Biochemical Characterization of S-Nitrosohemoglobin

MECHANISMS UNDERLYING SYNTHESIS, NO RELEASE, AND BIOLOGICAL ACTIVITY*

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S-Nitrosohemoglobin (SNO-Hb) has been suggested to act as an endogenous NO donor and physiological regulator of blood pressure. However, the mechanisms responsible for the formation of SNO-Hb and those underlying the release of NO and subsequent biological activity have yet to be elucidated. In the present study, a number of nitrosated oxyhemoglobin (HbO2) derivatives have been synthesized and characterized. HbO2 can be nitrosated at up to three distinct residues, one in the α-globin chain and two in the β-chain. A β-chain mononitrosated species (designated "SNO-Hb"), generated by the reaction of HbO2 and S-nitrosoglutathione, released NO via a thiol-dependent mechanism involving nucleophilic attack at the nitrosated thiol functionality of SNO-Hb; in the case of glutathione, this process was associated with the formation of a mixed disulfide. In contrast, multinitrosated hemoglobin species released NO and relaxed vascular smooth muscle by a thiol-independent mechanism. HbO2 scavenged potently NO released from SNO-Hb and inhibited its vasorelaxant properties. These data show that the predominant vasoactive species released from SNO-Hb is NO, with HNO a putative intermediate; the presence of a low molecular weight thiol is a prerequisite for this process. Such observations have important implications for the generation, metabolic fate, and biological activity of S-nitrosothiols.

The interaction of nitric oxide (NO)1 with specific proteins is fundamental to the regulation of many biological processes including neurotransmission, platelet aggregation, vascular smooth muscle relaxation, and the cytotoxic actions of immune cells (1). The best characterized target sites for NO are proteins possessing transition metal ion prosthetic groups; this is exemplified by the interaction of NO with the heme moiety of soluble guanylyl cyclase resulting in the formation of the intracellular second messenger cyclic guanosine 3’,5’-monophosphate (cGMP) (2). Recently, it has become apparent that modification of proteins by NO at cysteine or tyrosine residues may also play an important role in regulating function (3, 4). Nitration of tyrosine may have important pathological consequences in conditions such as amyotrophic lateral sclerosis and other neurodegenerative disorders (5). In contrast, nitrosation of cysteine residues in proteins, to yield S-nitrosothiols, may represent a physiological regulatory mechanism. This phenomenon is illustrated by nitrosation of the cardiac ryanodine receptor which results in a marked increase in Ca2+ flux (6) and by nitrosation of p21ras which acts as a molecular switch to regulate signal transduction (7). It has also been suggested that S-nitrosothiol formation may be a mechanism by which NO is transported to targets distant from its point of synthesis. S-Nitrosothiols are considerably more stable than NO itself (8) and reproduce the biological actions of NO (9). As such, incorporation of NO into a S-nitrosothiol may serve to preserve the biological activity of NO.

A significant physiological role has been proposed for a nitrosated derivative of hemoglobin, S-nitrosohemoglobin (SNO-Hb) (10, 11). SNO-Hb can be formed via a transnitrosation reaction between oxyhemoglobin (HbO2) and low molecular weight S-nitrosothiols (e.g. S-nitrosocysteine, CysNO, or S-nitrosoglutathione, GSNO). This process has been suggested to result in the transfer of an NO moiety to Cys93 on the β-globin chain (10). SNO-Hb is found in the systemic circulation, at concentrations approximately 10 times higher in the arterial (~300 nM) than in the venous (~30 nM) blood. This arterial-venous difference has led to the suggestion that SNO-Hb might act as an NO donor in the systemic circulation. It has also been proposed that the rate of release of NO from SNO-Hb is inversely related to the ambient oxygen tension and that the allosteric changes in Hb (shifts between the relaxed (R) and tense (T) states), which facilitate the delivery of oxygen to tissues, might also promote the release of NO. Such a system would preferentially deliver oxygen to tissues in which oxygen utilization was high and would dilate the vessels supplying such tissues to ensure adequate delivery of oxygenated blood.

The mechanism(s) by which SNO-Hb might be formed endogenously and the pathway by which the NO moiety of SNO-Hb exerts its biological activity have not been explained. Due to the potentially significant biological role of SNO-Hb, the biochemical and pharmacological characteristics of this compound have been investigated.

