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# Nitric oxide circulates in mammalian plasma primarily as an *S*-nitroso adduct of serum albumin

(*S*-nitrosothiols/endothelium-derived relaxing factor)

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ABSTRACT **We have recently shown that nitric oxide or authentic endothelium-derived relaxing factor generated in a biologic system reacts in the presence of specific protein thiols to form *S*-nitrosoprotein derivatives that have endothelium-derived relaxing factor-like properties. The single free cysteine of serum albumin, Cys-34, is particularly reactive toward nitrogen oxides (most likely nitrosonium ion) under physiologic conditions, primarily because of its anomalously low pK; given its abundance in plasma, where it accounts for ≈0.5 mM thiol, we hypothesized that this plasma protein serves as a reservoir for nitric oxide produced by the endothelial cell. To test this hypothesis, we developed a methodology, which involves UV photolytic cleavage of the S—NO bond before reaction with ozone for chemiluminescence detection, with which to measure free nitric oxide, *S*-nitrosothiols, and *S*-nitrosoproteins in biologic systems. We found that human plasma contains ≈7 μM *S*-nitrosothiols, of which 96% are *S*-nitrosoproteins, 82% of which is accounted for by *S*-nitroso–serum albumin. By contrast, plasma levels of free nitric oxide are only in the 3-nM range. In rabbits, plasma *S*-nitrosothiols are present at ≈1 μM; 60 min after administration of $N^G$-monomethyl-L-arginine at 50 mg/ml, a selective and potent inhibitor of nitric oxide synthetases, *S*-nitrosothiols decreased by ≈40% (>95% of which were accounted for by *S*-nitrosoproteins, and ≈80% of which was *S*-nitroso–serum albumin); this decrease was accompanied by a concomitant increase in mean arterial blood pressure of 22%. These data suggest that naturally produced nitric oxide circulates in plasma primarily complexed in *S*-nitrosothiol species, principal among which is *S*-nitroso–serum albumin. This abundant, relatively long-lived adduct likely serves as a reservoir with which plasma levels of highly reactive, short-lived free nitric oxide can be regulated for the maintenance of vascular tone.**

Endothelium-derived relaxing factor (EDRF), first described by Furchgott and Zawadzki (1), is a product of the normal endothelial cell having both vasodilatory (2) and antiplatelet (3, 4) properties. Pharmacologic studies suggest that disease states as varied as septic shock (5), atherosclerosis (6), and hypoxia-induced pulmonary hypertension (7) may be associated with abnormal concentrations of EDRF in the vascular milieu. As a result of the seminal work of two groups (8, 9), this bioactive substance is believed to be equivalent to nitric oxide or a chemical congener or adduct thereof. Among the species thought of potential importance as adducts of nitric oxide are *S*-nitrosothiols—adducts with the sulfhydryl groups of amino acids, peptides, and proteins. We have recently shown that nitric oxide and authentic EDRF react with free thiol groups of proteins under physiologic conditions *in vitro* to form *S*-nitrosoproteins (10)—nitric oxide adducts with bioactivities comparable to EDRF but with half-lives of the order of hours. Although the facile formation of such species is intrinsically interesting, the biochemical relevance of these protein derivatives remains to be elucidated.

We describe here an analytical method to determine plasma levels of free nitric oxide and *S*-nitrosothiols. By application of this method, we show that the concentration of *S*-nitrosothiols is 3–4 orders of magnitude greater than that of free nitric oxide in mammalian plasma. We further show that the *S*-nitrosothiol fraction is largely composed of the *S*-nitrosothiol adduct of serum albumin. Finally, we observe that the levels of *S*-nitroso–serum albumin change with inhibition of nitric oxide production in concert with changes in blood pressure. This methodology, then, represents a simple technique for the sensitive, specific, and reproducible measurement of nitric oxide and its thiol adducts in biologic specimens and should have broad application in analysis of the role of EDRF in normal and disease states.

