistochemistry**

RBC pellets were obtained by centrifuging washed RBCs for 5 mins at 500 × g followed by resuspension and fixing in 4% paraformaldehyde (5 ml w/v in PBS) for 20 min at room temperature. Subsequently, the cells were washed and centrifuged again at 500 × g for 5 min, and then suspended in warmed 2% agarose gel (w/v in distilled water). A hard pellet was formed on cooling which was covered in 70% ethanol. The following day, the pellet was embedded in a wax paraffin block. Sections (4µm) were incubated with a rabbit polyclonal antibody against bovine buttermilk XOR (1:50; Chemicon,UK), for 35 min; negative controls were incubated in PBS only. Non-specific secondary antibody binding was blocked by incubating with goat serum (1:25) in PBS for 15 min. All slides were then washed and incubated in biotinylated goat anti-rabbit antibody (1:300) for 35 min. Slides were developed in 3,3' diaminobenzidine for 2 min, washed and counterstained with hematoxylin or methyl green.
RESULTS

Aortic tissue homogenate, under hypoxic and pH 6.8 conditions, did not produce measureable levels of NO in the absence of nitrite treatment. However, upon the addition of nitrite (100µM) substantial NO synthesis was evident above that achieved by chemical acidification alone that was not inhibited by the NO synthase inhibitor, L-NAME (300µM).

**Online Figure** I Chemical acidification (pH 6.8) under hypoxic conditions of aortic homogenates is insufficient to produce measurable NO production using gas phase chemiluminescence. Values shown are net of the effect of chemical acidification of nitrite and are expressed as mean ± s.e.mean of n=8. Statistical significance shown as ** for P<0.01 using one-way ANOVA followed by Bonferroni post-test.
Reference