nature medicine

Nitrite reduction to nitric oxide by deoxyhemoglobin vasodilates the human circulation

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Nitrite anions comprise the largest vascular storage pool of nitric oxide (NO), provided that physiological mechanisms exist to reduce nitrite to NO. We evaluated the vasodilator properties and mechanisms for bioactivation of nitrite in the human forearm. Nitrite infusions of 36 and 0.36 μmol/min into the forearm brachial artery resulted in supra- and near-physiologic intravascular nitrite concentrations, respectively, and increased forearm blood flow before and during exercise, with or without NO synthase inhibition. Nitrite infusions were associated with rapid formation of erythrocyte iron-nitrosylated hemoglobin and, to a lesser extent, *S*-nitroso-hemoglobin. NO-modified hemoglobin formation was inversely proportional to oxyhemoglobin saturation. Vasodilation of rat aortic rings and formation of both NO gas and NO-modified hemoglobin resulted from the nitrite reductase activity of deoxyhemoglobin and deoxygenated erythrocytes. This finding links tissue hypoxia, hemoglobin allostery and nitrite bioactivation. These results suggest that nitrite represents a major bioavailable pool of NO, and describe a new physiological function for hemoglobin as a nitrite reductase, potentially contributing to hypoxic vasodilation.

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Nitrite is a vasodilator at high concentrations *in vitro*[1–6]. *In vivo* plasma levels of nitrite are in the range of 150–1,000 nM, and the concentration in aortic ring tissue is >10 μM (refs. 7–9). This potential storage pool for NO is in vast excess of plasma *S*-nitrosothiols, reported to be <10 nM in human plasma[9–12]. Mechanisms for the *in vivo* conversion of nitrite to NO have been proposed to involve either enzymatic reduction with xanthine oxidoreductase, or nonenzymatic disproportionation or acidic reduction[13–21]. Each mechanism would occur preferentially in vascular regions with low pH and low partial pressure of oxygen (pO_2). Indeed, consistent with oxygen- and pH-sensitive chemistry, hypoxia and acidosis potentiate NO generation and vasodilation from both nitrite and NO donors in aortic ring and lung perfusion bioassay systems[22–24]. However, the extremely low oxygen tension and pH necessary for nitrite reduction by xanthine oxidoreductase and disproportionation, as well as the high nitrite concentrations required for vasodilation in previous *in vitro* studies, have cast doubt on the role of this anion as a vasodilator. Indeed, no vasodilatory effects were reported when nitrite was infused into the forearm circulation of three human subjects for 1 min (ref. 25). That study suggested that under physiological conditions, nitrite would not function as an intravascular storage pool for NO and, thus, was not an intrinsic vasodilator.

Consistent with the bioconversion of intravascular nitrite to NO, we and others have observed arterial-to-venous gradients of nitrite across the human forearm at rest and during regional NO synthase inhibition, with increased consumption of nitrite occurring during exercise[8,26]. Other research groups have reported large arterial-to-venous gradients of nitrite also form across the human forearm during NO synthase inhibition[25]. Unlike the simpler case of oxygen extraction across a vascular bed, nitrite is both consumed—as evidenced by arterial-to-venous gradients during NO synthase inhibition and exercise—and produced in the vascular bed by endothelial NO synthase–derived reactions of NO with oxygen. Supporting the existence of an intravascular NO species capable of storage and distal delivery of NO bioactivity, multiple research groups have observed that red blood cells and plasma 'loaded' with NO, by exposure to NO solutions, NO gas or NO donors, can export an 'NO-like' bioactivity and induce vasodilation *in vitro* and *in vivo*[11,27–32]. We have previously evaluated the reaction products formed in human blood during inhalation of NO gas, and found significant increases in plasma nitrite and limited formation of plasma and erythrocyte *S*-nitroso-proteins, suggesting a role for nitrite in the transduction of NO bioactivity along the vasculature[29]. We therefore considered the possibility that nitrite,

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Published online 2 November 2003; doi:10.1038/nm954

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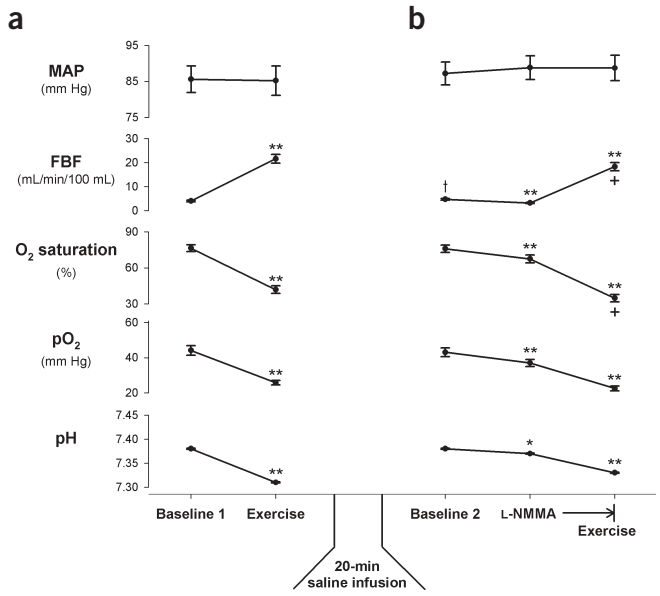

Figure 1 Hemodynamic and metabolic measurements at baseline and during exercise (protocol, part I). Measurements were taken without (**a**) and with (**b**) inhibition of NO synthesis in 18 subjects. Mean arterial pressure (MAP), forearm blood flow (FBF), venous oxyhemoglobin saturation (O_2 saturation), pO_2 and pH are shown for all experimental conditions. These interventions and measurements (part I of the protocol) served as a control for part II of the protocol, in which these interventions were performed during nitrite infusion. *, $P < 0.05$ versus baseline 2; **, $P < 0.01$ versus baselines 1 or 2, respectively; †, $P < 0.05$ versus baseline 1; +, $P < 0.01$ versus exercise. Error bars denote s.e.m.

rather than *S*-nitrosothiols, is the largest intravascular storage pool for NO, and that nitrite bioactivation to NO could vasodilate regions with tissue oxygen debt in the human circulation.