MATERIALS AND METHODS

Synthesis of S-Nitrosothiols

CysNO and GSNO were prepared by reaction of the appropriate thiol (L-isomer) with sodium nitrite at pH 2 (8). HbO2 was prepared by reacting commercially available hemoglobin (human) with a molar excess of sodium dithionite at 20 °C with subsequent purification through Sephadex G-25 columns. Nitrosated Hb derivatives were prepared by incubating HbO2 with a 10-fold molar excess of CysNO, GSNO, or...
DEA-NO for 45 min at room temperature and protected from light, with subsequent purification through Sephadex G-25 columns. The concentration of HbO2 and SNO-Hb in the column eluate was determined by UV-Vis spectroscopy ($\lambda_{max} = 415$ nm; $\varepsilon = 125$ molar). The concentrations of SNO-Hb (and other nitrosated hemoglobin derivatives) stated in the text are expressed on the basis of heme content and not the concentration of nitrosated subunits.

**Organ Bath Studies**

Male Harlan-Sprague Dawley rats (200–250 g) were killed by stunning and cervical dislocation. The thoracic aorta was dissected and cleared of connective tissue and blood products. Intact rings, 2–3 mm in width, were mounted in 25-mL glass organ baths containing Krebs bicarbonate buffer (composition mm: NaCl, 118.1; KCl, 4.7; MgSO4, 1.0; KH2PO4, 1.0; CaCl2, 2.5; NaHCO3, 25.0; glucose, 11.1) which was maintained at 37°C. Tissues were gassed continuously with 95% O2, 5% CO2, unless otherwise stated. A resting tension of 1 g was applied to each tissue and changes in tension recorded with a Grass FT03 force displacement transducer attached to a Rikadenki (R-64) chart recorder. Where indicated, the endothelium was removed from the tissues by displacement transducer attached to a Rikadenki (R-64) chart recorder. Where indicated, the endothelium was removed from the tissues by gently rubbing the luminal surface with forceps. The rings were allowed to equilibrate for 1 h prior to experimentation. Vessels that demonstrated an active tone of less than 1 g in response to a submaximal concentration of phenylephrine (1 $\mu$M) were discarded. To test the functional integrity of the endothelium, phenylephrine-precontracted vessels that showed >50% relaxation to 1 $\mu$M carbachol were deemed endothelium intact, those exhibiting <15% relaxation were regarded as endothelium denuded; vessels not satisfying either criterion were discarded.

In experiments investigating the vasorelaxant potency of SNO-Hb in the presence and absence of thiols, time-matched vessels were split into two groups. Concentration-response curves to SNO-Hb were constructed in the presence or absence of GSH, cysteine, or bovine serum albumin (added 15 min prior to the SNO-Hb). An identical protocol was followed to establish the effect of 13.7 $\mu$M dipropyldithiocarbamate on SNO-Hb-induced relaxations. In certain experiments a fixed concentration of SNO-Hb was added to control and test vessels and a concentration-response curve to cumulative additions of the tissue-saturating concentration of GSH or cysteine established in the test tissue. Studies examining the S-nitrosothiol-dependent component of relaxations to SNO-Hb involved incubation of nitrosated Hb preparations with HgCl2 (degrades S-NO bonds; 10 $\mu$M) except for DEA-NO (diethylamine-NONOate) and SPER-NO (spermine-NONOate; Cayman Chemicals, Ann Arbor, MI). Studies examining the pattern of NO release from SNO-Hb preparations with HgCl2 (degrades S-NO bonds; 10 $\mu$M; 15 min) prior to addition to the organ baths. In all cases, responses are expressed as a percentage of phenylephrine-induced tone.

In experiments investigating the vasorelaxant action of SNO-Hb were also studied in tissues exposed to different oxygen concentrations in order to alter the globin allosteric conformation. This was achieved by gassing the organ baths with the following gas mixtures: 21% O2, 4% CO2, in N2; 10% O2, 4% CO2 in N2; 5% O2, 3% CO2 in N2; these gas combinations resulted in calculated oxygen tensions (pO2) in the baths of approximately 160, 76, and 38 mm Hg, respectively.

**Quantification of S-Nitrosothiols**

**Biochemical Assay—S-Nitrosothiol concentrations were measured according to the method of Saville (12). Briefly, samples were mixed with 0.1% ammonium sulfamate in 0.4 N HCl and incubated for 10 min. Absorbance was read at 540 nm and S-nitrosothiol concentration calculated by comparison with a standard curve constructed by addition of increasing concentrations of NaNO2 under reducing conditions (acidified KI) to yield known concentrations of NO.**

