Oxygen Regulation of Tumor Perfusion by S-Nitrosohemoglobin Reveals a Pressor Activity of Nitric Oxide


Abstract—In erythrocytes, S-nitrosohemoglobin (SNO-Hb) arises from S-nitrosylation of oxygenated hemoglobin (Hb). It has been shown that SNO-Hb behaves as a nitric oxide (NO) donor at low oxygen tensions. This property, in combination with oxygen transport capacity, suggests that SNO-Hb may have unique potential to reoxygenate hypoxic tissues. The present study was designed to test the idea that the allosteric properties of SNO-Hb could be manipulated to enhance oxygen delivery in a hypoxic tumor. Using Laser Doppler flowmetry, we showed that SNO-Hb infusion to animals breathing 21% O₂ reduced tumor perfusion without affecting blood pressure and heart rate. Raising the pO₂ (100% O₂) slowed the release of NO bioactivity from SNO-Hb (ie, prolonged the plasma half-life of the SNO in Hb), preserved tumor perfusion, and raised the blood pressure. In contrast, native Hb reduced both tumor perfusion and heart rate independently of the oxygen concentration of the inhaled gas, and did not elicit hypertensive effects. Window chamber (to image tumor arteriolar reactivity in vivo) and hemodynamic measurements indicated that the preservation of tissue perfusion by micromolar concentrations of SNO-Hb is a composite effect created by reduced peripheral vascular resistance and direct inhibition of the baroreceptor reflex, leading to increased blood pressure. Overall, these results indicate that the properties of SNO-Hb are attributable to allosteric control of NO release by oxygen in central as well as peripheral tissues. (Circ Res. 2005;96:1119-1126.)

Key Words: nitric oxide ■ hemoglobin ■ oxygen ■ hemodynamics ■ blood flow

Hemoglobin (Hb) of red blood cells (RBC) is a tetrameric protein composed of 2 α and 2 β chains, each containing a heme prosthetic group. One α and 1 β chain is combined in stable αβ dimers, and 2 dimers are more loosely associated to form tetramers. The physiological role of Hb depends on its ability to reversibly bind O₂ at its heme iron centers. This transport capacity is governed by a cycle of allosteric transitions in which Hb assumes the R (relaxed, high O₂ affinity) form tetramers. The physiological role of Hb depends on its conformation to bind O₂ in the lungs and, on partial deoxygenation, the T (tense, low O₂ affinity) conformation to unload NO. This transition also efficiently delivers O₂ to peripheral tissues. The present study was designed to test the idea that the allosteric properties of SNO-Hb could be manipulated to enhance oxygen delivery in a hypoxic tumor. Using Laser Doppler flowmetry, we showed that SNO-Hb infusion to animals breathing 21% O₂ reduced tumor perfusion without affecting blood pressure and heart rate. Raising the pO₂ (100% O₂) slowed the release of NO bioactivity from SNO-Hb (ie, prolonged the plasma half-life of the SNO in Hb), preserved tumor perfusion, and raised the blood pressure. In contrast, native Hb reduced both tumor perfusion and heart rate independently of the oxygen concentration of the inhaled gas, and did not elicit hypertensive effects. Window chamber (to image tumor arteriolar reactivity in vivo) and hemodynamic measurements indicated that the preservation of tissue perfusion by micromolar concentrations of SNO-Hb is a composite effect created by reduced peripheral vascular resistance and direct inhibition of the baroreceptor reflex, leading to increased blood pressure. Overall, these results indicate that the properties of SNO-Hb are attributable to allosteric control of NO release by oxygen in central as well as peripheral tissues.
retain the vasoconstrictive activity of cell-free Hb.\textsuperscript{13} Ferrous Hb and oxy-Hb can react with NO to produce iron-nitrosyl Hb, and oxidized Hb and nitrate ions (met-Hb reaction), respectively.\textsuperscript{14,15} The Hb concentrations required to exert such activity have not been carefully examined, and the extent to which vasodilation is involved (under more physiological circumstances, Hb can also react with NO or S-nitrosothiols to yield SNO-Hb)\textsuperscript{16} remains a matter of debate. Lower doses of Hb-based blood substitutes do not typically raise blood pressure.

To date, SNO-Hb, which combines the capacity to carry oxygen and the potential to act as a hypoxia-activated NO donor, has not yet been fully tested as a RBC substitute.\textsuperscript{17} In the present work, we hypothesized that S-nitrosylation of cell-free Hb would reduce the vasoconstrictive effects of tetrameric Hb. We also hypothesized that blood oxygenation, through allosterically-regulated control of NO release, would be a key determinant of the biological responses to SNO-Hb. These hypotheses were investigated in vivo using a physiological model of vascular hypoxia (perfused tumor vessels), in which pO\textsubscript{2} was modulated in situ by varying inspired O\textsubscript{2}.

![SNO-Hb and Oxy-Hb IV Reduce Muscle and Tumor Perfusion in Normoxic Rats](https://example.com)

**Blood Gas and Hemoglobin Saturation Measurements in Tumor Microvessels**

Blood gases and Hb oxygen saturation were measured using a 1640 oximeter coupled to a 482 cooximeter (Instrumentation Laboratories, Lexington, Mass) from 0.5 mL samples of femoral arterial and venous blood. For tumor microvessels in window chambers, Hb saturation was calculated from transmission optical measurements of vascular absorbance using a hyperspectral imaging technique.\textsuperscript{21} A liquid crystal tunable filter (CRI, Inc., Woburn, Mass) placed in front of a CCD camera (DVC Company, Austin, Tex) was used for band-limited imaging.

**Blood Flow**

Blood flow was measured using Laser Doppler flowmetry. For flank tumor experiments, 300-μm diameter Laser Doppler probes (OxiFlo, Oxford Optronix, Oxford, UK) were simultaneously inserted into the tumor and quadriceps muscle of each animal. For window chamber experiments, Laser Doppler probes (LASERFLO, TSI, St. Paul, Minn) were positioned beneath the tumor window as previously described.\textsuperscript{22}

**SNO-Hb Assay**

The determination of SNO-Hb concentrations is based on a reaction where cleavage of S-nitrosothiols yields a nitrosant that activates 4,5-diaminofluorescein (DAF-2, Sigma, St. Louis, Mo). Data acquired before drug infusion allowed us to determine basal SNO levels. The plasma half-life of SNO-Hb was calculated from exponential decay curves fitting each experimental curve. Hb concentration in plasma samples was determined by visible spectrophotometry.Expanded method is available online.

**Vascular Reactivity In Vivo**

Tumor-feeding arterioles in window chambers were visualized using transillumination. Arteriolar diameters were measured from video-taped images using an image-shearing monitor (IPM Inc., San Diego, Calif).

**Mean Arterial Pressure and Heart Rate**

Mean arterial pressure (MAP) and heart rate (HR) were measured with a blood pressure analyzer (Digi-Med, Micro-Med, Louisville, Ky) connected to the femoral artery cannula, as previously described.\textsuperscript{20}

**Statistical Analyses**

All values are shown as means±S.E. Time curves were normalized to the baseline before infusion. ‘N’ refers to the number of animals per group and ‘n’ to individual measurements. Repeated-measure 2-way ANOVA or Student’s t tests were used as indicated.

**Results**

SNO-Hb and Oxy-Hb IV Reduce Muscle and Tumor Perfusion in Normoxic Rats

We first examined whether IV infusion of human cell-free oxy-Hb and SNO-ox-Hb (SNO-Hb) influenced muscle perfusion in the quadriceps of rats breathing room air (normoxia). SNO-Hb decreased muscle perfusion more than Hb when compared with albumin (Figure 1A, P=0.055 and 0.3 versus albumin, respectively, 2-way ANOVA). We then determined the pharmacological effects of these molecules in flank tumors of normoxic rats. In contrast to albumin, Hb...
induced a rapid and sustained decrease in tumor blood flow (Figure 1B, \(P<0.01\) versus albumin, 2-way ANOVA). Un-
expectedly, i.v. infusion of SNO-Hb also reduced perfusion (Figure 1C, \(P<0.05\) versus SNO-albumin, 2-way ANOVA). How-
ever, the effects of the 2 Hbs were quite different from the small increase in tumor perfusion that we observed following albumin or 
SNO-albumin infusion i.v. (Figure 1B and 1C). The effects of SNO-albumin and albumin were not different (\(P>0.05\), 2-way ANOVA).