RESULTS

Vasodilatory properties of nitrite *in vivo*

Eighteen healthy subjects (nine males and nine females, aged 21–50 years) were enrolled in a physiological study to determine whether nitrite is a vasodilator and to examine nitrite's *in vivo* chemistry. In part I of the protocol, the normal hemodynamic and metabolic responses to exercise and to inhibition of NO synthesis in the forearm were measured as controls for part II, in which these interventions were done during nitrite infusion (**Fig. 1a,b**; see **Supplementary Note** online for detailed description of control observations). Parts I and II were conducted in random order.

To determine whether nitrite has vasoactivity in humans, in part II of the protocol we infused sodium nitrite in bicarbonate-buffered saline (final concentration of 36 μmol/ml) into the brachial arteries of the 18 subjects, to achieve an estimated intravascular nitrite concentration of ~200 μM (ref. 25). After infusion of sodium nitrite at 1 ml/min for 5 min, nitrite levels in the ipsilateral antecubital vein increased to 221.82 ± 57.59 μM (**Fig. 2a**). Forearm blood flow increased by 175% over resting values; venous hemoglobin oxygen saturation, pO_2 and pH levels increased significantly (all $P < 0.01$) over preinfusion values, indicating increased perfusion of the forearm.

The systemic nitrite concentration was 16 μM, as measured in the contralateral arm, and was associated with a systemic decrease in mean blood pressure of ~7 mm Hg ($P < 0.01$). Consistent with immediate NO generation from nitrite during arterial-to-venous transit, iron-nitrosylated hemoglobin in the ipsilateral antecubital vein increased from 55.7 ± 11.4 to 693.4 ± 216.9 nM during nitrite infusion. During forearm exercise (with continued nitrite infusion), blood flow increased further. Metabolic stress was present, as evidenced by reduced forearm venous hemoglobin oxygen saturation, pO_2 and pH levels compared with baseline values. Venous nitrite levels declined, indicating that increased blood flow to the forearm was diluting the concentration of infused nitrite. Despite decreasing forearm nitrite concentration during exercise, iron-nitrosylated hemoglobin levels increased, indicating an augmented rate of NO generation from nitrite during exercise stress (**Fig. 2a**).

After cessation of nitrite infusion and substitution of saline as the intra-arterial infusate for 30 min, repeat baseline measurements showed persistent elevations in systemic levels of nitrite, iron-nitrosylated hemoglobin and methemoglobin (**Fig. 2b**) compared with values obtained almost 1 h previously, before the infusion of nitrite. A persistent vasodilator effect was also apparent, as forearm blood flow was significantly higher (4.79 ± 0.37 versus 3.94 ± 0.38 ml per min per 100 ml tissue; $P = 0.003$) and systemic blood pressure was significantly

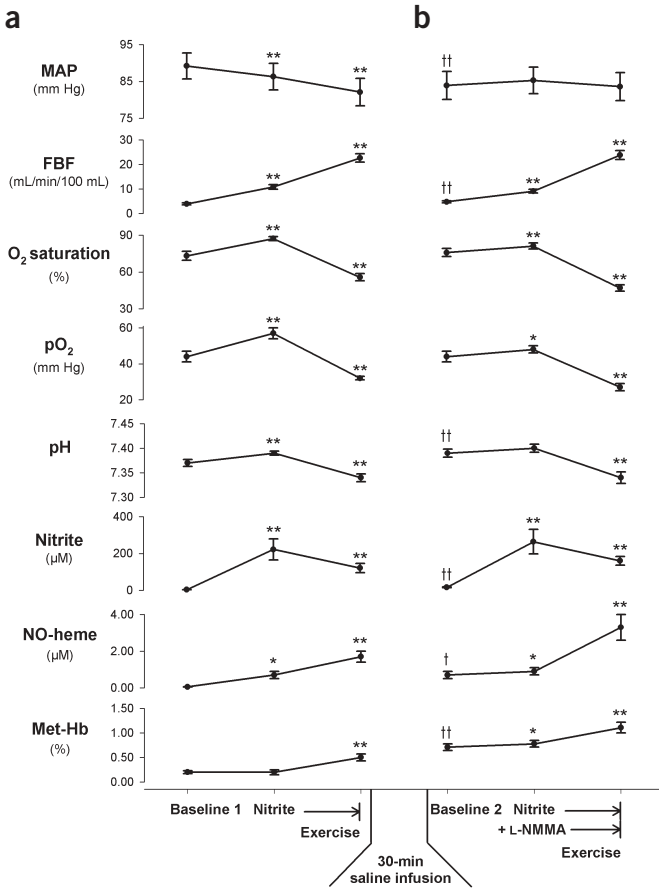

Figure 2 Effects of nitrite infusion. $NaNO_2$ was infused into the brachial arteries of 18 healthy subjects for 5 min at baseline and continued during exercise, without (**a**) or with (**b**) inhibition of NO synthesis with L-NMMA (protocol 1, part II). Values for mean arterial blood pressure (MAP), forearm blood flow (FBF), venous oxyhemoglobin saturation, pO_2, pH, venous nitrite, venous iron-nitrosylated hemoglobin (NO-heme) and venous methemoglobin (Met-Hb) are shown for all experimental interventions. *, $P \leq 0.06$ versus baselines 1 or 2, respectively; **, $P < 0.01$ versus baselines 1 or 2, respectively; †, $P < 0.05$ versus baseline 1; ††, $P < 0.01$ versus baseline 1. Error bars represent s.e.m.