**RESULTS**

**Biochemical Characterization of Nitrosated Hemoglobin Derivatives**

**Synthesis of S-Nitrosohemoglobin—Incubation of 0.5 mM HbO2 (based upon heme content) with a 10-fold molar excess of GSNO or CysNO at room temperature (light protected) for 45 min resulted in the formation of 127.4 ± 14.4 $\mu$M (25.5% conversion; n = 5) and 292.2 ± 13.7 $\mu$M (58.4% conversion; n = 5) nitrosated Hb products, respectively. Higher concentration of S-nitrosothiol (10 mM), increasing the incubation time (up to 2 h), or increasing the incubation temperature (37°C) did not increase the proportion of nitrosated products formed. Reaction of CysNO and HbO2 at a ratio of 1:1 also resulted in the formation of a small quantity of nitrosated Hb (86.36 ± 10.2 $\mu$M; 17.0% conversion; n = 3). In all cases, significantly less nitrosation occurred if the reaction mixture was exposed to light during the 45-min incubation period (data not shown). Incubation of HbO2 (0.5 mM) with a 10-fold molar excess of the NO donor DEA-NO also resulted in the formation of a nitrosated Hb derivative (246.5 ± 89.2 $\mu$M; 49.3% conversion; n = 4).**

**LC-MS Analysis—Mass spectrometric analysis revealed that incubation of HbO2 (0.5 mM) with a 10-fold molar excess of CysNO resulted in the formation of three new species. 51% of the $\beta$-globin chains were nitrosated at a single residue as indicated by an increase in M+ of +29; 43% of the $\beta$-chains became nitrosated at two residues ($\Delta M + 58$). Additionally, a small fraction (17%) of $\alpha$-globin chains were nitrosated at one residue ($\Delta M + 29$). Addition of GSH (10 mM) to this Hb preparation resulted in the loss of all nitrosated products detectable by MS (Table 1). At a HbO2:CysNO ratio of 1:1 (each 0.5 mM),...
35% of the β-chains became nitrosated at a single residue. Incubation of HbO₂ (0.5 mM) with a 10-fold molar excess of GSNO yielded markedly different products to those described above. 43% of the β-globin chains were nitrosated at a single residue (ΔMᵣ + 29) but, in addition, 28% had an increase in mass of +305, indicative of the formation of a mixed disulfide between GSH and Hb. Addition of GSH (10 mM) to this nitrosated Hb preparation had little effect on the disulfide component (31%) but completely removed the bound NO. However, dithiothreitol (10 mM) was able to remove the NO group and disulfide bond such that all the Hb returned to a native molecular mass. Incubation of HbO₂ and GSNO at a ratio of 1:1 (each 0.5 mM) did not result in the formation of any detectable nitrosated products.

The above observations are in close agreement with the biochemical analysis (Saville assay (12)). Synthesis of a nitrosated Hb preparation from a 10-fold molar excess of CysNO resulted in the formation of products possessing 1 to 3 NO moieties, representing (on average) 50% nitrosation based upon heme. Using a 1:1 ratio of HbO₂:CysNO, a small quantity of mononitrosated Hb was formed (35% of β-chains = 17.5% on the basis of heme). Likewise, SNO-Hb formed from GSNO exhibited nitrosation at ~50% of its β-globin chains, equivalent to ~25% of displaceable NO per Hb tetramer.

Therefore, since the Hb preparation obtained by incubating HbO₂ with GSNO resulted in a mononitrosated (β-chain) derivative, and that GSNO represents the most physiologically relevant S-nitrosothiol within erythrocytes (15), the compound synthesized by this method was termed S-nitrosohemoglobin (SNO-Hb); subsequent experimentation focused on this reagent.

**SNO-Hb Stability and Transnitrosation Reactions**—Incubation of SNO-Hb for 60 min at 25 or 37 °C in phosphate-buffered saline did not result in a significant decrease of the SNO content of the preparation (data not shown). However, in the presence of GSH (100 μM-10 mM), a time- and concentration-dependent loss of SNO-Hb was observed. The nitroso content of the SNO-Hb preparation, expressed as a percentage of the initial concentration, was 92.2 ± 4.1, 66.5 ± 2.0, and 58.2 ± 4.9% (mean ± S.E.; n > 5) in the presence of 100 μM, 1 mM, and 10 mM GSH, respectively (see also Fig. 1). GSNO alone did not significantly decompose over a 60-min incubation period at 25 or 37 °C (data not shown). However, in the presence of GSH (10 mM), decomposition of GSNO occurred in a time-dependent manner (Fig. 1). Following the 60-min incubation period, separation of low molecular weight thiols (Mᵣ < 30,000) from the hemoglobin derivatives revealed that low molecular weight S-nitrosothiols accounted for 5.2 ± 0.8% (n = 4) of the nitroso content loss from SNO-Hb. HPLC analysis of the reaction between SNO-Hb (0.5 mM) and a 10-fold molar excess of GSH revealed that GSNO formation peaked within 1 min (29.8 ± 2 μM; n = 3) and then declined in a linear fashion such that levels were not significantly above background after 90 min. Concomitantly, a rise in the level of glutathione disulfide was observed (GSSG; data not shown).