## METHODS

We linked a photolysis cell directly to the reaction chamber and detector portion (bypassing the pyrolyzer) of a chemiluminescence apparatus (model 543 thermal energy analyzer, Thermedix, Woburn, MA) (Fig. 1). A sample (5 to 100 μl) is either introduced directly or introduced as a chromatographic effluent from an attached high-performance liquid or gas chromatography system into the photolysis cell (Nitrolite, Thermedix, Woburn, MA). This cell consists of a borosilicate glass coil (3 m × 0.64 cm o.d. × 1 mm i.d., turned to a diameter of 6 cm and a width of 12 cm). The sample is introduced with a purge stream of helium (5 liters/min) and then irradiated with a 200-W mercury-vapor lamp (vertically mounted in the center of the photolysis coil on Teflon towers). The effluent from the photolysis coil is directed to a series of cold traps, where liquid and gaseous fractions less volatile than nitric oxide (such as nitrite and nitrate) are removed. Nitric oxide is then carried by the helium stream into the chemiluminescence spectrometer, in which free nitric oxide is detected by reaction with ozone. Signals are recorded on a digital integrator (model 3393A, Hewlett–Packard). Flow rates and illumination levels in the photolysis cell were designed to result in complete photolysis of the S—N bond of *S*-nitrosothiols, as confirmed by analysis of effluent from the cell according to the method of Saville (11).

To determine what fraction of the total nitric oxide detected in samples was derived from *S*-nitrosothiols, several

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Abbreviations: L-NMMA, $N^G$-monomethyl-L-arginine; EDRF, endothelium-derived relaxing factor.

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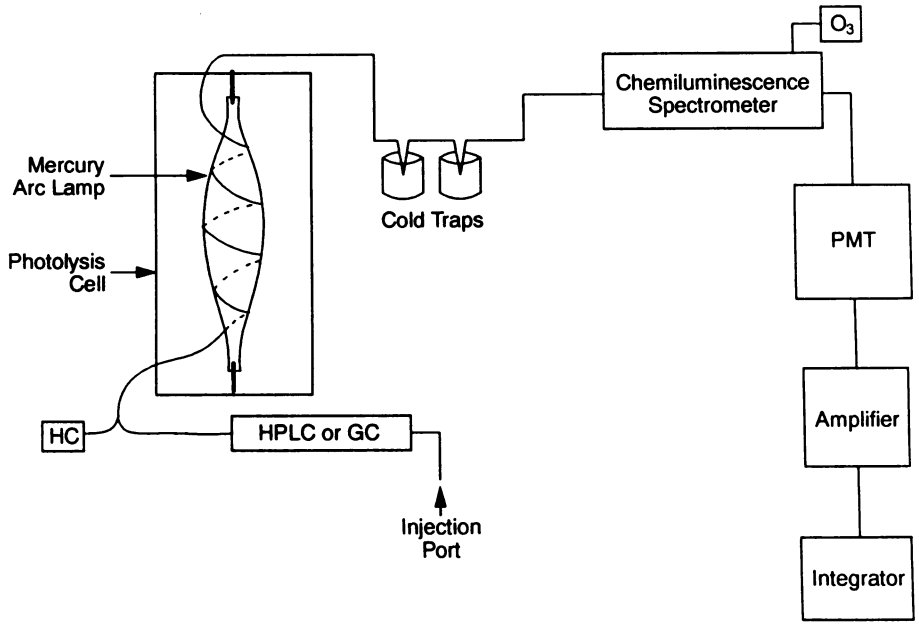


FIG. 1. Schematic representation of the GC/HPLC–photolysis–chemiluminescence system used to measure *S*-nitrosothiols in biologic samples. HC, helium canister; PMT, photomultiplier tube.

control measurements were performed. Mercuric ion was used to displace nitric oxide selectively from the *S*-nitrosothiols (11). Comparison of measured nitric oxide concentrations from samples alternatively pretreated or not pretreated with $HgCl_2$ ensured that nitric oxide obtained by photolysis was derived specifically from *S*-nitrosothiols. Similarly, as an added measure of confirmation, we distinguished between *S*-nitrosothiols and free nitric oxide by comparing nitric oxide concentrations in samples alternatively exposed or not exposed to photolyzing illumination.

The possibility that *S*-nitrosoproteins represented a naturally occurring pool of *S*-nitrosothiols in plasma was also assessed by quantifying the amount of total nitric oxide signal that was removable by precipitation with trichloroacetic acid. To eliminate the possibility that *S*-nitrosoproteins formed as a consequence of exposing the sample containing nitrite to protein thiols upon lowering the pH during acid precipitation, acetone, phenol, or ammonium sulfate was also used to precipitate protein without a pH change. In all cases, the results were similar to those for acid precipitation.