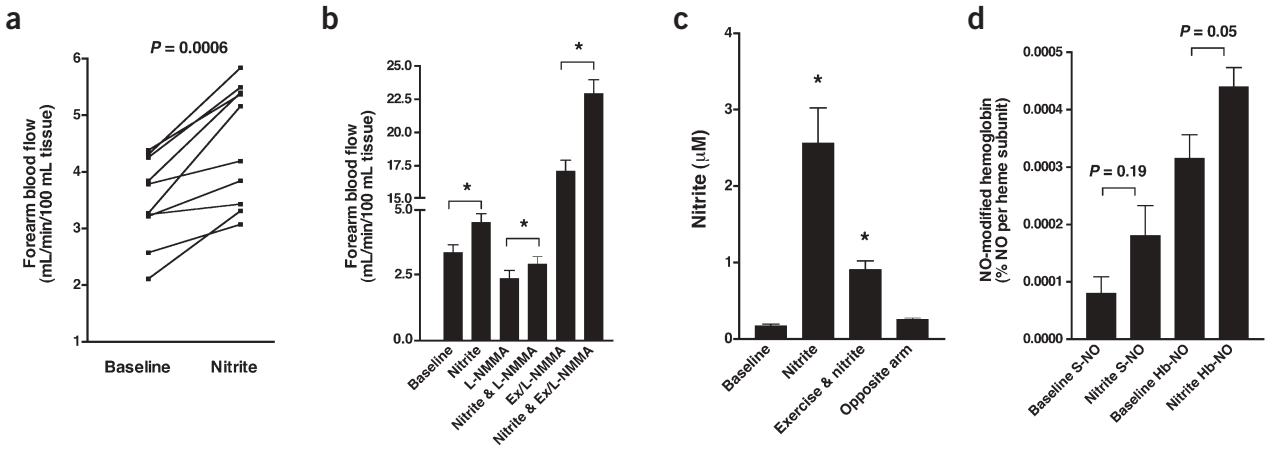

Figure 3 Effects of low-dose nitrite infusion. $NaNO_2$ was infused into the brachial arteries of ten healthy subjects at baseline and during exercise, without or with inhibition of NO synthesis. (**a**) Forearm blood flow at baseline and after a 5-min infusion of $NaNO_2$. (**b**) Forearm blood flow with and without low-dose nitrite infusion at baseline and during L-NMMA infusion, with and without exercise stress (Ex). (**c**) Venous levels of nitrite from forearm circulation at the time of blood flow measurements. (**d**) Venous levels of *S*-nitroso-hemoglobin (S-NO) and iron-nitrosylated hemoglobin (Hb-NO) at baseline and after nitrite infusion during exercise stress. *, $P < 0.05$ versus baseline. Error bars represent s.e.m.

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lower (82.1 ± 3.7 versus 89.2 ± 3.5 mm Hg; $P = 0.002$) than initial preinfusion values. We then reinfused the brachial artery with sodium nitrite (36 μmol/ml) and N^G-monomethyl-L-arginine (L-NMMA; 8 μmol/min) again to inhibit regional synthesis of NO. We observed vasodilator effects of nitrite on resting and exercise forearm blood flow similar to those observed during nitrite infusion without L-NMMA (**Fig. 2b**). This is in contrast to the vasoconstrictor effect of NO synthase inhibition with L-NMMA observed in part I of the protocol (**Fig. 1b**).

Vasodilatory properties of nitrite at physiological concentrations

As a test of the physiological relevance of vascular nitrite as a vasodilator, the concentrations of the nitrite infusions were decreased by 2 logs to 400 nmol/ml. An infusion of 400 nmol/ml nitrite at 1 ml/min for 5 min significantly increased forearm blood flow in all ten subjects from 3.49 ± 0.24 to 4.51 ± 0.33 ml per min per 100 ml tissue (**Fig. 3a**; $P = 0.0006$). Blood flow significantly increased at rest and during NO synthase inhibition, with or without exercise (**Fig. 3b**; $P < 0.05$ under all conditions). Mean venous nitrite levels increased from 176 ± 17 nM to 2,564 ± 462 nM after a 5-min infusion, and exercise venous nitrite levels decreased to 909 ± 113 nM (secondary to the diluting effects of increased blood flow during exercise; **Fig. 3c**). Again, the vasodilator effects of nitrite were paralleled by an observed formation of both iron-nitrosylated hemoglobin and *S*-nitroso-hemoglobin across the forearm circulation (**Fig. 3d**). These data suggest that basal levels of nitrite, from 150–1,000 nM in plasma to 10,000 nM in vascular tissue[7–9], are likely to contribute to resting vascular tone and hypoxic vasodilation.

The vasodilatory property of nitrite during basal blood flow conditions, when tissue pO_2 and pH are not exceedingly low, was unexpected. These results suggest that the previously hypothesized mechanisms for nitrite reduction, nitrite disproportionation and xanthine oxidoreductase activity, all of which require extremely low pO_2 and pH values not typically encountered in normal physiology, must be complemented *in vivo* by additional factors that catalyze nitrite reduction. We now report that deoxyhemoglobin effectively reduces nitrite to NO, a mechanism described by Doyle *et al.* in 1981 (ref. 33), within one half-circulatory time from artery to vein. This mechanism provides graded production of NO along the physiological oxygen gradient, tightly regulated by hemoglobin oxygen desaturation.

Intravascular formation of NO and *S*-nitrosothiol

Before and during nitrite infusions, blood was drawn from the brachial artery and antecubital vein, and the whole blood was immediately (at the bedside to minimize processing time) lysed 1:10 in an NO-hemoglobin 'stabilization solution'. The iron-nitrosylated hemoglobin and *S*-nitroso-hemoglobin content was determined by tri-iodide–based reductive chemiluminescence and electron paramagnetic resonance (EPR) spectroscopy (described in Methods). As previously reported[30] and recently confirmed[9], the baseline levels of *S*-nitroso-hemoglobin and iron-nitrosyl-hemoglobin were at the limits of detection (<50 nM or 0.0005% NO per heme), with no artery-to-vein gradient. After nitrite infusion as in part II of the protocol, venous levels of iron-nitrosylated hemoglobin and *S*-nitroso-hemoglobin rose substantially (**Fig. 4a–c**). This formation of iron-nitrosylated hemoglobin across the forearm circulation was confirmed by EPR spectroscopy (**Fig. 4b**). The formation of both NO-hemoglobin adducts occurred across the vascular bed, with a half-circulatory time of less than 10 s. The rate of NO formation was measured as iron-nitrosylated and *S*-nitroso-hemoglobin content and quantified by subtraction of the arterial from the venous levels with the difference being multiplied by blood flow. The NO formation rate increased greatly during exercise, despite a significant decrease in the venous concentration of nitrite secondary to the dilution of regional nitrite concentration by increased blood flow (**Fig. 4d**; $P = 0.006$ for iron-nitrosylated hemoglobin and $P = 0.02$ for *S*-nitroso-hemoglobin, by repeated-measures ANOVA).

