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S-Nitrosothiol Formation in Blood of Lipopolysaccharide-Treated Rats

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The administration of the gram-negative bacterial cell wall component lipopolysaccharide (LPS) to experimental animals results in the dramatic up-regulation of the inducible form of nitric oxide synthase (iNOS). The resulting sustained overproduction of nitric oxide (NO) is thought to contribute to the septic shock-like state in these animals. