# Role of circulating nitrite and *S*-nitrosohemoglobin in the regulation of regional blood flow in humans

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Edited by Louis J. Ignarro, University of California, Los Angeles, CA, and approved August 7, 2000 (received for review June 13, 2000)

**To determine the relative contributions of endothelial-derived nitric oxide (NO) vs. intravascular nitrogen oxide species in the regulation of human blood flow, we simultaneously measured forearm blood flow and arterial and venous levels of plasma nitrite, LMW-SNOs and HMW-SNOs, and red cell S-nitrosohemoglobin (SNO-Hb). Measurements were made at rest and during regional inhibition of NO synthesis, followed by forearm exercise. Surprisingly, we found significant circulating arterial-venous plasma nitrite gradients, providing a novel delivery source for intravascular NO. Further supporting the notion that circulating nitrite is bioactive, the consumption of nitrite increased significantly with exercise during the inhibition of regional endothelial synthesis of NO. The role of circulating *S*-nitrosothiols and SNO-Hb in the regulation of basal vascular tone is less certain. We found that low-molecular-weight S-nitrosothiols were undetectable and S-nitroso-albumin levels were two logs lower than previously reported. In fact, S-nitroso-albumin primarily formed in the venous circulation, even during NO synthase inhibition. Whereas SNO-Hb was measurable in the human circulation (brachial artery levels of 170 nM in whole blood), arterial-venous gradients were not significant, and delivery of NO from SNO-Hb was minimal. In conclusion, we present data that suggest (*i*) circulating nitrite is bioactive and provides a delivery gradient of intravascular NO, (*ii*) S-nitroso-albumin does not deliver NO from the lungs to the tissue but forms in the peripheral circulation, and (*iii*) SNO-Hb and *S*-nitrosothiols play a minimal role in the regulation of basal vascular tone, even during exercise stress.**

Nitric oxide (NO) is a soluble gas synthesized in endothelial cells from the amino acid L-arginine by the constitutive calcium and calmodulin-dependent enzyme NO synthase (1). In their seminal experiment, Furchgott and Zawadzki (2) found that strips of rabbit aorta with intact endothelium relaxed in response to acetylcholine but constricted in response to the same agonist when the endothelium had been rubbed off. The substance responsible for acetylcholine-stimulated relaxation was initially called endothelium-derived relaxant factor but was subsequently found to include NO (3, 4). The importance of endothelium-derived NO in the regulation of coronary and systemic vasodilator tone has been demonstrated experimentally by regional inhibition of its synthesis with $N^G$-monomethyl-L-arginine (L-NMMA), which competes with L-arginine as the substrate for NO synthase (5, 6).

Because of the instability and short half-life of NO, there has been considerable interest in the role of more stable NO adducts and metabolites that could circulate and regulate vascular tone *in vivo*. It has recently been proposed that NO is stabilized by covalent bonding with thiols such as glutathione, cysteine, albumin, and hemoglobin (7–10). These low- and high-molecular-weight *S*-nitrosothiols (LMW-SNOs and HMW-SNOs) are believed to play a role in the stabilization and delivery of NO to the vascular bed, where the NO may modify vascular tone. In fact, there is discussion about how much endothelium-derived relaxant factor activity may be due to *S*-nitrosothiols vs. NO gas (11). Nitrite, generated from the reaction of NO and oxygen, can be converted to NO by reaction with protons (12–14) or enzymatic conversion by xanthine oxidase (15–18). Therefore, intravascular nitrite may provide an additional stable source of bioavailable NO.

The most studied *S*-nitrosothiols are *S*-nitroso-albumin (SNO-albumin) and *S*-nitroso-hemoglobin (SNO-Hb). Stamler and colleagues reported in 1992 that human venous plasma contains 7 $\mu$M *S*-nitrosothiols, of which 80% was SNO-albumin (7). This level of *S*-nitrosothiols would represent a large pool of bioavailable NO with significant potential vascular effects. It has also been reported that NO is transported on hemoglobin by binding to the highly conserved $\beta$-chain Cys-93 residue, forming SNO-Hb (8–10, 19). According to this theory, NO carried on hemoglobin $\beta$-Cys-93 is delivered from the lungs to the microvasculature, whereupon, during deoxygenation in the tissues, the NO molecules, free or complexed to small thiols, diffuse through the erythrocytes to the vascular walls, promoting vasodilation.

However, the postulated roles of circulating *S*-nitrosothiols in the regulation of vascular tone have been subject to practical and theoretical criticism. First, Marley and colleagues measured the levels of venous plasma total *S*-nitrosothiols, using a well-validated chemiluminescent-based reductive assay and reported that they did not exceed 30 nM (20). Samouilov and Zweier reported that *S*-nitrosothiols in plasma were undetectable (21). Second, it is known that NO gas is a poor nitrosating agent at physiological pH, suggesting that minimal *S*-nitrosation would occur from NO generated by NO synthase. The reaction of NO and oxygen to form nitrosative intermediates ($N_2O_3$), which can nitrosate thiol groups and produce nitrite (22–25), is limited by the low concentrations of oxygen and NO dissolved in blood. (This low oxygen concentration results in an increased half-life and bioavailability of NO, supporting its candidacy as endothelium-derived relaxant factor.) Finally, the high affinity of NO for heme groups suggests that the predominant reaction of NO gas at the alveolar-vascular interface will be with oxyhemoglobin to form nitrate and methemoglobin, rather than with thiol groups. In other work, we have demonstrated that during NO breathing, heme reaction pathways predominate, and that minimal *S*-nitrosation occurs (26).