The amounts of iron-nitrosylated and *S*-nitroso-hemoglobin formed *in vivo* in this study are notable. With a transit time of less than 10 s through the forearm circulation during exercise, infused nitrite (200 μM regional concentration) produced ~750 nM iron-nitrosylated hemoglobin and 200 nM *S*-nitroso-hemoglobin (**Fig. 4b,c**). The formation of both NO-hemoglobin adducts was inversely correlated with hemoglobin-oxygen saturation, which fell during exercise stress, as measured from the antecubital vein by co-oximetry ($r = –0.7$ and $P < 0.0001$ for iron-nitrosylated hemoglobin; $r = –0.45$ and $P = 0.04$ for *S*-nitroso-hemoglobin; **Fig. 4e**). Addition of 200 μM nitrite to whole blood at different oxygen tensions (0–100%) recapitulated the *in vivo* data, with increasing concentrations of iron-nitrosylated hemoglobin being formed at lower oxygen tensions

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(r = –0.968 and P < 0.0001 for iron-nitrosylated hemoglobin; r = –0.45 and P = 0.07 for *S*-nitroso-hemoglobin; data not shown). This strongly suggests that iron-nitrosylated hemoglobin and *S*-nitroso-hemoglobin formation was dependent on the reaction of nitrite with deoxyhemoglobin.

These data are consistent with the previous characterization of the reaction of nitrite with deoxyhemoglobin to form NO and iron-nitrosylated hemoglobin[33]. Nitrite is first reduced to form NO and methemoglobin, with a rate constant of 2.9 $M^{-1}s^{-1}$ (measured at 25 °C, pH 7.0)[33]. This reaction is pseudo-first order, governed by the vast amounts (20 mM) of intraerythrocytic hemoglobin, and limited by the rate of nitrite uptake by the erythrocyte membrane. NO then either binds to deoxyhemoglobin to form iron-nitrosylated hemoglobin, escapes the erythrocyte (discussed below) or reacts with other higher oxides (such as NO_2, to form N_2O_3 and *S*-nitroso-hemoglobin; discussed later). These reactions are represented by the following equations:

NO_2^- (nitrite) + $HbFe^{2+}$ (deoxyhemoglobin) + H^+ → $HbFe^{3+}$ (methemoglobin) + NO + OH^-

NO + $HbFe^{2+}$ (deoxyhemoglobin) → $HbFe^{2+}NO$ (iron-nitrosylated hemoglobin)

We confirmed that the reaction of deoxyhemoglobin and nitrite is second-order in nitrite and hemoglobin by conducting kinetic measurements, first with a molar excess of nitrite to hemoglobin, and then with an excess of hemoglobin to nitrite. We found the same bimolecular rate constant, 0.47 ± 0.07 $M^{-1}s^{-1}$, for both conditions at 25 °C and pH 7.4. This rate constant is similar to that found by Doyle *et al.* at this pH (1 $M^{-1}s^{-1}$)[33].

To explore the effects of red blood cell membrane nitrite uptake rate on the formation of intraerythrocytic iron-nitrosylated hemoglobin, we examined the kinetics of the reaction of 200 μM nitrite with deoxygenated whole blood at 37 °C. Iron-nitrosylated hemoglobin formed at an observed rate constant (k) of 0.0035 ± 0.006 s^{-1} (**Fig. 4f,g**). Assuming a concentration of 20 mM for the concentration of hemoglobin in the red blood cell, this corresponds to a bimolecular rate of 0.18 ± 0.03 $M^{-1}s^{-1}$, which is substantially lower than the rate expected by measurements made by Doyle *et al.*, and indicates that the *in vivo* rate is limited by erythrocyte nitrite uptake. Using this rate and a 10-s artery-to-vein transit time (with the equation (0.28)(200 μM)$(1 - e^{-kt})$), we would expect 1.9 μM of iron-nitrosylated hemoglobin formation *in vivo*. This result would be similar in magnitude to the observed formation of ~750 nM iron-nitrosylated hemoglobin across the arterial-to-venous gradient (**Fig. 4**).

We also observed the formation of significant amounts of *S*-nitroso-hemoglobin *in vivo* during nitrite infusion. It was recently proposed that nitrite reacts with deoxyhemoglobin to make iron-nitrosylated hemoglobin, and that the subsequent 'transfer' of the NO to the cysteine 93 of the β-chain of hemoglobin to form *S*-nitroso-hemoglobin is mediated by reoxygenation and the quaternary T-to-R structural transition state of hemoglobin[34]. However, a direct transfer of NO from the heme to the thiol would require NO oxidation to NO^+, and such 'cycling' has not been reproduced by other research groups[35]. It has recently been suggested that nitrite catalyzes the reductive nitrosylation of methemoglobin by NO, a process that generates the intermediate nitrosating species dinitrogen trioxide (N_2O_3)[36]. Additional reactions of nitrite with hemoglobin produce reactive oxygen metabolites (such as superoxide and hydrogen peroxide[37–39]). Such reactions of NO radicals with oxygen radicals will provide competitive pathways