**Release of NO from S-Nitrosothiols**—SNO-Hb (100 mM) did not release NO under control conditions. However, in the presence of a low molecular weight thiol (GSH or L-cysteine), an increase in NO release from SNO-Hb was observed (Fig. 2). The pattern of release of NO from SNO-Hb was specific for each low molecular weight thiol studied, although both evoked release of NO immediately upon addition. In the presence of cysteine, NO release peaked sharply and returned to baseline within 10 min. In the presence of GSH, however, a slow long-lasting release of NO was observed (Fig. 2). The presence of HbO₂ significantly affected the detectable levels of NO released from SNO-Hb as measured by the NO electrode. The detectable NO release from a mixture of SNO-Hb (100 mM) and GSH (100 μM) was reduced, in a concentration-dependent manner, by HbO₂ (0.1–10 μM; Fig. 2)

**Pharmacological Characterization of S-Nitrosohemoglobin**

**Effect of SNO-Hb on Isolated Rat Aorta**—In endothelium-intact vessels, addition of SNO-Hb to pre-contracted aortic rings resulted in a further contraction, which was not observed in denuded vessels (data not shown). To avoid this confounding effect on tone (presumably due to scavenging of NO released basally from the endothelium), subsequent studies were conducted in denuded rings. SNO-Hb did not relax rat aortic rings unless a low molecular weight thiol was also present (Fig. 3) and the vasorelaxant potency of SNO-Hb could be enhanced by increasing the concentration of thiol (Fig. 4). The biological activity of authentic SNO-Hb synthesized from GSNO differed markedly from the nitrosated Hb preparations synthesized with CysNO or DEA-NO. The derivative prepared by incubating HbO₂ with CysNO caused a concentration-dependent relaxation of pre-contracted rat aortic rings that was not affected by the presence of GSH (Fig. 3); an almost identical pattern of vasorelaxant activity was observed with a nitrosated Hb preparation synthesized from DEA-NO (data not shown). Interestingly, the potency of SNO-Hb was some 10 times less than the nitrosated Hb derivative synthesized with CysNO (Fig. 3).
These observations are consistent with the LC-MS data which indicate that CysNO and DEA-NO are capable of nitrosating multiple thiol- (and possibly non-thiol-) sites within HbO2, whereas GSNO appears to transnitrosate a specific cysteine residue (presumably Cysb93). This thesis was supported by experiments in which the different nitrosated Hb preparations were incubated with HgCl2 (10 μM; 15 min) prior to administration to the organ bath. In this case, SNO-Hb (0.3 μM) lost all vasorelaxant activity (control relaxation: 64.1 ± 5.0%; relaxation following HgCl2 treatment: 2.1 ± 2.1%; mean ± S.E.; n = 3; p < 0.05) whereas the nitrosated product prepared from CysNO (0.1 μM) was still able to elicit 80% of its control response (control relaxation: 77.4 ± 7.2%; mean ± S.E.; n = 3). The product of the reaction between HbO2 and GSNO at a ratio of 1:1, which lacked any detectable nitrosated residues (by Saville assay and LC-MS), had no pharmacological activity (data not shown).

Effect of Thiols on Relaxations to SNO-Hb—SNO-Hb induced relaxations were dependent entirely on the availability of a free -SH moiety since in the presence of GSSG (glutathione disulfide) SNO-Hb lacked vasorelaxant activity (Fig. 5). Furthermore, only low molecular weight thiols could facilitate SNO-Hb-induced relaxations since no response was observed in the presence of bovine serum albumin (up to 1 mM; data not shown). SNO-Hb was also able to elicit vascular smooth muscle relaxation in the presence of cysteine; this effect was not stereoselective since D-cysteine was at least as potent as L-cysteine (Fig. 5).

Effect of HbO2 on Relaxations to SNO-Hb—Concentration-response curves to SNO-Hb (30 nM-30 μM) in the presence of 100 μM GSH, had no pharmacological activity (data not shown).
again, this effect was absent in tissues in which the phenyl-

The present study demonstrates that HbO₂ can be nitrosated by incubation with low molecular weight S-nitrosothiols, such as GSNO and CysNO; however, the characteristics of this process are particular to the nitrosating agent employed. In the case of GSNO, the product formed is nitrosated solely at a free

Derivatives—Nitrosation and nitration of proteins are emerging as important mechanisms regulating protein activity and cellular function. It has been suggested previously that SNO-

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ecrine concentration was increased to elicit a contraction equivalent to that observed in control tissues (data not shown).