To elucidate the role of circulating *S*-nitrosothiols and nitrite versus regional endothelium-derived NO in the regulation of regional basal vascular tone, we have now measured forearm arterial and venous plasma levels of nitrate, nitrite, LMW-SNOs

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: SNO-Hb, *S*-nitrosohemoglobin; $HbFe^{II}NO$, nitrosyl(heme)hemoglobin; SNO-albumin, *S*-nitroso-albumin; HMW-SNO, high-molecular-weight *S*-nitrosothiol; LMW-SNO, low-molecular-weight *S*-nitrosothiol; L-NMMA, $N^G$-monomethyl-L-arginine.

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and HMW-SNOs, and red cell SNO-Hb in normal volunteers, using highly sensitive and specific chemiluminescent assays. To date, the basal arterial and venous levels of these nitrogen oxide species have not been published for humans. These measurements were performed at rest, during L-NMMA infusion into the brachial artery to achieve regional blockade of NO synthesis, and during L-NMMA infusion with exercise. Simultaneous blood flow measurements were obtained. We hypothesized that with reduced forearm endothelial synthesis of NO, *S*-nitrosothiol and/or nitrite transport of NO from the lungs to the peripheral circulation would assume greater significance, resulting in increases in their consumption. Furthermore, we hypothesized that inasmuch as exercise during NO synthase inhibition would cause relative regional acidosis, further release of NO from both SNO-Hb and nitrite would be promoted.

## Materials and Methods

**Protocol.** The study protocol was approved by the Institutional Review Board of the National Heart, Lung and Blood Institute, and all subjects gave written informed consent. All volunteers had a normal hemoglobin concentration and hemoglobin A documented by electrophoresis. Seven men and three women were studied. Their mean age was 42 years (range: 30–61 years). All volunteer subjects fasted after midnight and during the study day, except for water. In addition, five subjects were placed on a nitrate-restricted diet (<15 mg/day) for 3 days before the study. Brachial artery and antecubital vein catheters were placed. The intraarterial catheter was connected by a three-way stopcock to a pressure transducer for blood pressure measurements and to an infusion pump delivering 5% dextrose in water at 1 ml/min. At 20 min of rest after catheter placement, arterial and venous blood samples were obtained for NO measurements (see below).

Forearm blood flow measurements were then performed by strain gauge venous occlusion plethysmography, as previously reported (5). Briefly, a mercury-filled Silastic strain gauge was placed around the widest portion of the forearm and connected to a plethysmograph calibrated to measure the percentage change in volume (model EC-4; D. E. Hokanson, Inc.). The plethysmograph was connected to a strip chart recorder for forearm blood flow measurements. After inflation of a wrist cuff to suprasystolic pressure to exclude the hand circulation, a blood pressure cuff on the upper arm was inflated to 40 mmHg for 7 s with a rapid cuff inflator to occlude venous outflow, but not arterial inflow, into the forearm. This set-up causes nondiscernible distention of the forearm at a rate proportionate to arterial inflow. A series of seven blood flow measurements were averaged for each blood flow value determination.

pH was measured at the bedside with the i-STAT system (i-STAT Corporation, East Windsor, NJ).

**Determination of Vascular Response to NO Blockade and Exercise.** Five volunteers were studied at an L-NMMA dose of 4 $\mu$mol/min, as generally done in our laboratory (5). An additional five subjects were studied at a dose of 8 $\mu$mol/min to ensure that pharmacological inhibition of NO synthesis was as complete as possible (a lack of further reduction in blood flow suggested maximal NO synthesis inhibition at these concentrations). Before and after 5 min of L-NMMA infusion, arterial and venous blood samples were obtained, and forearm blood flow was measured. Forearm exercise was then initiated in that arm during continued L-NMMA infusion, using a hand-grip dynamometer (Technical Products International, St. Louis), which is similar to a protocol previously used in this laboratory (27). Exercise was performed by repetitive hand grip at 50% of the predetermined maximum grip strength, individualized for each subject at the start of the study. Each contraction lasted for 10 s, followed by relaxation for 5 s. After 5 min of exercise, arterial and venous blood samples were obtained and forearm blood flow was measured. After a 2-h rest period, with D5W replacing L-NMMA infusion into the brachial artery, the entire experiment was repeated.