*S*-nitroso-L-cysteine, *S*-nitrosoglutathione, *S*-nitroso-N-acetyl-L-cysteine, and *S*-nitroso–bovine serum albumin were synthesized as described (10, 12), and standard curves were determined. Similarly, standard curves were derived for nitric oxide generated from acidified $NaNO_2$ or from a saturated solution of nitric oxide gas serially diluted immediately before measurement in air-tight syringes. Concentration–response curves were linear with correlation coefficients of $\geq 0.98$ in all cases. Limits of sensitivity were $\approx 0.1$ pM, and intraassay variability was $\pm 3\%$.

Blood samples, anticoagulated with 0.13 M trisodium citrate, were obtained for analysis of plasma from normal volunteers. Samples were also obtained from rabbits, anesthetized with ketamine (50 mg/kg i.m.) and sodium pentobarbital (5–10 mg/kg i.v.), before and 15, 30, 60, and 180 min after i.v. administration of $N^G$-monomethyl-L-arginine (L-NMMA) (50 mg/kg, i.v. bolus). Each of these plasma specimens was injected directly into the photolysis cell, bypassing the HPLC apparatus. In addition, each sample was compared with a corresponding control that included measurement without photolyzing illumination, a determination after protein precipitation, as well as measurement after adding 8.9 mM $HgCl_2$ to the sample.

## RESULTS

The response of this system to pure *S*-nitrosothiols was first examined using *S*-nitroso-L-cysteine as a standard. The concentration of the stock solution from which serial dilutions were made was determined by the method of Saville (11) and confirmed by measuring the OD of the solution at 340 nm (10, 12). Serial dilutions were prepared with concentrations from 100 $\mu$M to 0.1 pM. The chemiluminescence signal was linear over this concentration range (correlation coefficient 0.98). As additional controls, *S*-nitrosoglutathione and *S*-nitroso-N-acetyl-L-cysteine were also synthesized, and responses were measured. Without photolysis, the nitric oxide signal was below the limits of detectability. Similarly, $HgCl_2$ pretreatment followed by air incubation to oxidize (and render undetectable) the liberated nitric oxide equivalent lost >99% of the chemiluminescence signal in all cases.

The fasting plasma of five normal volunteers (mean age, 30 $\pm$ 3 yr) was next obtained for measurement of nitric oxide by this protocol. Blood samples were obtained in an evacuated sample tube containing 13 mM trisodium citrate, the tube was centrifuged at 700 $\times$ *g* for 10 min, and the supernatant plasma was removed in an air-tight syringe for transfer to the detection system. Mean plasma levels (Table 1) were 7.19 $\pm$ 5.73 $\mu$M, only 10.01% of which was not displaceable by $Hg^{2+}$ and 0.047% of which was detectable without photolysis. Furthermore, protein precipitation (with 5% trichloroacetic acid or 50% acetone) led to a loss of 96.25% of the signal.

Inasmuch as we have previously shown that *S*-nitroso–albumin can form under physiologic conditions, that this

Table 1. Mean plasma levels of nitric oxide and *S*-nitrosothiols in humans

| | Mean level, $\mu$M |
|---|---|
| Free nitric oxide | 0.0034 $\pm$ 0.00058 |
| *S*-Nitrosothiol | 7.19 $\pm$ 5.73 |
| *S*-Nitrosoprotein | 6.92 $\pm$ 5.45 |

Values represent the mean $\pm$ SD of five different donors. Intraassay variability was $\pm 3.0\%$.

Table 2. Changes in mean blood pressure and plasma S-nitrosothiols in rabbits after administration of L-NMMA

| Time | Mean blood pressure, mmHg | S-Nitrosothiol, μM | S-Nitrosoprotein, μM |
|---|---|---|---|
| Pretreatment | 64 ± 5 | 1.29 ± 0.57 | 1.22 ± 0.55 |
| Treatment | | | |
| 15 min | 74 ± 6* | 1.11 ± 0.51 | 1.03 ± 0.44* |
| 60 min | 78 ± 11* | 0.78 ± 0.26* | 0.73 ± 0.23* |

*$P \leq 0.05$ by two-way analysis of variance and Newman–Keuls comparison to the pretreatment value.

bioactive S-nitrosoprotein is very stable, and that it may represent a carrier molecule in plasma for nitric oxide (10), we attempted to define the fraction of nitric oxide borne in human plasma by serum albumin. We subjected plasma specimens (three) to affinity chromatography by using an Econo-Pac Blue 5-ml column cartridge (Bio-Rad) (to which both serum albumin and S-nitroso-serum albumin bound) and found that 82% of the total (protein) precipitable nitric oxide signal could be eliminated by this procedure.