**Hyperoxia Prolongs the Half-life and Modulates the Pressor Activity of Cell-Free SNO-Hb**

Based on previous observations,\(^2\) we reasoned that an increase in plasma oxygenation would stabilize SNO-Hb, increase peripheral delivery and thereby increase the bioac-
tivity in tumors. We aimed to determine whether the plasma half-life of SNO-Hb i.v. was prolonged in rats breathing 100% O\(_2\) (hyperoxia) versus room air. Blood gas measure-
ments showed that breathing 100% O\(_2\) induced significant increases in venous and arteriolar blood pO\(_2\), pCO\(_2\), and endogenous Hb oxygen saturation. Changes in Hb saturation were identical in femoral vein and tumor venules. Changes were less pronounced in tumor-feeding arterioles versus the femoral artery, as expected from blood deoxygenation along the arterial tree and at the tumor margin (see Table in the online supplement).

Because of low concentration (\(\approx 6.5\) \(\mu\)mol/L) and alteration in the tetramer/dimer ratio as a function of concentration and O\(_2\) saturation (dimer/tetramer equilibrium is 10000-
fold lower in deoxy-Hb), it was not possible to directly measure the oxygen saturation of exogenous Hbs. Instead, we measured plasma SNO-Hb concentration versus time follow-
ing infusion using a DAF-2 assay. The fitting of individual experimental data with exponential decay curves (\(R^2=0.87\pm0.05\) and 0.90±0.03 for normoxic and hyperoxic conditions, respectively) revealed a 2-fold increase in the half-life of the S-nitrosyl group in SNO-Hb in the plasma of hyperoxic versus normoxic rats (Figure 2, \(P<0.01\), Student’s \(t\) test). We found no influence of O\(_2\) on the rate of SNO-Hb protein clearance (data not shown). Spectrophotometric measure-
ments revealed that hyperoxia did not affect the rate of protein clearance during the period of the experiments (half-
life \(\approx 30\) minutes, independent of O\(_2\) supply) (data not shown). Thus, our results, together with previous observa-
tions,\(^2,3\) suggest that the effects of O\(_2\) are mediated by an allosteric mechanism that promotes the R structure.

The effects of hyperoxia alone on tumor and muscle perfusion ranged from a transient decrease (that resumed within 5 minutes, ie, before drug infusion) to no change. As expected from these observations (and in agreement with prior publications),\(^2,3\) hyperoxia abolished the reduction in tumor perfusion that we observed after i.v. infusion of SNO-Hb in normoxic rats (Figure 3A, \(P<0.005\), 2-way ANOVA). In contrast to SNO-Hb, and native Hb reduced tumor perfusion independently of O\(_2\) supply (Figure 3B, \(P>0.05\), 2-way ANOVA).
Hyperoxia Unmasks the Systemic Pressor Activity of Cell-Free SNO-Hb

We then sought to identify the relative contribution of changes in vascular activity versus systemic hemodynamics in the tumor perfusion response to SNO-Hb. In window chambers, i.v. infusion of SNO-Hb induced no significant change in the diameter of feeding arterioles in normoxic or hyperoxic rats (Figure 4A, \( P > 0.05 \), 2-way ANOVA). As reported in our previous studies,\(^{20,22} \) hyperoxia alone had no vascular effect (compare t5 to t=0 in Figure 4A, white boxes). Interestingly, in the same rats, we simultaneously documented that SNO-Hb induced a potent decrease in tumor perfusion in normoxic animals, and that this decrease was prevented in hyperoxic rats (data not shown). Thus, these results were identical to what we observed in flank tumors (Figure 3A).

Changes in the tumor blood flow were paralleled by changes in the perfusion of the leg quadriceps muscle: the decrease in muscle blood flow after delivery of SNO-Hb under normoxia was prevented under hyperoxia (Figure 4B, ***\( P < 0.005 \) between t=0 and t+20 (2-way ANOVA).
P<0.05, 2-way ANOVA). In contrast, hyperoxia did not modify the effect of oxy-Hb in muscles (data not shown).