**Processing of Blood.** Blood samples were drawn into EDTA collection tubes and centrifuged at 750 $\times$ *g* for 5 min. Plasma aliquots were removed and stored at −80°C until assayed for nitrate, nitrite, and low-molecular-weight *S*-nitrosothiols as described below. A 500-$\mu$l aliquot of the plasma was immediately run on a 9.5-ml bed volume Sephadex G25 sizing column in a dark room within 30 min of collection. The 3–3.5-ml elution volume was collected, which contained plasma high-molecular-weight proteins (albumin and immunoglobulins), and 200 $\mu$l was reacted in $I_3^-$ as described below. The value was corrected for the dilution in the G25 column. The red blood cell pellet was removed, washed one time in 20 vol of PBS, flash-frozen on dry ice, and stored at −80°C until it was assayed for SNO-Hb. SNO-Hb is stable under these conditions, even at $1 \times 10^{-5}$ mol of SNO-Hb/mol of unpurified hemoglobin from lysed red cells (26, 28).

**Ozone-Based Chemiluminescent Detection of SNO-Hb.** Red cell samples were rapidly thawed and added to a 1:4 dilution of 0.2 M KCN and 0.2 M $K_3Fe(CN)_6$ in 0.5 mM EDTA distilled water. Pretreatment with a 100-fold molar excess of KCN and $K_3Fe(CN)_6$ was used to selectively remove the NO from heme while preserving the *S*-nitrosothiol bond. In fact, within 3 min KCN/$K_3Fe(CN)_6$ (0.2 M) oxidizes the $Fe^{II}NO$ to an intermediate species not detectable in the chemiluminescent $I_3^-$ reaction, while preserving and stabilizing the S-NO bond of SNO-Hb (26). The stability of the *S*-nitroso linkage in KCN/$K_3Fe(CN)_6$ has been tested for up to 2 h ($n = 5$) with less than 10% loss of NO signal. After 30 min of incubation, 500 $\mu$l was passed through a Sephadex G25 column (9.5-ml bed volume) to remove nitrite, small thiols, and KCN/$K_3Fe(CN)_6$. We validated that the collected fraction (from the 3–3.5-ml fraction) was free of nitrite and small nitrosothiols by adding nitrite and *S*-nitrosoglutathione to hemoglobin and sampling serial elution fractions ($n =$ 3 experiments each). Samples (200 $\mu$l) were then immediately drawn into 250-$\mu$l Hamilton syringes and reacted with $I_3^-$, which stoichiometrically releases NO from SNO-hemoglobin for chemiluminescent detection (26, 28).

This technique was validated using pure synthesized species of SNO-Hb (specificity of $\beta$-Cys-93 modification confirmed by HPLC electrospray mass spectrometry after enzymatic digestion) and nitrosyl(heme)hemoglobin ($HbFe^{II}NO$) (100% heme nitrosylation confirmed by visible absorption spectroscopy). Mass spectrometry was used to confirm that $HbFe^{II}NO$ standards were not *S*-nitrosated as well. Synthesized standard preparations of SNO-Hb contained a mean 1.96 $\pm$ 0.17 mol S-NO/mol hemoglobin tetramer (measurements performed using SNO-glutathione standards), consistent with nitrosation of the $\beta$-Cys-93 residues on each $\beta$-chain. Standards of SNO-Hb and $HbFe^{II}NO$ were diluted in fresh purified oxyhemoglobin to levels of $1 \times 10^{-5}$ (0.001%) mol NO/mole heme. We found linear sensitivity for total nitrosylated hemoglobin, SNO-Hb, and $HbFe^{II}NO$ from 0.001% to 100% (SNO-Hb: $r^2 = 0.996$, $P < 0.001$, $n = 5$; $HbFe^{II}NO$: $r^2 = 0.999$, $P < 0.001$, $n = 5$). The assay is capable of measuring differences between control hemoglobin and 0.001% nitrosylated hemoglobin ($n = 8$, $P = 0.007$). The result is expressed as a percentage of mol NO/mol heme subunit. This value is calculated by dividing the concentration of NO released from hemoglobin in $I_3^-$ (after subtracting the background NO concentration generated by a 200-$\mu$l injection of the water from the Sephadex G25 column) by the concentration of the hemoglobin, measured by conversion to cyanomethemoglobin ($\varepsilon_{540} = 11$ for heme) (28).

**Ozone-Based Chemiluminescent Determination of Serum Nitrate, Nitrite, and High and Low-Molecular-Weight *S*-Nitrosothiols.** Blood samples were drawn into EDTA collection tubes and centrifuged at 750 × *g* for 5 min. Plasma aliquots were removed and stored at −80°C until they were assayed for nitrate, nitrite, and low-molecular-weight *S*-nitrosothiols as described below. To measure the HMW-SNO, a 500-μl aliquot of the plasma was run on a Sephadex G25 sizing column (9.5-ml bed volume) in a dark room within 30 min of collection. The 3–3.5-ml elution volume, which contained plasma high-molecular-weight proteins (albumin and immunoglobulins), was collected, and 200 μl was reacted in $I_3^-$. Injection of samples into this $I_3^-$ reductant stoichiometrically produces NO from nitrosothiols (21, 28).