6). At the highest concentration of HbO₂, the vasorelaxant effect of SNO-Hb was virtually abolished.

Effect of Oxygen Tension on Relaxations to SNO-Hb—Concentration-response curves to SNO-Hb (0.1–30 μM; in the presence of 100 μM GSH) were conducted at various oxygen tensions by exposing the tissues to different gas mixtures 1 h prior to and during experimentation. A gas mixture containing 21 or 10% oxygen did not significantly affect the vasorelaxant activity of SNO-Hb. A gas mixture containing 5% oxygen caused a significant increase in potency of SNO-Hb, but also decreased the phenylephrine-induced tone by 22 ± 9% (n = 5). However, when the concentration of phenylephrine was increased to give an equivalent tone to that observed under control conditions (i.e. 95% O₂, 5% CO₂), no significant difference in the potency of SNO-Hb was detected (Fig. 7). Thus, a greater degree of relaxation at low pO₂ by virtue of the starting contraction being of a lesser magnitude, can be easily misinterpreted as an apparent increase in potency of SNO-Hb due to functional antagonism. In control experiments, an almost identical leftward shift was observed for the concentration-response curves to the NO donor, SPER-NO, when tissues were gassed with 5% oxygen; again, this effect was absent in tissues in which the phenyl-


dilation. As a consequence, the potency of SNO-Hb always appeared enhanced in these tissues. In the presence of 2,3-diphosphoglycerate (100 μM), which facilitates the allosteric transformation of hemoglobin from the T to R state, the vasorelaxant potency of SNO-Hb was unaffected at any of the oxygen tensions studied (Fig. 8).

Fig. 4. Effect of different concentrations of glutathione (1–100 μM) on the concentration-response curve to SNO-Hb in endothelium-denuded rings of rat aorta. Control, open circles; +1 μM GSH, closed circles; +10 μM GSH, open squares; +30 μM GSH, closed squares; +100 μM GSH, open triangles. Results are expressed as percentage relaxation of the phenylephrine-induced contraction and shown as mean ± S.E. for n = five experiments.

Fig. 5. Vasorelaxation to 100 nM SNO-Hb in the presence of increasing concentrations of oxidized GSSG (open circles), glutathione (closed circles), l-cysteine (open squares), and d-cysteine (closed squares) on endothelium-denuded rings of rat aorta. Results are expressed as % relaxation of the phenylephrine-induced contraction and shown as mean ± S.E. for n = six experiments.

Fig. 6. Effect of HbO₂ (0.1–30 μM) on the concentration-response curve to SNO-Hb in the presence of 100 μM GSH in endothelium-denuded rings of rat aorta. Control, open circles; +0.1 μM HbO₂, closed circles; +1 μM HbO₂, open squares; +10 μM HbO₂, closed squares; +30 μM HbO₂, open triangles. Results are expressed as percentage relaxation of the phenylephrine-induced contraction and shown as mean ± S.E. for n = six experiments.

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ephrine-induced contraction and shown as mean ± S.E. for n = six experiments.

6). 95% O₂, 5% CO₂, no significant difference in the potency of SNO-Hb was observed between tissues even at millimolar concentrations of phenylephrine. As a consequence, the potency of SNO-Hb always appeared enhanced in these tissues. In the presence of 2,3-diphosphoglycerate (100 μM), which facilitates the allosteric transformation of hemoglobin from the T to R state, the vasorelaxant potency of SNO-Hb was unaffected at any of the oxygen tensions studied (Fig. 8).