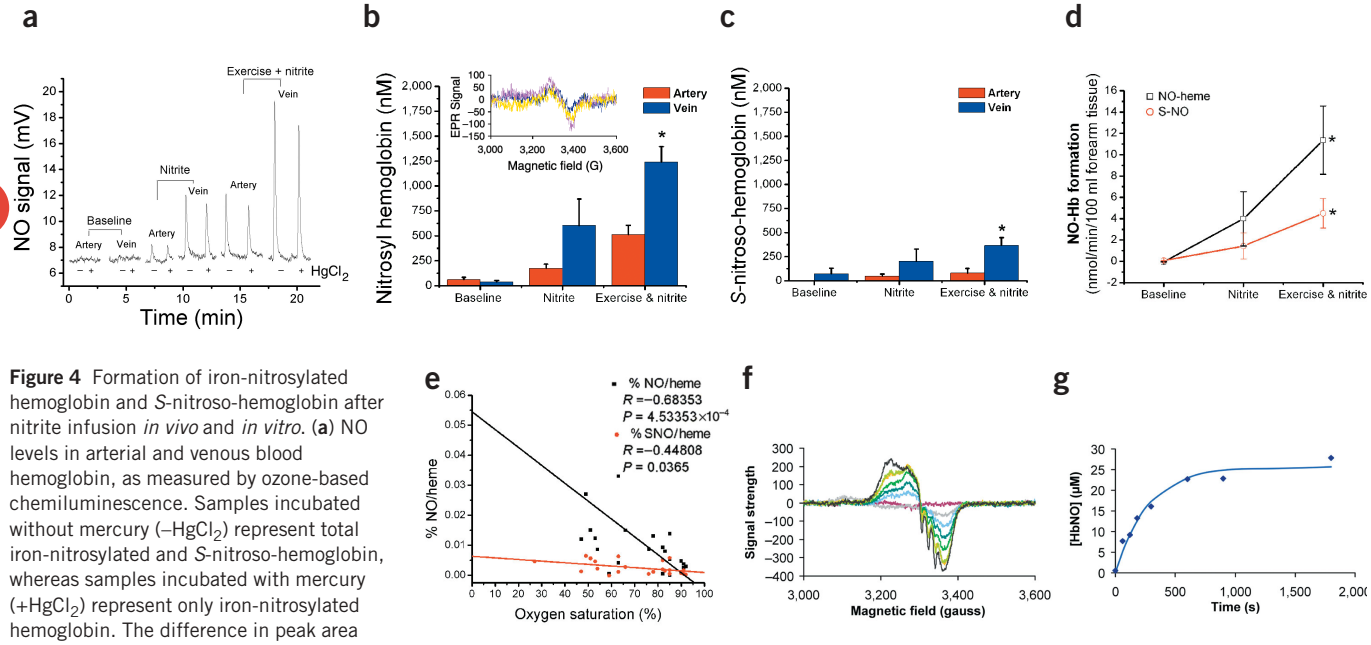

Figure 4 Formation of iron-nitrosylated hemoglobin and *S*-nitroso-hemoglobin after nitrite infusion *in vivo* and *in vitro*. (**a**) NO levels in arterial and venous blood hemoglobin, as measured by ozone-based chemiluminescence. Samples incubated without mercury (–$HgCl_2$) represent total iron-nitrosylated and *S*-nitroso-hemoglobin, whereas samples incubated with mercury (+$HgCl_2$) represent only iron-nitrosylated hemoglobin. The difference in peak area represents *S*-nitroso-hemoglobin. (**b**,**c**) Levels of iron-nitrosylated hemoglobin (**b**) and *S*-nitroso-hemoglobin (**c**) increased from artery to vein, indicating formation across the vascular bed after nitrite infusion. Inset in **b** shows arterial blood EPR spectra subtracted from venous blood EPR spectra, showing an increase in iron-nitrosylated hemoglobin from artery to vein. Difference spectra from three patients during exercise with nitrite infusion are shown. (**d**) Formation of iron-nitrosylated hemoglobin (NO-heme) and *S*-nitroso-hemoglobin (S-NO) at baseline, during nitrite infusion and during nitrite infusion with exercise, quantified by subtracting arterial from venous levels and multiplying the result by blood flow. (**e**) Formation of both NO-hemoglobin adducts was inversely correlated with hemoglobin oxygen saturation during nitrite infusion. (**f**,**g**) Representative EPR spectra (**f**) and kinetic traces (**g**) for reaction of nitrite with hemoglobin in venous blood at 37 °C, with deoxygenation performed under argon. *, P < 0.05 compared with baseline (**b**–**d**) and arterial levels (**b**,**c**).