For the measurement of nitrite, nitrate, and LMW-SNO, frozen plasma samples were later thawed and filtered through a prewashed (four times with nitrite-free water) filtration unit (molecular weight cut-off: 30,000). Samples were then injected in different reductants in line with 1 M NaOH and the Sievers NO analyzer (model 280). Nitrite was measured by reduction in acidified KI (7 ml of glacial acetic acid, 2 ml of distilled water, 50 mg of KI) (21). Nitrate was measured by reduction in vanadium (III) at 90°C (29). Low-molecular-weight *S*-nitrosothiols were measured in $Cu^+$/L-cysteine, which does not release NO from nitrate or nitrite (30). These assays demonstrated linear sensitivity above 1.0 pmol ($r^2 > 0.99$, $P < 0.001$) for standards of nitrite, nitrate, and SNO-glutathione. Characteristics of the assays for standards in plasma are described in the Results section.

**Statistical Analysis.** Comparison of arterial with venous levels of SNO-Hb, LMW-SNOs and HMW-SNOs, nitrite, and nitrate for the three experiments (baseline, L-NMMA, and L-NMMA with exercise) were performed by two-tailed paired *t* test and repeated-measures ANOVA. Interactions between arterial-venous gradients and experiment, and between the gradients and the morning and afternoon studies, separated in time by 2 h, were tested with this model (SAS Institute, Cary, NC). When interactions were statistically significant ($P < 0.05$), post hoc testing was performed by the Bonferroni method. Because there were no significant differences between the morning and afternoon studies, the results were averaged. Similarly, because the effects of L-NMMA at 4 μmol/min and at 8 μmol/min infusions were similar (approximately 30% reduction in forearm blood flow), blood flow data from all 10 subjects were averaged. Differences between arterial and venous concentrations and consumption (arterial minus venous concentrations multiplied by flow) of SNO-Hb, LMW-SNOs and HMW-SNOs, nitrite, and nitrate for the three experiments (baseline, LNMMA, exercise) were also evaluated by two-tailed paired *t* test and repeated-measures ANOVA. Data are shown with standard errors of the mean.

## Results

**Physiological Effect of NO Synthase Inhibition and Exercise on Forearm Blood Flow.** L-NMMA infusion results in a physiologically significant reduction in basal forearm blood flow (Fig. 1). Basal forearm blood flow was 2.79 ± 0.34 ml/min per 100 ml of forearm tissue and, during both 4 and 8 μmol/min L-NMMA infusions, was reduced to 2.04 ± 0.22 ml/min per 100 ml of tissue ($P = 0.001$). Exercise during continued L-NMMA infusion significantly increased blood flow to 14.50 ± 1.71 ml/min per 100 ml of tissue. Exercise during L-NMMA infusion reduced venous pH in the forearm from 7.37 ± 0.01 to 7.31 ± 0.02 ($P = 0.015$).

**Basal Circulatory Levels of Plasma Nitrate and Nitrite.** Filtered plasma was reacted in vanadium (III) to detect nitrate. Circulatory values for these nitrogen oxide species are shown in Table 1. Five individuals were put on a 3-day nitrate-restricted diet, and their nitrate levels were approximately 20 μM lower than those individuals who simply fasted overnight. Significant arterial-venous gradients were not found in either group. The stability of the plasma nitrate levels over 1 day of measurement in fasting individuals on a nitrate-restricted diet suggests continued nitrate production during the day, consistent with low-level NO reactions with oxyhemoglobin to produce methemoglobin and nitrate (26, 28).

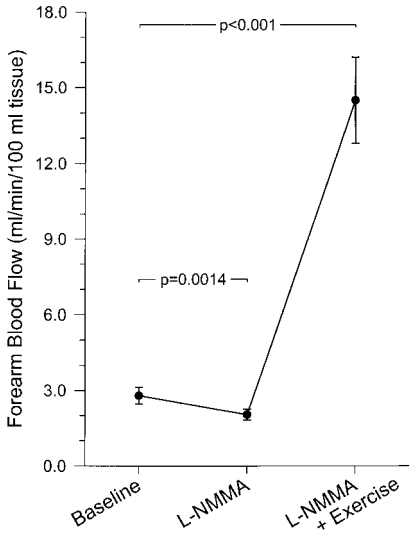


**Fig. 1.** Physiological effect of NO synthase inhibition and exercise on forearm blood flow. Forearm blood flow measurements, expressed as ml/min/100 g tissue, were performed at baseline, during L-NMMA infusion, and during L-NMMA infusion with hand-grip exercise. Basal forearm blood flow was significantly reduced from 2.79 ± 0.34 ml/min per 100 ml of tissue to 2.04 ± 0.22 ml/min per 100 ml of tissue ($P = 0.001$) during L-NMMA infusion (data shown represent the average of measurements made during the morning and the repeated afternoon study in 10 individuals). Exercise, during L-NMMA infusion, significantly increased blood flow to 14.50 ± 1.71 ml/min per 100 ml of tissue ($P < 0.001$). Data are expressed as the mean ± SEM.