Together, these observations suggest that SNO-Hb might indirectly modulate tumor perfusion in an O2-dependent manner as a consequence of changes in mean arterial pressure (MAP) and the heart rate (HR). We observed no change in MAP (Figure 4C) or HR (Figure 4D) when SNO-Hb was infused i.v. in normoxic animals (Student’s t test versus values at t=0). However, under hyperoxia, we measured a significant increase in the MAP as soon as 10 minutes after the infusion of SNO-Hb i.v. (Figure 4C, P<0.05 from t=10 to t=20, Student’s t test versus value at t=0). There was no significant effect on HR (Figure 4D, P>0.05 for all values versus value at t=0, Student’s t test). Interestingly, in the same conditions, oxy-Hb had no effect on MAP during normoxia or hyperoxia (Figure 4E, P>0.05 for all values versus value at t=0, Student’s t test). Also, in contrast to SNO-Hb, oxy-Hb induced bradycardia as soon as 5 minutes after infusion (Figure 4D, P<0.05 from t=5 to t=20, Student’s t test versus value at t=0), which was more severe in hypoxic than in normoxic rats. Hyperoxia alone induced no significant changes in MAP (+1%) or HR (-3%) (P<0.05, N=19, Student’s t test).

The Route of Administration Impacts the Pressor Activity of Cell-Free SNO-Hb

The exquisite O2 dependence of the bioactivity of SNO-Hb prompted us to check whether the physiological difference of blood oxygenation between veins (femoral vein infusion) and arteries (left carotid artery infusion) was sufficient to modulate the pressor activity of SNO-Hb. In normoxic animals, despite a trend toward preservation of the flow, the decrease in tumor perfusion after i.a. infusion of SNO-Hb was not significantly different compared with i.v. infusion (Figure 5A, P=0.09, 2-way ANOVA). However, in contrast to i.v. SNO-Hb, the pressor activity of i.a. SNO-Hb was not altered when this experiment was repeated in hyperoxic rats (P>0.05 versus SNO-Hb i.a. room air, 2-way ANOVA). The lack of O2 dependence contrasted with the strong O2-dependent changes in tumor perfusion for i.v. infusions (these curves are provided in Figure 5A for comparison).

Furthermore, O2 concentration in the breathing gas did not modify the perfusion of the quadriceps muscle when SNO-Hb was infused i.a. (Figure 5B, P>0.05, 2-way ANOVA). As with the tumor results, the effects contrasted with the changes observed following i.v. injection (Figure 4B). Muscle blood flow remained unaltered after i.a. infusion of SNO-Hb, independently of O2 delivery (Figure 5B).

MAP was highly sensitive to SNO-Hb i.a.; we documented a significant increase in MAP as soon as 5 minutes and 10 minutes after drug delivery to normoxic and hyperoxic rats, respectively (Figure 5C, P<0.05 versus value at t=0, Student’s t test). The degree of hypertension, however, was unaffected by blood pO2. In the same set of experiments, HR remained unchanged (Figure 5D). In control experiments, i.a. and i.v. infusion of albumin (used to monitor volume effects on the baroreceptor) did not induce any significant or differential change in tumor perfusion, MAP, or HR of tumor-bearing rats (data not shown, all P>0.05 for albumin i.a. versus albumin i.v., 2-way ANOVA).

Discussion

Despite molecular modifications that successfully overcome the toxicity of unstable Hb tetramers,12 vasoconstriction remains a major limitation in the clinical use of Hb-based blood substitutes.13 Cell-free Hb preparations are devoid of the SNO that normally serves to counter their vasoconstrictive effects in vivo.23 We therefore investigated whether SNO reconstitution of Hb could reverse the vasoconstrictor activity of oxy-Hb, and whether SNO-Hb could be manipulated allosterically to maximize O2 delivery. In rats breathing room air (normoxia), SNO-Hb induced a greater decrease in tissue...
perfusion than native Hb (Figure 1A and Table 1). These results suggested that oxy-Hb and SNO-Hb operate by different mechanisms. Whereas the effects of oxy-Hb could be readily explained by NO scavenging (the “oxyhemoglobin,” or met-Hb forming-reaction), those of SNO-Hb were consistent with other NO donors, which paradoxically also decrease tissue perfusion. NO-mediated dilation of healthy blood vessels upstream of tumors creates shunts that divert blood away from the tumors (vascular steal). By raising the concentration of the inhaled O\textsubscript{3}, SNO-Hb bioactivity can be targeted to more distally blood vessels, and elicits improvements in tumor blood flow.

