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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.

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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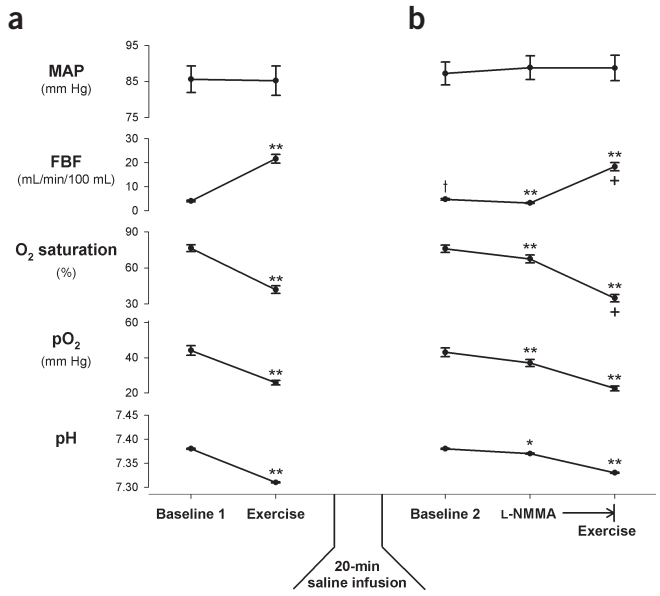

Figure 1 Hemodynamic and metabolic measurements at baseline and during exercise (protocol, part I). Measurements were taken without (**a**) and with (**b**) inhibition of NO synthesis in 18 subjects. Mean arterial pressure (MAP), forearm blood flow (FBF), venous oxyhemoglobin saturation (O_2 saturation), 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

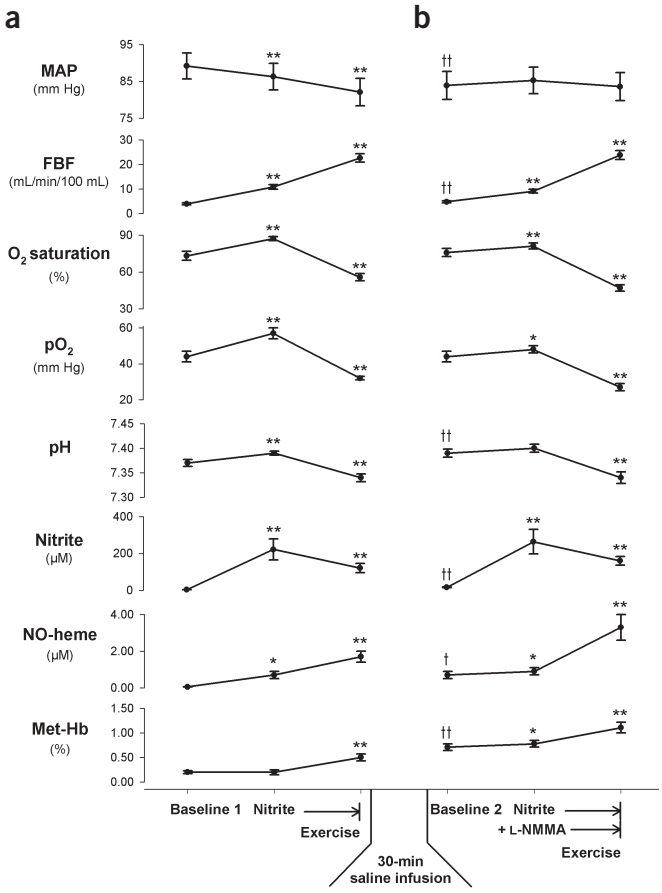

Figure 2 Effects of nitrite infusion. $NaNO_2$ was infused into the brachial arteries of 18 healthy subjects for 5 min at baseline and continued during exercise, without (**a**) or with (**b**) inhibition of NO synthesis with L-NMMA (protocol 1, part II). Values for mean arterial blood pressure (MAP), 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.