Filtered plasma was reacted in KI in acetic acid to measure nitrite. Arterial nitrite levels were 540 ± 74 nM and venous levels 466 ± 79 nM. Interindividual volunteer subject variations in nitrite levels were large, with values ranging from 923 nM to 209 nM, but intraindividual variations were minimal (Table 1 and Fig. 2).

**Basal Circulatory Levels of Plasma LMW-SNOs and HMW-SNOs and Red Cell SNO-Hb.** Filtered plasma samples were injected in $Cu^+$/L-cysteine (100 μl of sample) to measure LMW-SNO. All samples were rapidly thawed and filtered in the dark (<20 min from processing to injection). LMW-SNO levels were undetectable in arterial and venous blood in all subjects studied. To determine the stability of LMW-SNO in plasma, standards of *S*-nitrosoglutathione were added to patient plasma samples and processed under the same conditions. There is an approximately 25% loss of LMW-SNO in plasma over 30 min, even in the presence of 0.5 mM EDTA, resulting in a measured limit of sensitivity of the $Cu^+$/L-cysteine–chemiluminescent assay for *S*-nitrothiols in plasma of 25 nM. Therefore we can conclude that the levels of LMW-SNO in human plasma are less than 25 nM.

Fresh plasma from volunteer subjects was immediately run through a molecular sizing column, and 100 μl of the high-molecular-weight fraction was injected into the $I_3^-$ reductant. Circulatory values for HMW-SNO are shown in Table 1. Arterial and venous HMW-SNO levels were detectable but much lower than previously reported. Arterial levels of HMW-SNO have never previously been reported and are 44.9 ± 14 nM. Inter-

**Table 1. Circulatory levels of nitrogen oxide molecules at rest and during regional NO synthase inhibition followed by exercise**

| | | Baseline | L-NMMA | L-NMMA + exercise |
|---|---|---|---|---|
| Nitrate, $\mu$M | Artery | 40.7 ± 4.5 | 41.4 ± 4.9 | 40.4 ± 5.8 |
| Standard diet | Vein | 41.3 ± 5.5 | 39.3 ± 6.4 | 37.3 ± 5.0 |
| $n = 5$ | | | | |
| Nitrate, $\mu$M | Artery | 22.9 ± 1.2 | 23.6 ± 2.8 | 25.0 ± 3.9 |
| Low nitrate diet | Vein | 21.3 ± 1.5 | 23.5 ± 3.0 | 24.8 ± 4.4 |
| $n = 5$ | | | | |
| Nitrite, nM* | Artery | 540 ± 74 | 569 ± 93 | 618 ± 86 |
| $n = 10$ | Vein | 466 ± 79 | 499 ± 73 | 531 ± 91 |
| HMW-SNO, nM** | Artery | 45 ± 14 | 44 ± 8 | 44 ± 11 |
| $n = 10$ | Vein | 63 ± 13 | 67 ± 10 | 57 ± 9 |
| SNO-Hb, nM† | Artery | 161 ± 42 | 173 ± 49 | 123 ± 30 |
| $n = 10$ | Vein | 142 ± 29 | 115 ± 34 | 75 ± 33 |

Data = mean ± SEM. *, $P < 0.05$ for arterial-venous gradients during study. **, $P < 0.05$ for venous-arterial gradients during study.
†Expressed as concentration in whole blood.

estingly, venous levels (63.4 ± 13 nM) were higher than arterial levels (Table 1).

Red cells were washed, lysed, and reacted in KCN/$K_3Fe(CN)_6$, and the hemoglobin was purified through a sizing column. Two hundred-milliliter samples of purified hemoglobin were then injected into $I_3^-$ to measure SNO-Hb. Arterial SNO-Hb levels were $1.6 \times 10^{-5} \pm 0.000004$ mol of NO/mol of heme subunit (161 ± 42 nM in whole blood), and venous levels were $1.4 \times 10^{-5} \pm 0.000003$ mol of NO/mol of heme subunit (142 ± 29 nM in whole blood) (Table 1).

**Arterial-Venous Gradients Are Observed for Nitrite, HMW-SNOs, and SNO-Hb.** Arterial levels of nitrite are significantly higher than venous levels in all three experimental situations, suggesting delivery or metabolism in the peripheral circulation (Fig. 2) ($P <$ 0.05 for baseline, L-NMMA treatment, and L-NMMA treatment with exercise).