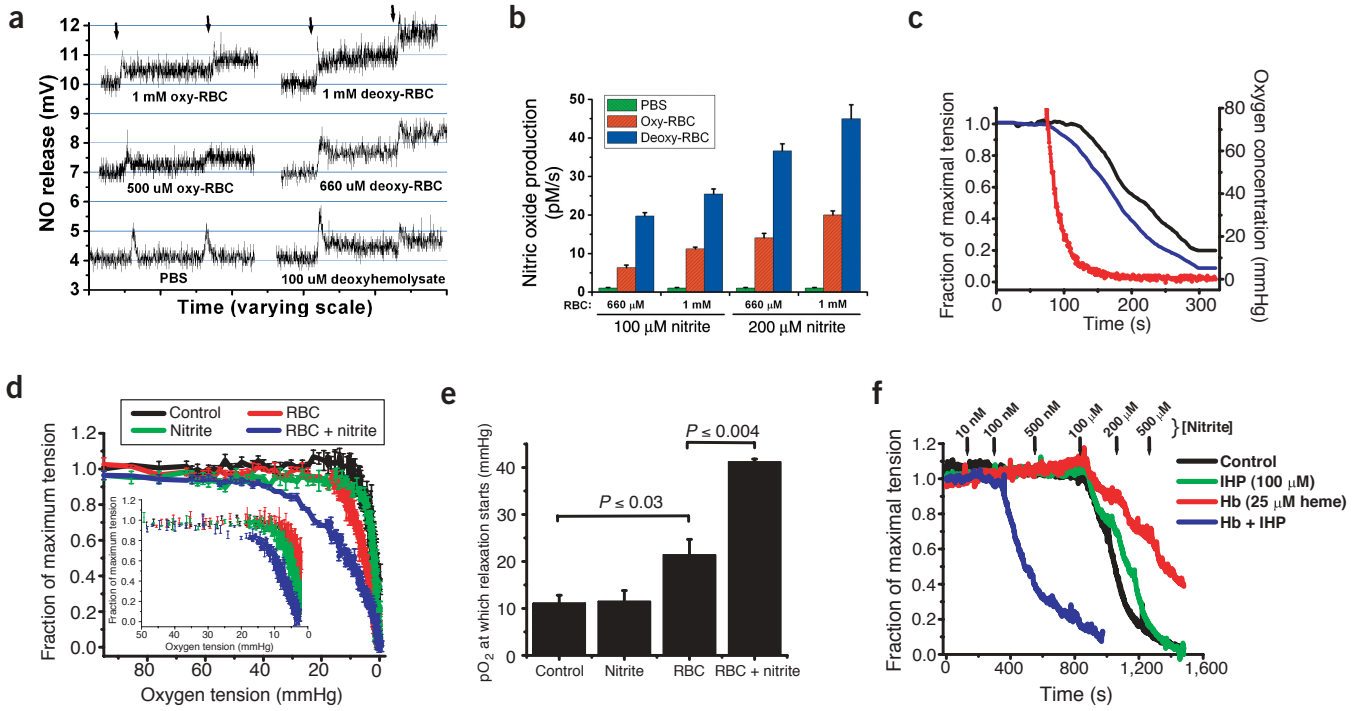

Figure 5 Production of NO gas and vasodilation are augmented by nitrite reaction with deoxyhemoglobin. (**a**) NO release after injection of nitrite into PBS and deoxygenated and oxygenated red blood cells. Arrows indicate time of nitrite injection into the system. (**b**) Rate of NO formation from nitrite mixed with PBS and oxygenated and deoxygenated red blood cells. (**c**) Representative time courses for pO_2 (red trace) and vessel tension during deoxygenation of vessels treated with control (rat erythrocytes alone; black trace) or rat erythrocytes and nitrite (blue trace). (**d**) Aortic ring bioassay threshold curves showing the relationship between pO_2 and vessel tension during different treatments. Inset shows data under same conditions with 500 nM nitrite. (**e**) Oxygen tensions at which relaxation began were determined from individual traces. Data represent mean ± s.e.m. ($n = 3$). (**f**) Representative traces ($n = 3$) showing effects of sodium nitrite (added at concentrations indicated) of relaxation of rat thoracic aorta at 15 mm Hg O_2 under control conditions (black trace) or in the presence of 100 μM IHP (green trace), human hemoglobin (red trace) or IHP and human hemoglobin together (blue trace).

for *S*-nitrosothiol formation in the presence of high-affinity NO sinks such as hemoglobin and deserve further study.

Nitrite reductase activity of deoxyhemoglobin

To determine whether free NO radicals can be formed from the reaction of nitrite and deoxyhemoglobin, we reacted 100 and 200 μM nitrite with deoxygenated erythrocytes (5-ml volume containing a total of 660 and 1,000 μM in heme) in a light-protected, helium-purged reaction vessel in line with a chemiluminescent NO analyzer. The injection of nitrite into a solution of deoxygenated erythrocytes resulted in the liberation of NO into the gas phase (**Fig. 5a**,**b**). There was no release from nitrite in a buffer control under the same conditions, and significantly ($P < 0.05$) less NO was released upon addition of nitrite to oxygenated erythrocytes (100% oxygen). The observed rate (area under the curve of increased steady-state NO generation after nitrite injection, calculated over 120 s) of NO production in the 5-ml reaction volume was consistent with an NO production rate of 47 pM/s (corresponding to an estimated rate of 300–500 pM/s in whole blood). Although NO formation rates in this experimental system cannot be extrapolated to rates of NO formation *in vivo*, the experiments illustrate two important concepts. First, a fraction of the free NO can escape autocapture by the remaining heme groups; this is likely to be possible only because nitrite is only converted to NO by reacting with deoxyhemoglobin, and because its 'leaving-group' is the met(ferric)heme protein that will limit scavenging and inactivation of NO[33]. Second, the rate of NO production is increased under anaerobic conditions, which indicates a nitrite-deoxyhemoglobin reaction.

We next evaluated whether the vasodilator properties of nitrite could be reproduced in an aortic ring bioassay system, and whether this vasodilation is potentiated by deoxygenated erythrocytes. Rat aortic rings were suspended in custom-made vessel baths that were sealed and fitted with an oxygen electrode, allowing simultaneous measurement of vessel tension and pO_2. **Figure 5c** shows representative tracings of vessel tension and pO_2, measured during deoxygenation of rat aortic rings exposed to red blood cells (0.3% hematocrit, 300 μM heme) and nitrite (2 μM). To determine the relationship between oxygen tension, vessel tone and the impact of erythrocytes and nitrite, vessel tension was plotted as a function of pO_2 (**Fig. 5d**). Control vessels spontaneously relaxed after reaching a pO_2 of ~10 mm Hg (**Fig. 5e**). Addition of 2 μM nitrite did not affect this process. Addition of rat erythrocytes alone increased the oxygen tension at which dilation was initiated (**Fig. 5e**), and addition of nitrite and erythrocytes together significantly ($P < 0.004$) left-shifted the vessel tension– pO_2 threshold curve (**Fig. 5c**,**d**) such that vasodilation was observed at oxygen tensions <40 mm Hg ($P < 0.004$). Lower concentrations of nitrite (500 nM) that fall within the physiological range also significantly promoted vasodilation in the presence of red blood cells, compared with red blood cells or nitrite alone (**Fig. 5d**) ($P < 0.05$).