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molecular weight thiol. The ability of CysNO and DEA-NO, but not GSNO, to add an NO moiety to multiple functional sites on HbO₂ can be explained by the rate of release of NO from these compounds. In the case of GSNO, the release of NO is comparatively slow and the prevailing NO concentration is low. Here, it is the transnitrosation process which predominates, resulting in the formation of SNO-Hb, nitrosated preferentially at Cys^693. In the case of CysNO and DEA-NO, which release NO at faster rates, the reaction of NO with O₂ becomes of greater significance (since the rate of reaction is dependent on the square of the NO concentration: 2NO + O₂ → 2NO₂), resulting in the formation of powerful nitrosating species (e.g. N₂O₃); subsequently, these can nitrosate HbO₂ at multiple amino acid residues. DEA-NO releases NO spontaneously and is not an S-nitrosothiol, which prevents its participation in transnitrosation reactions. Hence, in this circumstance it is likely that a product of the reaction of NO with O₂ is responsible for the formation of the nitrosated Hb derivative. The ability of CysNO to nitrosate one residue on the α-chain and two residues on the β-chain corresponds to the distribution of cysteine residues in human hemoglobin (Cys^104, Cys^93, and Cys^112). Cys^104 and Cys^112 have been shown to be considerably less reactive than Cys^93 with regard to thiol-modifying agents (16); however, it is possible that when exposed to a sufficiently high concentration NO (or related species) each cysteine becomes accessible to nitrosation. This is supported by the selective nitrosation of one residue on the β-chain by a lower concentration of CysNO (CysNO:HbO₂, 1:1) compared with the three nitrosated residues at higher CysNO concentrations (CysNO:HbO₂, 10:1). Moreover, addition of a thiol to both these preparations removes all NO moieties. A similar pattern of thiol reactivity is observed with bovine serum albumin, which can only be nitrosated in the presence of CysNO and not GSNO (17). Notably, however, HgCl₂ does not displace entirely the biologically active NO groups from the tri-nitrosated Hb preparation, suggesting NO may also be bound to a non-thiol site. Alternative amino acid candidates, which may form thiol-displaceable adducts with NO, should not be excluded; for example, tryptophan has been shown to possess vasorelaxant activity following nitrosation by NO oxidation products (18). Intriguingly, the tryptophan distribution in human hemoglobin is identical to that of cysteine (i.e. one α-chain residue, Trp^144, and two β-chain residues, Trp^913 and Trp^957).

The above observations have particular significance for the study of the biological activity of SNO-Hb. Previous studies have utilized “SNO-Hb” prepared from HbO₂ and CysNO (10). It is clear from the present investigation that this method of synthesis results in the formation of mixed species which makes interpretation of results difficult. However, the extent of HbO₂ nitrosation by CysNO is dependent on the ratio of CysNO:HbO₂, such that at a 1:1 ratio, a mononitrosated (β-chain) Hb derivative can be formed; this is presumably analogous to SNO-Hb synthesized from GSNO (although this species also possesses a mixed disulfide). Reaction of HbO₂ with GSNO resulted in an approximately 25% yield of SNO-Hb with respect to the starting heme concentration. This represents a conversion of 1 of 2 thiol groups per Hb tetramer, consistent with previous findings (10, 11). Interestingly, this preparation also possesses a mixed disulfide between GSH and the β-globin chain. The reactive nature of these SNO groups was confirmed by their removal following addition of GSH or DTT.

SNO-Hb was found to be stable at 25 and 37 °C for at least 4 h in phosphate-buffered saline, with only a small (<5%) decrease in SNO content. In the presence of GSH, the nitroso content of SNO-Hb was lost rapidly, being almost completely degraded within 30 min in the presence of GSH. This is con-
sistent with previous observations in which incubation with the parent (or similar) thiol dramatically enhances the breakdown of S-nitrosothiols (8). Nevertheless, SNO-Hb itself is stable in the absence of added thiol. GSNO was also found to be relatively stable at 25 and 37 °C, with a comparable, small loss of nitrosothiol content over 4 h. Again, the rate of decomposition of GSNO was markedly enhanced in the presence of GSH. However, the rate of breakdown of equivalent concentrations of SNO-Hb and GSNO in the presence of GSH was significantly different; SNO-Hb decomposition was considerably faster such that at 37 °C less than 50% remained intact after 30 min whereas more than 80% of GSNO was present at the same time point. It has been suggested that interaction of SNO-Hb with GSH results in a transnitrosation reaction yielding HbO₂ and GSNO; subsequent NO release from GSNO would thereby account for the bioactivity of SNO-Hb (10, 11). However, for this hypothesis to be correct, the breakdown of SNO-Hb in the presence of GSH could only proceed as rapidly as the breakdown of GSNO itself; the present study has demonstrated clearly that this is not the case.