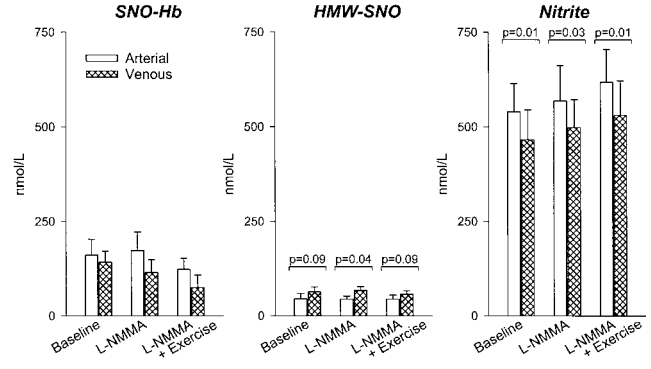


**Fig. 2.** Plasma circulatory levels of nitrite, HMW-SNO, and SNO-Hb at rest and during NO synthase inhibition followed by exercise. SNO-Hb: *S*-nitrosohemoglobin in arterial (empty bars) and venous (hatched bars) red blood cells at rest and during L-NMMA infusion, followed by exercise. The arterial and venous levels were close to the limits of detection by chemiluminescent technology at $1.6 \times 10^{-5}$ mol NO/mol heme subunit (161 nM in whole blood) and $1.4 \times 10^{-5}$ mol of NO/mol of heme subunit (141 nM in whole blood), respectively. We observed an insignificant arterial-venous gradient in SNO-Hb ($P = 0.53$ at baseline, $P = 0.17$ during L-NMMA infusion, and $P = 0.31$ during exercise, by paired *t* test, and $P = 0.24$ by repeated-measures ANOVA). HMW-SNO: plasma passed through a G25 Sephadex sizing column was reacted in $I_3^-$ to measure HMW-SNO in arterial and venous plasma. HMW-SNO displayed a reversed gradient with venous levels (63.4 ± 13 nM) greater than arterial levels (44.9 ± 14 nM) (*P* values for paired *t* tests shown and $P < 0.05$ for venous-arterial differences by repeated-measures ANOVA). Nitrite: filtered plasma was reacted in KI in acetic acid to measure nitrite in arterial and venous plasma. Although nitrite levels varied widely between individuals, within individuals the arterial levels of nitrite (540 ± 74 nM) were significantly higher than venous levels (466 ± 79 nM), suggesting delivery or metabolism in the peripheral circulation ($P < 0.05$ for all experiments by paired *t* test).

Interestingly, HMW-SNO displays a reversed gradient with venous levels greater than arterial levels in all three groups of experiments (Fig. 2). These gradients achieved significance only during L-NMMA infusion ($P = 0.09$ at baseline, $P = 0.04$ during L-NMMA infusion, and $P = 0.09$ during L-NMMA infusion with exercise, by paired *t* test) but were significant by repeated-measures ANOVA ($P < 0.05$). The observed formation of HMW-SNO in the peripheral circulation is consistent with an intravascular transfer of NO from nitrite or SNO-Hb to albumin. The lack of a significant effect of NO synthase inhibition on HMW-SNO formation suggests that the *S*-nitrosation of albumin derives from an intravascular source rather than from endothelial NO. The amount of HMW-SNO formed is significantly less than the amount of nitrite consumed, suggesting that the majority of nitrite is converted to NO, which may be bioactive, or metabolized to nitrate by cellular or hemoglobin reactions.

We observed an insignificant arterial-venous gradient in SNO-Hb under all experimental conditions (Fig. 2). The levels are close to the limits of detection by chemiluminescent technology and at 161 nM are lower than those reported in rats (8).

**Nitrite Consumption Increases Significantly During Exercise with NO Synthase Inhibition; No Significant Change in Production or Consumption Occurs for HMW-SNO or SNO-Hb, Respectively.** To determine the role of circulating nitrogen oxide species in the regulation of blood flow, we first inhibited regional NO synthesis and then stressed the vascular bed with hand-grip exercise during continued NO synthesis inhibition. Inhibiting NO synthase ensures that any change in consumption or production of nitrite or *S*-nitrosothiols represents intravascular consumption or production. Arterial and venous nitrite and *S*-nitrosothiol gradients (arterial nitrite concentration minus venous nitrite concentration) were multiplied times flow measurements to determine consumption or, in the case of HMW-SNO, production. All units are expressed as picomol consumed or produced per ml of blood per min per 100 ml of tissue; the results are shown in Fig. 3. Nitrite consumption significantly increased with exercise, from 156 ± 59 pmol/ml/min/100 ml of tissue during L-NMMA infusion to 1583 ± 517 pmol/ml/min/100 ml of tissue during L-NMMA infusion with exercise, suggesting that intravascular nitrite is used, particularly during physiological stress. Interestingly, a trend toward increased formation of HMW-SNO was observed, suggesting transfer of a fraction of the NO produced from nitrite to albumin. SNO-Hb consumption increased non-