L-NMMA is a specific competitive inhibitor of the nitric oxide synthetases, the family of enzymes responsible for elaboration of nitric oxide from L-arginine. Infusion of L-NMMA has been shown to increase blood pressure in rabbits and dogs (13, 14), implying a role for steady-state levels of EDRF in maintaining blood pressure. To identify a potential relationship between blood pressure and depletion of plasma S-nitrosothiols, we infused L-NMMA at 50 mg/kg as an i.v. bolus into three 3- to 4-kg female New Zealand White rabbits. Table 2 shows that within 15 min the mean blood pressure (measured by a cannula in the femoral artery) increased 16% [from 64 ± 5 to 74 ± 6 mmHg (1 mmHg = 133 Pa), $P < 0.05$], and by 1 hr the mean pressure increased by 22% (to 78 ± 11, $P < 0.05$). Concomitant with this increase in blood pressure, total S-nitrosothiols in plasma decreased by 14% at 15 min after infusion (1.29 ± 0.57 μM to 1.11 ± 0.51 μM), and by 40% at 1 hr (to 0.78 ± 0.26 μM, $P < 0.02$). These changes occurred primarily in the S-nitrosoprotein fraction of plasma, wherein 94.6% of total S-nitrosothiols was found before infusion of L-NMMA, and 92.9% of total S-nitrosothiols was found by 1 hr after infusion. The time course of a typical experiment is shown in Fig. 2, where changes in mean blood pressure and total S-nitrosothiols are plotted. Initially after L-NMMA administration, mean arterial pressure increased with a time course similar to that for the complementary decrease in plasma S-nitrosothiols. Peak effects (maximum and minimum) were noted by 60 min, and by 180 min both parameters returned to pretreatment values. Importantly, cross-over points are noted at 30 and 120 (interpolated) min, suggesting (but not proving) a mechanistic association between these two parameters. Again of note is the observation that only 0.26% of the total chemiluminescence signal could be accounted for by free nitric oxide (data not shown). Linear-regression analysis of the change in mean arterial pressure and change in S-nitrosoproteins at each time point in each rabbit yielded $R^2 = 0.74$ ($P < 0.01$).

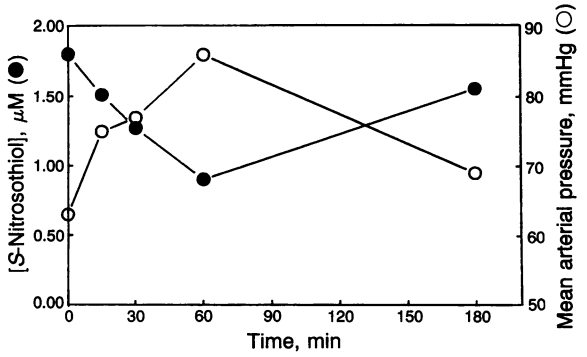


FIG. 2. Representative response in mean arterial pressure (○) and plasma S-nitrosothiols (●) in a rabbit administered L-NMMA at 50 mg/kg as an i.v. bolus infusion at $t = 0$. Note that by 180 min after L-NMMA administration, blood pressure had returned to pretreatment values, as had plasma S-nitrosothiols.

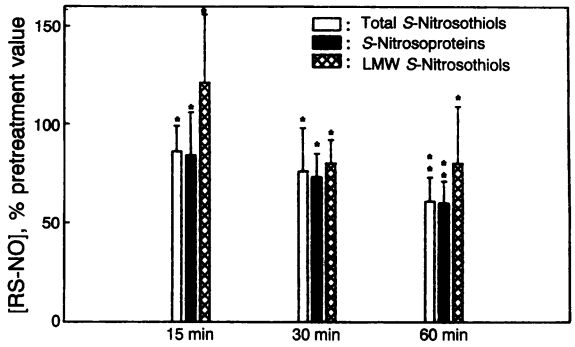


FIG. 3. Percentage changes in S-nitrosothiols (RS-NO) in rabbit plasma after administration of L-NMMA at 50 mg/kg at $t = 0$. Total S-nitrosothiols (clear bars), S-nitrosoproteins (filled bars), and low-molecular-weight (LMW) S-nitrosothiols (hatched bars) were measured at 15, 30, and 60 min after L-NMMA infusion and plotted relative to their measured values at $t = 0$. *, $P < 0.05$; **, $P < 0.02$.