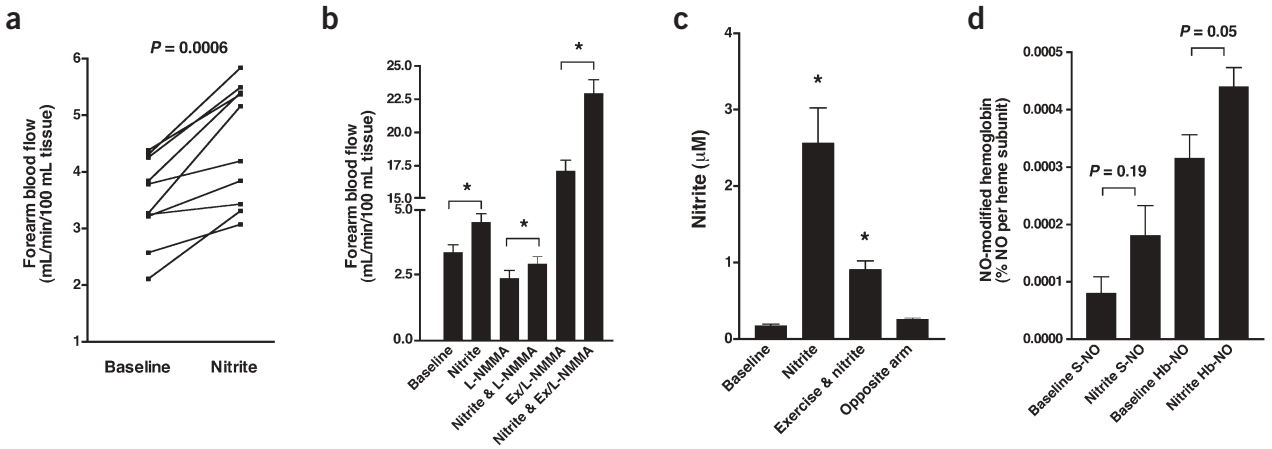

Figure 3 Effects of low-dose nitrite infusion. $NaNO_2$ was infused into the brachial arteries of ten healthy subjects at baseline and during exercise, without or with inhibition of NO synthesis. (**a**) Forearm blood flow at baseline and after a 5-min infusion of $NaNO_2$. (**b**) Forearm blood flow 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.

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

(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) + $H^+ \rightarrow HbFe^{3+}$ (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

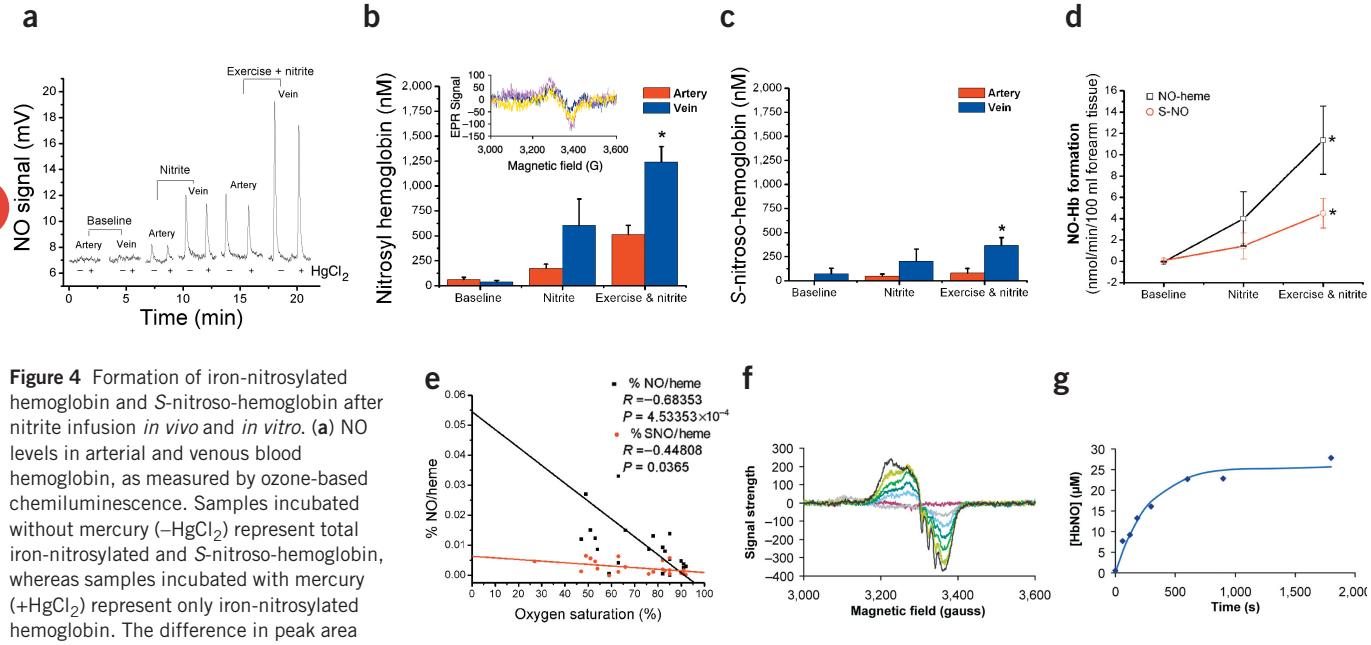

Figure 4 Formation of iron-nitrosylated hemoglobin and *S*-nitroso-hemoglobin after nitrite infusion *in vivo* and *in vitro*. (**a**) NO levels in arterial and venous blood hemoglobin, as measured by ozone-based chemiluminescence. Samples incubated without mercury (–$HgCl_2$) represent total iron-nitrosylated 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**).

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