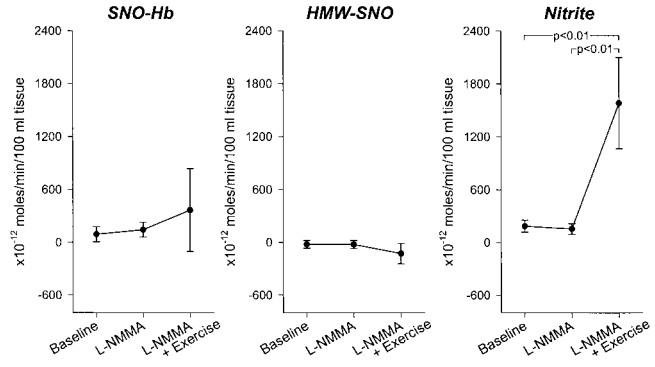


**Fig. 3.** Consumption of nitrogen oxide species at baseline, during NO synthase inhibition, and during exercise with NO synthase inhibition. Arterial and venous nitrite and *S*-nitrosothiol gradients were multiplied by flow measurements to determine consumption (positive value on the *y* axis) or, in the case of HMW-SNO, production (negative value on the *y* axis). SNO-Hb: Basal SNO-Hb consumption was 92 ± 86 pmol/ml/min/100 ml of tissue. SNO-Hb consumption increased nonsignificantly from 143 ± 83 pmol/ml/min/100 ml of tissue during L-NMMA infusion to 366 ± 471 pmol/ml/min/100 ml of tissue during L-NMMA infusion with exercise. Data are expressed as the mean ± SEM. HMW-SNO: At baseline, HMW-SNO forms (negative value for consumption) in the peripheral circulation at a rate of 23 ± 14 pmol/ml/min/100 ml of tissue. Although there was no significant change in the formation of HMW-SNO during L-NMMA infusion (25 ± 8 pmol/ml/min/100 ml of tissue), a trend toward increased formation of HMW-SNO was observed during exercise and L-NMMA infusion (127 ± 117 pmol/ml/min/100 ml of tissue). Nitrite: Nitrite consumption significantly increased with exercise, from 156 ± 59 pmol/ml/min/100 ml of tissue during L-NMMA infusion to 1583 ± 517 pmol/ml/min/100 ml of tissue during L-NMMA infusion with exercise, suggesting that intravascular nitrite is used even during physiological stress.

significantly from 143 ± 83 pmol/ml/min/100 ml of tissue during L-NMMA infusion to 366 ± 471 pmol/ml/min/100 ml of tissue during L-NMMA infusion with exercise.

## Discussion

Our results represent a comprehensive survey of circulating arterial and venous levels of all bioactive nitrogen oxide species in humans. These experiments were designed to study the role of circulating nitrite and *S*-nitrosothiols vs. endothelium-derived NO in the regulation of basal vascular tone. Consistent with our previous observations (5), regional pharmacological inhibition of NO synthase significantly reduces forearm blood flow, demonstrating the importance of regional synthesis of NO in the regulation of regional microvascular flow. Importantly, we found circulating arterial-venous nitrite gradients, providing a novel delivery source of intravascular NO. Further supporting the notion that nitrite is bioactive, the consumption of nitrite increases significantly with exercise and inhibition of regional endothelial synthesis of NO. The role of circulating *S*-nitrosothiols and SNO-Hb in the regulation of basal vascular tone is less certain. Using well-validated methodology in 10 normal volunteers, we report here that the basal circulatory levels of low-molecular-weight *S*-nitrosothiols are unmeasurable, and HMW-SNO levels (likely representing SNO-albumin) are two logs lower than previously reported. In fact, SNO-albumin appears to form in the venous circulation, even during NO synthase inhibition, providing evidence for intravascular NO transfer to albumin and suggesting that NO is not delivered on albumin from the lungs to the tissue. Finally, we demonstrate that SNO-Hb does exist in the human circulation, that trends (albeit not statistically significant) toward arterial-venous gradients are apparent, but that delivery is minimal under basal conditions and during stress (L-NMMA and exercise).

Nitrite is a relatively stable oxidation product of NO and oxygen and exists in the circulation at concentrations of approximately 500 nM. Because nitrite levels are below the sensitivity of the classic colorimetric Griess reaction, it has been less studied than nitrate. However, unlike nitrate, which is thought to be a biologically inactive end product of NO reactions with oxyhemoglobin, recent data suggest that nitrite remains bioactive. Under physiological levels of acidity, nitrite forms nitrous acid with spontaneous decomposition to NO (12–14). Although these processes have been shown to occur under more acidic conditions (pH 5 in airway lining fluid and pH 5–6 in ischemic tissue), these reactions should continue to occur, albeit to a lesser extent, at higher pH. Furthermore, recent studies suggest that xanthine oxidase, which is present in abundance in vascular endothelium (31–33), contains a molybdenum center similar to the bacterial enzyme nitrate reductase. Xanthine oxidase has been reported to reduce nitrite to NO, an effect that increases with increasing NADH concentration and hypoxia (15–18). It is therefore possible that nitrite, from NO produced in the lungs reacting with oxygen, is carried in the plasma and reduced in the peripheral vasculature to bioactive NO. Under hypoxic and acidic conditions, evident during exercise, as demonstrated in these studies, or pathological tissue ischemia, the conversion of nitrite to NO could reduce NO scavenging by red cells in stasis and increase blood flow to these critical regions.