We and recently others\textsuperscript{1,3,8} have proposed that SNO-Hb is formed in concert with O\textsubscript{2} uptake in the lung, and that the release of vasodilator NO from SNO-Hb is favored at low pO\textsubscript{2} (ie, in deoxygenated tissues and arterioles). The presence of diffusional barriers that attenuate endothelium-derived NO entry into the RBC represents an additional mechanism by which NO bioactivity may be preserved in large vessels.\textsuperscript{4} But whereas diffusional barriers are biologically important in limiting overall NO consumption, they play a little role in hypoxic vasodilation, which is subserved by erythrocytic SNOM-Hb in microvessels\textsuperscript{3} (and is unaffected by NOS inhibitors and preserved in eNOS\textsuperscript{-/-} animals).\textsuperscript{6} Thus, in the microcirculation (where RBC are in close contact with the vessel wall), the barrier cannot provide an explanation for NO bioactivity. As reported in our previous studies,\textsuperscript{20,22} normobaric 100% oxygen breathing (hyperoxia) induced a significant increase in venous and arteriolar pO\textsubscript{2}. Although the delivery of pure oxygen sometimes reduced tumor and muscle perfusion (see Figure 3A, 3B, 5A, and 5B between t=-5 and t=0), this effect was transient and returned to baseline within <5 minutes. Thus, the vasoactive properties of oxygen per se did not affect the response to SNO-Hb or oxy-Hb. On i.v. infusion in hyperoxic animals, SNO modified Hb successfully opposed the reduction in perfusion created by Hb in tumors (compare Figure 3A and 3B). Increases in MAP were seen in response to SNO-Hb during 100% O\textsubscript{2} breathing, but there was no change in tumor/muscle perfusion or HR (Table 1). By comparison, the reduction in tumor perfusion by native Hb remained unaffected by hyperoxia (Figure 3B). This is likely accounted for by lowered systemic perfusion and bradycardia, not by changes in MAP (Table 1).

Hb dissociation from tetramers to dimers depends on Hb concentration and oxygen tension, and NO release from dimers is unresponsive to allosteric effectors (eg, O\textsubscript{2}).\textsuperscript{27} Assuming a plasma concentration of \approx 6.5 \mu mol/L, \approx 35% of SNO-Hb would be tetramers (K\textsubscript{o} for R and T conformers are 3 \mu mol/L and 3 nmol/L, respectively) at room air and the amount would increase precipitously as O\textsubscript{2} tension declines - the majority would be tetramers at tissue pO\textsubscript{2}. Is there enough tetrameric SNO-Hb to sense oxygen and regulate NO delivery? Evidently yes, as we documented a finely tuned regulation of SNO-Hb bioactivity by oxygen tension (NO release depends on local pO\textsubscript{2}) that contrasted with the O\textsubscript{2}-independent behavior of oxy-Hb. We further demonstrated that the plasma half-life of SNO in Hb was more than doubled during hyperoxia whereas protein clearance remained unaffected. Thus, the prolonged half-life of SNO at high pO\textsubscript{2} is consistent with the expectation that release of NO bioactivity is disfavored in the R structure.\textsuperscript{1,2} Collectively these data provide a definitive demonstration of allosteric regulation by O\textsubscript{2} of NO delivery from SNO-Hb.

During hyperoxia SNO-Hb is greatly stabilized, surviving arteriovenous transit. SNO-Hb bioactivity was thus indistinguishable following hyperoxic i.v. and normoxic i.a. infusions (Table 1). To address a point of confusion, we note that SNO-Hb will exert activity at both high and low pO\textsubscript{2} but that this activity will be potentiated at low pO\textsubscript{2}.\textsuperscript{23} Effects will
Therefore manifest in deoxygenated vessels. Consistent with this interpretation, hyperoxia had no effect on i.a. infusions of SNO-Hb but markedly altered i.v. responses.