Interestingly, when the percentage changes in total S-nitrosothiols, S-nitrosoproteins, and low-molecular-weight S-nitrosothiols are compared in these experiments, a transient increase in low-molecular-weight S-nitrosothiols occurs at 15 min (Fig. 3). These data suggest that transfer of nitric oxide (probably in a trans-nitrosation reaction involving nitrosonium and thiolate anions under physiologic conditions) may occur from S-nitrosoproteins to low-molecular-weight thiols during the course of reequilibrating nitric oxide among thiol pools as EDRF production decreases. Importantly, *in vitro* experiments suggest that low-molecular-weight thiols can facilitate release of nitric oxide from S-nitrosoproteins and, thereby, enhance the vasodilator or platelet-inhibitory activity of the protein-bound nitric oxide. Glutathione added to a solution containing an equivalent of S-nitroso–bovine serum albumin in phosphate-buffered saline at pH 7.4, for example, led to the rapid formation of stoichiometric concentrations of S-nitrosoglutathione, measured as acid- or acetone-soluble S-nitrosothiol by the photolysis–chemiluminescence method described here or directly by a capillary-zone electrophoretic method we have recently developed (12).

## DISCUSSION

Under normal circumstances, the concentration of nitric oxide in blood or plasma is believed to be quite low—in the 1 nM range—and its reactivity toward oxygen and redox metals is believed to be high. The latter situation makes the routine measurement of blood levels in normal and disease states difficult (15, 16) by standard methods, such as chemiluminescence spectroscopy (17), electron paramagnetic resonance spectroscopy (18), or differential absorbance spectroscopy of hemoglobin (19). Chemiluminescence spectroscopy has been used, in particular, to detect nitric oxide in studies of EDRF *in vitro*. By the standard analysis, however, samples to be tested are subjected to extensive chemical pretreatment that precludes discrimination of free nitric oxide from labile adducts or from higher oxidation states of nitrogen. In view of this serious and important limitation, we have developed a method that both avoids the source-masking

chemical pretreatment and permits discrimination of free nitric oxide and *S*-nitrosothiols.

Results obtained by using this method show that (*i*) nitric oxide and its related biologically active adducts can be measured simply and with minimal sample preparation in plasma; (*ii*) *S*-nitrosothiols circulate in plasma primarily as *S*-nitrosoproteins, the predominant species of which is *S*-nitroso–serum albumin; and (*iii*) pharmacologic interventions that modulate nitric oxide generation change plasma levels of *S*-nitrosothiols. The abundance of *S*-nitrosothiols in plasma compared with that of free nitric oxide 3 to 4 orders of magnitude lower in concentration suggests that plasma *S*-nitrosothiols may serve as a reservoir for nitric oxide, effectively buffering its concentration. Protein thiol adducts of nitric oxide are particularly well-suited for this role because of their relatively long half-lives under physiologic conditions (10) compared with free nitric oxide or *S*-nitrosothiol adducts of low-molecular-weight thiols. Release of nitric oxide from *S*-nitrosoprotein stores may be brought about by mixed disulfide formation with low-molecular-weight thiols or facilitated by the transfer of nitric oxide from protein thiols to low-molecular-weight thiols, such as glutathione or cysteine (unpublished observation). That albumin circulates in plasma as a mixed disulfide with low-molecular-weight thiols (20) further supports this mechanism of nitric oxide release and transfer *in vivo*.

The role that *S*-nitrosothiols play under various normal conditions that may require moment-to-moment modulation of nitric oxide levels in the vascular milieu (such as exercise or food intake) or under various pathophysiologic states (such as septic shock, atherosclerosis, or hypertension) remains to be demonstrated. The methodology presented here may, however, provide a means to address these important issues.

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