HPLC analysis showed that GSNO formation accounted for <10% of the SNO content lost from SNO-Hb in the presence of excess GSH. Moreover, less than 6% of the total SNO content was accounted for by low molecular weight (M₀ < 30,000) compounds. These experiments indicate that an intermediate low molecular weight S-nitrosothiol is not a prerequisite for the release of NO; rather, thiol react with SNO-Hb via nucleophilic attack at the sulfur atom to displace NO directly with the concurrent formation of a mixed disulfide (1). In fact, this reaction will yield nitrosoyl (HNO) (19) (and possibly other metabolites including NH₃ (20)) and it is likely that subsequent reaction of HNO with S-nitrosothiols, thiols, or metal ions (19, 21) results in the liberation of NO. A small quantity of low molecular weight S-nitrosothiol is formed during the above process (2), but this species will react immediately with excess thiol to give a disulfide and HNO (3). Intracellularly (i.e. within erythrocytes), the concentration of GSH is in the millimolar range and therefore in great excess to SNO-Hb. Consequently, any GSNO formed will likely encounter a second GSH molecule resulting in the formation of GSSG and HNO (3). A mixed disulfide is also generated during the synthesis of SNO-Hb from GSNO. Here, transnitrosation of HbO₂ by GSNO results in a transnitrosation reaction yielding HbO₂ and GSNO; the latter species can then attack the SNO-Hb (1). This pattern of reactivity also pertains to other proteins possessing reactive sulfydryl groups; for both carboxic anhydrase III and H-ras, interaction with GSNO results in S-nitrosation of the protein and the production of a mixed disulfide (22). The effect that mixed disulfide formation might have on the physiological activity of these proteins, for instance, the oxygen binding characteristics of HbO₂, is unclear. However, the mixed disulfide between GSH and SNO-Hb cannot be broken by excess GSH. This implies that if this mixed disulfide linkage occurs in vivo, SNO-Hb might accumulate irreversibly bound GSH. In this case, it may be that alternative reducing agents possessed by erythrocytes (e.g. cysteine) may be responsible for preventing and/or reversing mixed disulfide formation with SNO-Hb.

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\text{SNO-Hb} + \text{GSH} \rightarrow \text{GS-HbO}_2 + \text{HNO} \quad \text{(Eq. 1)}
\]

\[
\text{SNO-Hb} + \text{GSH} \rightarrow \text{HbO}_2 + \text{GSNO} \quad \text{(Eq. 2)}
\]

\[
\text{GSNO} + \text{GSH} \rightarrow \text{GSSG} + \text{HNO} \quad \text{(Eq. 3)}
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Data from the present study are in accord with observations made with a nitrosated thiol-Sepharose 4B derivative (SNO-4B) which has similar characteristics to SNO-Hb in that it is stable in a physiological solution (estimated t₀₂₀ is > 7 days at 37 °C) but decomposes in the presence of low molecular weight thiols (15). Incubation with GSH resulted in complete loss of HgCl₂-displaceable NO from SNO-4B but less than 30% was converted to GSNO. It is likely the remainder is lost as HNO (as is the case for SNO-Hb).

Pharmacological Analysis of S-Nitrosohemoglobin—Nitrosated hemoglobin derivatives synthesized from HbO₂ and CysNO were found to possess inherent vasorelaxant activity on rat aortic rings. In contrast, SNO-Hb exerted no direct effects on pre-contracted, endothelium-denuded rat aorta, but relaxed rings in the presence of low molecular weight thiols such as GSH or cysteine. The requirement for a reduced low molecular weight thiol was confirmed by the inability of SNO-Hb to relax aortic rings in the presence of GSSG. Furthermore, SNO-Hb was without effect in the presence of bovine serum albumin, unless a low molecular weight thiol was also present, suggesting that there is a limit to the size and/or nucleophilicity of thiol which can participate in the release of NO. The vasorelaxant action of SNO-Hb does not appear to be stereospecific with regard to the low molecular weight thiol requirement, since D-cysteine was at least equipotent to L-cysteine. This lack of stereospecificity exhibited in vitro does argue against an enzymatically facilitated NO transfer being involved in the process of smooth muscle relaxation by SNO-Hb; however, the study of isolated SNO-Hb under artificial conditions in vitro does not mimic the in vivo environment within erythrocytes in which the presence of additional proteins may alter the kinetics of NO release.

HbO₂ dramatically reduced relaxations to SNO-Hb and scavenged the NO released from SNO-Hb as measured with a specific NO electrode. The finding that at the higher concentrations studied (e.g. 30 μM), HbO₂ all but abrogated relaxations to SNO-Hb, has significant implications for the suggestion that SNO-Hb may be an endogenous vasodilator. The concentration of hemoglobin in erythrocytes is in the order of 1–2 mM; there is also a smaller but significant concentration of free hemoglobin in the circulation (~20 μM) (23). Since the concentration of SNO-Hb in the arterial circulation is ~300 nM (10), the ratio of native hemoglobin to SNO-Hb is approximately 10,000 to 1. In the present study, the vasorelaxant activity of SNO-Hb was virtually abolished at a ratio of HbO₂ to SNO-Hb of 30 to 1. Considering this ratio is more than 2 orders of magnitude smaller than that predicted in vivo, it questions the possible physiological role of SNO-Hb as a local regulator of blood flow. Nevertheless, it cannot be discounted that a mechanism which protects NO from the scavenging effects of HbO₂ is present in vivo. For instance, the rate of metabolism of NO by erythrocytes is some 600 times slower than that observed with an equivalent concentration of free HbO₂ (24). The partition coefficient for NO predicts that its concentration is some 10 times greater in the lipid phase than the aqueous phase. Thus, the tendency of NO to accumulate in the erythrocyte membrane may alter the kinetics of its metabolism by HbO₂. Moreover, the mechanism of release of NO from SNO-Hb, as described by this study, suggests that HNO might be an intermediate; this process may also diminish the scavenging effects of HbO₂ since HNO will not react rapidly with Fe(II). The presence of such a protective mechanism is supported by studies utilizing erythrocytes in which the native HbO₂ has been nitrosated artificially (following incubation with CysNO) (25). These cells are able to inhibit platelet aggregation, suggesting that it is possible to elicit an NO-dependent biological effect from intra-erythrocytic SNO-Hb.