On close inspection, our data unravel a central effect of SNO-Hb on control of hemodynamics. An increase in MAP was observed following i.a. infusion during normoxia, and both i.v. and i.a. infusions during hyperoxia. Native Hb (hyperoxia, i.v.) did not produce increases in MAP (Table 1) under any condition. Baroreceptor activity opposes increases in MAP and resistance to flow. Native Hb activated the baroreceptor, which opposed changes in MAP by decreasing the HR. In contrast, strikingly, SNO-Hb raised the MAP without altering perfusion (in the organs that we monitored) or HR. The systemic pressor activity of SNO-Hb results from a direct inhibition of the baroreceptor reflex. Indeed, at higher concentrations (1 μmol/kg, i.v., normoxia), it has been reported that both SNO-Hb and oxy-Hb overcome baroreceptor buffer capacities (presumably indirectly through NO scavenging and peripheral vasoconstriction). Thus, SNO-Hb activity in hyperbaric hyperoxia is sufficient to inhibit the baroreceptor. As a neurotransmitter, NO is known to regulate baroreceptor activity through sympathetic afferent nerve fibers. Exogenous NO has an inhibitory action on the baroreceptor, albeit only at high concentrations, and under such circumstances that the baroreceptor activity recovers within seconds. Unlike classical NO activity, SNO-Hb bioactivity is mediated by species distinct from NO itself (eg, low-mass SNOs). It is noteworthy that other endogenous S-nitrosothiols (eg, SNO-cysteine) can suppress baroreceptor activity independently of cGMP generation (the mediator of classical nitrososaldilator activity). Stereoselective recognition sites in the baroreceptor vasculature could mediate baroreceptor inhibition by S-nitrosylated species such as SNO-cysteine and SNO-Hb. Expanded discussion is available online.

In conclusion, we have shown that SNO reconstitution of Hb successfully overcomes the reduction in tumor perfusion created by Hb itself. This activity involves the allosteric control of NO release by O2. The hemodynamic effects of SNO-Hb are a composite of central activity (baroreceptor inhibition) and allosterically facilitated NO release in the peripheral circulation. In contrast, native Hb, at concentrations characteristic of hemolytic states, has no central pressor effect. These results are summarized in Table 2.

Whereas the concentration of Hb we used is low from the O2 delivery standpoint, it greatly exceeds the concentration of any endogenous NO. In light of our results, development of safe Hb-based blood substitutes might include approaches that not only preserve Hb allosterity (eg, crosslinking to prevent dissociation into dimers) but also reconstitute SNO content. Reoxygenation of hypoxic tissues may benefit from both the central pressor and blood flow increasing effects elicited by SNO-Hb. Further, the treatment of trauma or septic patients might involve manipulation of SNO-Hb allostery to limit the excessive release of NO.

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Oxygen Regulation of Tumor Perfusion Unravels the Central Pressor Activity of Cell-Free Human S-Nitrosohemoglobin

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**Expanded methods.**

**SNO-Hb synthesis**

Solutions of purified human hemoglobin A0 (Apex Bioscience, Durham, NC) were dialyzed overnight at 4°C against 2% (w/v) aerated sodium tetraborate pH 9.2, 0.5 mmol/L EDTA. For SNO-Hb synthesis, L-cysteine hydrochloride (0.55 mol/L in 0.5 mol/L HCl, 0.5 mol/L EDTA) was first reacted with an equal volume of 0.5 mol/L NaNO2 to yield Cys-NO.1 Then, a 10-fold molar excess of Cys-NO was reacted with dialyzed oxy-Hb at a 1:20 (v/v) ratio for 10 min. The reaction was terminated by centrifugation through a fine Sephadex G-25 chromatography column (Pharmacia Biotech, Uppsala, Sweden) equilibrated with PBS, pH 7.4, 0.5 mmol/L EDTA. Under these conditions, the yield of oxy-Hb S-nitrosylation is consistently 1.9 S-nitroso-cysβ93 per tetramer.1,2