To test whether the effect of red blood cells on nitrite-dependent vasodilation could be mediated by deoxyhemoglobin, vessel dilation experiments were conducted at a pO_2 of 15 mm Hg, with cell-free hemoglobin, in the absence or presence of inositol hexaphosphate (IHP; **Fig. 5f**). IHP was used to modulate the oxygenation state of hemoglobin in this system. In the absence of IHP, hemoglobin

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remained saturated with oxygen at a pO_2 of 15 mm Hg (p50 = 9 mm Hg oxygen; p50 is the pO_2 at which hemoglobin is 50% saturated with oxygen), but was deoxygenated in the presence of IHP (p50 = 45 mm Hg oxygen). At a pO_2 of 15 mm Hg and using a Hill coefficient of $n = 2.8$, the oxygen saturation would be 81% for hemoglobin with p50 = 9 mm Hg, and 4% for hemoglobin with p50 = 45 mm Hg. With a heme concentration of 25 μM, ~82% of oxyheme is tetrameric. Addition of nitrite alone stimulated vasodilation, with a half-maximal effective concentration of 100–200 μM. IHP alone slightly inhibited this effect, whereas hemoglobin alone right-shifted the dose-dependence of nitrite, indicating an oxyhemoglobin-dependent oxidation of nitrite to nitrate. However, in the presence of IHP and hemoglobin at a pO_2 of 15 mm Hg, nitrite-dependent vasodilation was potentiated by three orders of magnitude (half-maximal effective concentration of 100–200 nM). These results support a physiological model for nitrite reduction to NO by reaction with erythrocyte deoxyhemoglobin.

DISCUSSION

We show that nitrite-induced vasodilation in humans is associated with reduction of nitrite to NO by deoxyhemoglobin. Systemic levels of 16 μM resulted in systemic vasodilation and decreased blood pressure, and regional forearm levels of only 1–2 μM significantly increased blood flow at rest and with exercise-induced stress. In addition, conversion of nitrite to NO and *S*-nitrosothiol was mediated by reaction with deoxyhemoglobin, providing a mechanism for hypoxia-regulated catalytic NO production by erythrocytes or endothelial or tissue heme proteins. A nitrite-hemoglobin chemistry would support a role for the red blood cells in oxygen-dependent NO homeostasis, a concept first advanced by Stamler *et al.* but ascribed to *S*-nitroso-hemoglobin[40,41]. It would also provide a mechanism for the observation that red blood cells and plasma 'loaded' with NO, by exposure to high concentrations in solution or to NO gas or donors (invariably in equilibrium with high concentrations of nitrite), can export NO and induce vasodilation *in vitro* and *in vivo*[11,27–32]. We realize that the high concentrations of hemoglobin in red blood cells, coupled with the near-diffusion-limited reaction rates (~10^7 $M^{-1}s^{-1}$) of NO with hemoglobin, seem to prohibit NO from being exported from the red blood cell. However, our data (**Fig. 5**) argue to the contrary. Perhaps the unique characteristics of the erythrocyte membrane, with its submembrane proteins and methemoglobin-rich microenvironment[42], and the relatively lipophilic nature of NO, allow compartmentalized NO production at the red blood cell membrane. This, coupled with the small amounts of NO necessary for vasodilation, could account for the export of NO despite these kinetic constraints. Further study will be required to determine whether this reaction occurs primarily in the erythrocyte, as data in **Figure 5** suggest, with subsequent export of NO or of *S*-nitrosothiol, or whether this is a primary reaction of nitrite with endothelial or smooth muscle heme proteins, such as myoglobin, soluble guanylyl cyclase, cytochrome P450 or mitochondrial cytochromes.

Three factors uniquely position nitrite, rather than *S*-nitrosothiol, as the major vascular storage pool of NO. First, nitrite is present in substantial concentrations in plasma, erythrocytes and tissues[7]. Second, nitrite is relatively stable because it is not readily reduced by intracellular reductants (as are *S*-nitrosothiols[30]), and its reaction rate with heme proteins is 10,000 times less than that of authentic NO. Third, nitrite is only converted to NO by reacting with deoxyhemoglobin (or presumably deoxymyoglobin, deoxycytoglobin, deoxyneuroglobin or other oxygen-binding heme proteins), and its leaving-group is the met(ferric)heme protein that limits scavenging and inactivation of NO[33]. The nitrite pool therefore provides the ideal substrate for NO generation along the physiological oxygen gradient, potentially providing a new mechanism for hypoxic vasodilation. Therapeutic application of nitrite should result in selective vasodilation to hypoxemic tissue, and could be used to treat diseases associated with ischemic tissue, neonatal pulmonary hypertension and hemolytic conditions such as sickle-cell disease, where free hemoglobin released during hemolysis scavenges NO and disrupts NO-dependent vascular function[43]. Nitrite would not only inhibit the ability of free hemoglobin (by oxidizing it to methemoglobin) to scavenge NO, but would actually generate NO in tissue beds with low oxygen tension.

METHODS

Protocol for human subjects. The protocol was approved by the Institutional Review Board of the National Heart, Lung and Blood Institute, and informed consent was obtained from all volunteer subjects. Nine men and nine women, with an average age of 33 years (range 21–50 years), participated in the study. An additional ten subjects returned 3–6 months later for a second series of experiments with low-dose nitrite infusion. Detailed inclusion and exclusion criteria are described in **Supplementary Methods** online.

Forearm blood flow measurements. Forearm blood flow measurements were made by strain-gauge venous-occlusion plethysmography, as previously described[44]. A series of measurements, termed parts I and II (detailed in Results, **Figs. 1** and **2** and **Supplementary Methods** online), were conducted in randomized order to minimize the effect of time on forearm blood flow response during nitrite infusion. Part I of the protocol was designed as a control for part II, and is described in **Figure 1** and **Supplementary Methods** online.

Nitrite infusions. Nitrite ($NaNO_2$; M_r = 69; 36 μmol/ml in 0.9% saline) was infused at 1 ml/min. Sodium nitrite for use in humans was obtained from Hope Pharmaceuticals (300 mg in 10 ml water), and 286 mg was diluted in 100 ml of 0.9% saline by the Pharmaceutical Development Service to a final concentration of 36 μmol/ml. For the final nine subjects studied, 0.01–0.03 mM sodium bicarbonate was added to the normal saline, so as to titrate the pH to 7.0–7.4. The nitrite solution was light-protected, and nitrite levels and free NO gas in solution were measured by reductive chemiluminescence after all experiments[30]. NO was present at a concentration of 50.5 ± 40.5 nM in nitrite solutions, and was unaffected by bicarbonate buffering. There was no correlation between NO levels in nitrite solutions and blood flow effects of nitrite ($r = -0.23$; $P = 0.55$). The total dose of sodium nitrite infused in our study participants was 1.08 mmol, or 75 mg (36 μmol/min × 15 min × 2 infusions), which is approximately one-third of the dose used in humans for emergency treatment of cyanide poisoning. In additional studies in ten subjects, the same stages of parts I and II of the protocol were followed with infusion of low-dose nitrite (0.36 μmol/ml $NaNO_2$ in 0.9% saline, infused at 1 ml/min).