One of the intriguing aspects of NO release from SNO-Hb is its putative regulation by the allosteric configuration of the
globin chains. In the present study, the vasorelaxant potency of SNO-Hb was examined at various oxygen tensions. By exposing tissues to gas mixtures containing 21, 10, and 5% O₂ (fixed 5% CO₂; remainder N₂), approximate pO₂ values of 160, 76, and 38 mm Hg were attained; the latter figure represents a pO₂ similar to that found in capillaries (26, 27). However, such conditions had no significant influence on the magnitude or pattern of vasorelaxation to SNO-Hb provided appropriate levels of pre-contraction were achieved. To investigate further a possible role for allosteric changes in SNO-Hb facilitating the transfer of NO, the effect of 2,3-diphosphoglycerate was examined on SNO-Hb-induced relaxations. 2,3-Diphosphoglycerate is produced during anaerobic glycolysis and accentuates the conformational shift in hemoglobin to promote oxygen delivery to the tissue. As such, this compound should also enhance the release of NO from SNO-Hb. However, 2,3-diphosphoglycerate had no effect on the vasorelaxant activity of SNO-Hb, even at low pO₂. Thus, these results do not support the suggestion that the vasorelaxant activity of SNO-Hb is enhanced at low oxygen tensions. The lack of allosteric regulation of NO release from SNO-Hb is supported by a recent report that following nitrosation of Cys^633 the oxygen affinity of Hb is increased (as indicated by a leftward shift of the O₂ dissociation curve) (28). Since the concentration of S-nitrosated Hb is a very small percentage of the total, and erythrocytes only exchange approximately 50% of their carried O₂ during arteriovenous transit, it could be hypothesized that any SNO-Hb would be more likely to retain its associated O₂ and thereby withhold its bound NO. This interpretation of the effect of Cys^633 nitrosation is supported by observations with a Hb variant (Cys^693 → Arg) (29), which has an elevated O₂ affinity. In native Hb, His^114 forms salt bridges with Lys^91 and Asp^98; this conformation contributes 40% to the Bohr effect. Modification of Cys^683 with thiol-oxidizing agents inhibits salt bridge formation and raises O₂ affinity by destabilizing the deoxy (T) state. NO-mediated modification of Cys^683 (i.e., nitrosation) would be expected to produce a similar transformation.

In conclusion, the interaction of SNO-Hb in the presence of a low molecular weight thiol results in the formation of NO, with HNO a likely intermediate; a mixed disulfide between Hb and GSH is also formed. Small quantities of S-nitrosothiols may be produced as a consequence of this, but it is free NO (via HNO) which accounts for the vast majority of nitrogen oxide given up by SNO-Hb, and for its vasorelaxant activity. The transnitrosation of low molecular weight thiols does not appear to be a prerequisite for SNO-Hb to be vasoactive and changes in pO₂ do not alter the potency of SNO-Hb. As such, the present study has important implications for the mechanism of NO release from different molecular weight S-nitrosothiols.

Do these observations preclude SNO-Hb from acting as a vasodilator in vivo? Release of S-nitrosothiols from erythrocytes is slow (in the order of minutes) (10), a time course inconsistent with the thesis that this process is involved in the local regulation of vascular tone exerted by SNO-Hb (10); such molecules would not be released at the required location but at a considerably more distal point. Theoretically, the release of NO from SNO-Hb would be far easier to reconcile with a genuine physiological effect. NO has similar characteristics to O₂ with respect to solubility and diffusion, so would possess the ability to diffuse from an erythrocyte to the vascular smooth muscle and activate soluble guanylyl cyclase. It is clear that O₂ can be delivered precisely upon demand and it is easy to conceive how NO might do the same. However, for such a process to have any physiological significance, an efficient mechanism by which NO is protected from avid scavenging by the native hemoglobin would have to be in place.

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