Recent innovative work by Stamler and his colleagues has suggested that NO binding to cysteine groups on amino acids and proteins, to form *S*-nitrosothiols, may play an important role in the regulation of blood flow in vertebrates. They have presented data suggesting that NO is transported on hemoglobin by binding to the highly conserved $\beta$-chain Cys-93 residue, forming SNO-Hb (8–10, 19) and on albumin, as SNO-albumin (7). SNO-Hb levels in the Sprague–Dawley rat were reported to be 311 nM, and the levels of venous plasma *S*-nitrosothiols in humans were reported to be 7 $\mu$M, of which 80% was SNO-albumin. This level of *S*-nitrosothiols would represent a large pool of bioavailable NO with significant vascular effects.

The amount and significance of these nitrosative species *in vivo* have been controversial for a number of reasons. NO gas is a poor nitrosating agent at physiological pH, suggesting that minimal *S*-nitrosothiols will form from NO generated by NO synthase. The high affinity of NO for heme groups suggests that the predominant reaction of NO gas at the alveolar-vascular interface will be with oxyhemoglobin to form nitrate and methemoglobin, while the reaction of NO and oxygen to form nitrosative intermediates ($N_2O_3$), which can nitrosate thiol groups and produce nitrite (22–25), may be limited by the low concentration of oxygen and NO dissolved in blood. The limited reactivity of NO and oxygen in blood is due to the fact that the reaction is second order in relation to NO and first order in relation to oxygen. When the initial concentration of NO is 1 $\mu$M, the half-life is 800 s, whereas at 100 $\mu$M, the half-life is 8 s (34). Previous work demonstrating increases in SNO-Hb levels in the Sprague–Dawley rat arterial and venous blood exposed to 100% oxygen at a pressure of 3 atm (9) may reflect an increase in the concentration of dissolved oxygen (approximately 0.13 mM in arterial blood, increasing to 2.85 mM at 100% oxygen, at 3 atm), resulting in increased autooxidation of NO, rather than a specific oxygen-mediated allosteric effect on hemoglobin *S*-nitrosation. This increase in autooxidation of NO would result in an increased nitrosative environment, particularly in the hydrophobic erythrocyte cell membrane, favoring nitrosation of thiols.

It is therefore not surprising that the basal circulatory levels of *S*-nitrosated proteins are very low and nitrosative chemistry is limited in the human circulation. Our previous work demonstrated that during NO inhalation, reactions with heme groups predominate (26). The very high affinity of NO for both oxyhemoglobin and deoxyhemoglobin ($K_a = 10^7\ M^{-1}\ s^{-1}$)

observed *in vitro* is manifest *in vivo* as well. We reported mean baseline methemoglobin levels of 0.24 ± 0.02% (22.8 $\mu$M heme), which increased to 0.9 ± 0.1% (84 $\mu$M heme) during NO breathing. Basal and NO breathing levels of methemoglobin were approximately 70-fold higher than Hb($Fe^{II}$)NO levels (1.2 $\mu$M heme), which in turn were nearly 5-fold higher than the SNO-Hb levels (0.26 $\mu$M heme) (26). Therefore, the *in vivo* reactions of NO with hemoglobin are consistent with the known kinetics of NO-hemoglobin interactions *in vitro* and the relative greater abundance of oxygenated compared with deoxygenated hemoglobin in the lung.

Furthermore, despite significant changes in blood flow and tissue pH, there is no observed extraction of NO from *S*-nitrosothiol intravascular sources under conditions of exercise, suggesting that these species do not deliver significant amounts of NO for the regulation of vascular flow during this stress. However, it remains possible that across other vascular beds (organ specific or more ischemic) the extraction rate of NO may increase. Recent studies demonstrate that SNO-Hb has an increased oxygen affinity with intact Bohr and phosphate effects, suggesting that it would only deliver its oxygen and thus release its NO to regions with low pH and/or very low oxygen tension (19, 35). Thus hemoglobin *S*-nitrosation may be a "salvage" pathway, providing the red cell with a mechanism to deliver NO only in regions with significant stress (reduced blood flow, tissue hypoxia, and acidosis), where hemoglobin-NO scavenging would be deleterious. It is also possible that Cys-93 evolved as a nucleophile acceptor of NO released from $HbFe^{II}NO$. $NO^+$ release from heme could undergo geminate recombination with intramolecular Cys-93 or intermolecular transfer to a neighboring hemoglobin molecule. However, this recombination or transfer would require an electron acceptor. It remains possible that reduction of oxygen in the heme pocket to superoxide (36, 37) or reduction of other metals such as $Cu^{2+}$ (38, 39) or methemoglobin could facilitate this transfer. The NO could then be transported out of the erythrocyte by transnitrosation with glutathione.

In conclusion, these results establish the basal circulatory levels of nitrite and *S*-nitrosothiols in the human circulation and provide evidence of a significant arterial-venous gradient in nitrite, a pool of bioavailable intravascular NO. These data suggest that intravascular NO transfer to high-molecular-weight thiols occurs, creating SNO-albumin in the peripheral circulation. Finally, although SNO-Hb and SNO-albumin are measurable in the human circulation, they have a limited role in the maintenance of basal vascular tone, even during NO synthase inhibition.

We gratefully acknowledge the assistance of Melissa Bryant, RN, in the performance of forearm blood flow studies, and William H. Schencke in the statistical analysis of data and the production of figures.

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