Measurement of red blood cell *S*-nitroso-hemoglobin and iron-nitrosylated hemoglobin. *S*-nitroso-hemoglobin and iron-nitrosylated hemoglobin were assayed as previously described[30,45]. The assay was conducted on whole blood to prevent *ex vivo* chemistry from occurring during sample processing. The detailed protocol is described in **Supplementary Methods** online.

EPR spectroscopy of whole blood. EPR spectroscopy was carried out at 137 K with a Bruker 4111 VT controller and ER-200 D ESR spectrometer set at 9.43 GHz, 10 mW, 5 G modulation, 0.1 s time constant and 100 s scans over 600 G. Each curve represents the average of ten 100-s scans. Arterial blood spectra were subtracted from venous blood spectra, indicating an increase in iron-nitrosylated hemoglobin from artery to vein.

Kinetics of reaction of nitrite with deoxygenated erythrocytes. Venous blood was deoxygenated under a vacuum of <0.5 torr and cycled under 100% argon or medical gas (95% N_2, 5% CO_2), to give a minimum of 75% deoxygenation of total hemoglobin (average deoxygenation was 81 ± 4%; pH 7.4). Deoxygenated PBS was added as needed to bring the total hemoglobin concentration to 10 mM. The maximum hemolysis measured was 2% of heme. EPR spectroscopy of free hemoglobin in the lysate and packed red blood cell fractions after reaction

with nitrite confirmed that the measured iron-nitrosylated hemoglobin signals were from the hemoglobin in the red blood cells. After equilibrating to 37 °C in a water bath, baseline EPR samples were extracted to deoxygenated tubes and immediately frozen in liquid nitrogen. Deoxygenated 0.03 M potassium nitrite stock solution was added to a final sample concentration of 200 μM nitrite and mixed by inversion, with subsequent EPR samples taken and frozen at 0, 1, 2, 3, 5, 10, 15 and 30 min. The concentration of iron-nitrosylated hemoglobin was determined by comparing the double integral of the measured spectrum to that of a standard.

Chemiluminescent detection of NO gas released from deoxyhemoglobin and deoxygenated erythrocytes after nitrite addition. To determine whether free NO radicals can be formed by the reaction of nitrite and deoxyhemoglobin, we mixed 100 and 200 μM nitrite with 5 ml of 660 and 1,000 μM deoxygenated erythrocytes, respectively, in a light-protected reaction vessel purged with helium or oxygen in line with a chemiluminescent NO analyzer (Seivers). After allowing equilibration for 5 min, nitrite was injected and the rate of NO production was measured. Nitrite was injected into PBS as a control, and into 100 μM hemoglobin to control for hemolysis in the 660 and 1,000 μM deoxygenated erythrocyte solutions. At the end of all experiments, the visible absorption spectra of the supernatant and erythrocyte reaction mixtures were analyzed, and hemoglobin composition was deconvoluted using a least-squares algorithm. There was <100 μM hemolysis in the system, no hemoglobin denaturation and significant formation of iron-nitrosylated hemoglobin.

Aortic ring bioassay experiments. Isometric tension was measured as described previously using rat thoracic aorta treated with indomethacin (5 μM) and precontracted with phenylephrine (100 nM) and L-N^G-nitroarginine (100 μM)[46]. To investigate the effect of oxygen tension on vasodilation responses stimulated by nitrite and/or red blood cells, a vessel bath was custom designed (Radnoti) so that it could be sealed and accommodate an oxygen electrode to allow simultaneous monitoring of pO_2 and vessel tone. Vessels were equilibrated in Krebs buffer at 37 °C at 609 mm Hg oxygen (achieved by perfusion of 95% O_2 and 5% CO_2). Oxygen tension was calibrated as previously described[46]. After reaching a stable tone, various treatments described in Results were added, and perfusion with 95% N_2, 5% CO_2 and helium were initiated (inclusion of helium was necessary to reduce oxygen tension to 0 mm Hg). Because the rates of deoxygenation varied in different vessel chambers, measurements were made only under conditions that allowed vessel tension and oxygen concentration to be determined simultaneously, in the same bath. The oxygen tension at which relaxation starrted was defined as the intersection between tangents drawn along the line formed at maximum tension as a function of oxygen concentration (i.e., when little change in tension occurs at high oxygen tension) and after relaxation responses were initiated. For experiments using cell-free hemoglobin, vessel baths were perfused with 95% N_2 and 5% CO_2, which decreased oxygen tension to 15 mm Hg. Nitrite-dependent vasodilation was then assessed by addition of incremental concentrations of nitrite in the presence and absence of hemoglobin (25 μM) and IHP (100 μM).

Statistical analysis. An *a priori* sample size calculation determined that 18 subjects would be necessary to detect a 25% improvement in forearm blood flow during nitrite infusion, when forearm NO synthesis had been inhibited by L-NMMA, compared with normal saline infusion control values (alpha = 0.05, power = 0.80). Two-sided *P* values, repeated-measures ANOVA and Pearson correlations were used as indicated. Measurements shown represent mean ± s.e.m.

Note: Supplementary information is available on the Nature Medicine website.

ACKNOWLEDGMENTS

This work was supported by Clinical Center and National Heart, Lung and Blood Institute intramural funds (R.O.C. and M.T.G.), National Institutes of Health grant HL58091 (D.B.K.-S.), RO1HL70146 (R.P.P.) and Medical Scientist Training Program T32GM08361. We thank V. Annavajjhala for helpful laboratory assistance.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

Received 18 July; accepted 1 October 2003
Published online at http://www.nature.com/naturemedicine/

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