**SNO-Hb Assay**

Briefly, blood samples were collected in heparinized tubes just before infusion and at 1 min, 5 min, and every 5 min after infusion of SNO-Hb i.v. for 30 min. Plasma (100 µl) was immediately mixed with DAF-2 (30 µmol/L final concentration). Half of this solution was then reacted for 10 min with an equal volume of HgCl2 1.2 mmol/L. The reaction was terminated by centrifugation in filter tubes (Nanosep, Pall, East Hills, NY). Upon light excitation at 485 nm, DAF-2-associated light emission was read at 520 nm. SNOs were quantified as the difference in fluorescence signals generated in the presence and absence of HgCl2, which specifically cleaves SNOs, yielding nitrite.
**Expanded Results.**

Although the method we used for the preparation of SNO-Hb was designed to selectively nitrosylate thiols\(^1\), reaction in alkaline buffer did not fully prevent heme oxidation. By comparison to unreacted oxy-Hb which is typically 100% oxy-Hb(FeII), SNO-Hb solutions contain small amounts of SNO-Hb(FeIII). In our hands, the extent of heme oxidation had fallen further under normoxic and hyperoxic conditions as soon as 30 minutes after i.v. SNO-Hb administration \((P > 0.05, n=5, \text{ Student's } t \text{ test})\). The rate of heme reduction was independent of the oxygen concentration delivered to rats. Moreover, there was no correlation between SNO content and FeII/FeIII ratio in Hb over time \((R^2 = 0.31)\).

**Supplemental Table. Blood Gas and Hemoglobin Saturation Measurements.**

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<tr>
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<th>Femoral artery</th>
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<tr>
<td></td>
<td>pO(_2) (mm Hg)</td>
<td>pCO(_2) (mm Hg)</td>
<td>HbO(_2) (%)</td>
<td>pO(_2) (mm Hg)</td>
<td>pCO(_2) (mm Hg)</td>
<td>HbO(_2) (%)</td>
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<td>Room air</td>
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<td>49.6±1.3</td>
<td>88.8±0.4</td>
<td>57.5±1.2</td>
<td>50.6±1.7</td>
<td>67.7±2.4</td>
<td>76±2</td>
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<tr>
<td>100% O(_2)</td>
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<td>56.9±1.7*</td>
<td>98.2±0.1*</td>
<td>69.8±3.0*</td>
<td>72.0±2.1*</td>
<td>75.8±3.4*</td>
<td>89±2*</td>
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* \(P < 0.05\) between value and corresponding room air value (paired Student’s \(t\) test, \(N=4\)). HbO\(_2\), endogenous hemoglobin oxygen saturation.
Expanded discussion.

In regard to the direct inhibition of the baroreceptor reflex by SNO-Hb, one can further envisage a mechanism independent of SNO generation. Indeed, unlike oxy-Hb, synthesized SNO-Hb contains small amounts of SNO-Hb(FeIII), which can scavenge NO•. Few studies have addressed the role of NO• present in the vasculature on the baroreceptor in systems independent of peripheral hemodynamics. Recently however, Meyrelles3 et al showed that gene transfer of the endothelial NO• synthase to the carotid sinus induced NO•-mediated vasodilation that inhibited the baroreceptor reflex; these effects were reversed by local NO• synthase inhibition. If local changes in the availability of NO• species are the main trigger for direct baroreceptor inhibition by SNO-Hb, it likely results from SNO donation rather than NO• scavenging. Furthermore, the lack of correlation between SNO and FeIII content in SNO-Hb indicates that SNO donation is independent of the oxidation state in Hb.

References.


Online data supplement.

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Expanded methods.

SNO-Hb synthesis

Solutions of purified human hemoglobin A₀ (Apex Bioscience, Durham, NC) were dialyzed overnight at 4°C against 2% (w/v) aerated sodium tetraborate pH 9.2, 0.5 mmol/L EDTA. For SNO-Hb synthesis, L-cysteine hydrochloride (0.55 mol/L in 0.5 mol/L HCl, 0.5 mol/L EDTA) was first reacted with an equal volume of 0.5 mol/L NaNO₂ to yield Cys-NO.¹ Then, a 10-fold molar excess of Cys-NO was reacted with dialyzed oxy-Hb at a 1:20 (v/v) ratio for 10 min. The reaction was terminated by centrifugation through a fine Sephadex G-25 chromatography column (Pharmacia Biotech, Uppsala, Sweden) equilibrated with PBS, pH 7.4, 0.5 mmol/L EDTA. Under these conditions, the yield of oxy-Hb S-nitrosylation is consistently 1.9 S-nitroso-cysβ93 per tetramer.¹²

SNO-Hb Assay

Briefly, blood samples were collected in heparinized tubes just before infusion and at 1 min, 5 min, and every 5 min after infusion of SNO-Hb i.v. for 30 min. Plasma (100 µl) was immediately mixed with DAF-2 (30 µmol/L final concentration). Half of this solution was then reacted for 10 min with an equal volume of HgCl₂ 1.2 mmol/L. The reaction was terminated by centrifugation in filter tubes (Nanosep, Pall, East Hills, NY). Upon light excitation at 485 nm, DAF-2-associated light emission was read at 520 nm. SNOs were quantified as the difference in fluorescence signals generated in the presence and absence of HgCl₂, which specifically cleaves SNOs, yielding nitrite.
**Expanded Results.**

Although the method we used for the preparation of SNO-Hb was designed to selectively nitrosylate thiols\(^1\), reaction in alkaline buffer did not fully prevent heme oxidation. By comparison to unreacted oxy-Hb which is typically 100% oxy-Hb(FeII), SNO-Hb solutions contain small amounts of SNO-Hb(FeIII). In our hands, the extent of heme oxidation had fallen further under normoxic and hyperoxic conditions as soon as 30 minutes after i.v. SNO-Hb administration \((P>0.05, n=5, \text{ Student's } t \text{ test})\). The rate of heme reduction was independent of the oxygen concentration delivered to rats. Moreover, there was no correlation between SNO content and FeII/FeIII ratio in Hb over time \((R^2 = 0.31)\).

**Supplemental Table. Blood Gas and Hemoglobin Saturation Measurements.**

<table>
<thead>
<tr>
<th></th>
<th>Femoral artery</th>
<th></th>
<th>Femoral vein</th>
<th></th>
<th>Tumor artery</th>
<th></th>
<th>Tumor vein</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pO(_2)  (mm Hg)</td>
<td>pCO(_2)  (mm Hg)</td>
<td>HbO(_2)  (%)</td>
<td>pO(_2)  (mm Hg)</td>
<td>pCO(_2)  (mm Hg)</td>
<td>HbO(_2)  (%)</td>
<td>HbO(_2)  (%)</td>
<td>HbO(_2)  (%)</td>
</tr>
<tr>
<td>Room air</td>
<td>82.4±0.4</td>
<td>49.6±1.3</td>
<td>88.8±0.4</td>
<td>57.5±1.2</td>
<td>50.6±1.7</td>
<td>67.7±2.4</td>
<td>76±2</td>
<td>69±2</td>
</tr>
<tr>
<td>100% O(_2)</td>
<td>435.5±34.7*</td>
<td>56.9±1.7*</td>
<td>98.2±0.1*</td>
<td>69.8±3.0*</td>
<td>72.0±2.1*</td>
<td>75.8±3.4*</td>
<td>89±2*</td>
<td>78±3*</td>
</tr>
</tbody>
</table>

* \(P<0.05\) between value and corresponding room air value (paired Student’s \(t\) test, \(N=4\)). HbO\(_2\), endogenous hemoglobin oxygen saturation.
Expanded discussion.

In regard to the direct inhibition of the baroreceptor reflex by SNO-Hb, one can further envisage a mechanism independent of SNO generation. Indeed, unlike oxy-Hb, synthesized SNO-Hb contains small amounts of SNO-Hb(FeIII), which can scavenge NO’. Few studies have addressed the role of NO’ present in the vasculature on the baroreceptor in systems independent of peripheral hemodynamics. Recently however, Meyrelles et al showed that gene transfer of the endothelial NO’ synthase to the carotid sinus induced NO’-mediated vasodilation that inhibited the baroreceptor reflex; these effects were reversed by local NO’ synthase inhibition. If local changes in the availability of NO’ species are the main trigger for direct baroreceptor inhibition by SNO-Hb, it likely results from SNO donation rather than NO’ scavenging. Furthermore, the lack of correlation between SNO and FeIII content in SNO-Hb indicates that SNO donation is independent of the oxidation state in Hb.

References.

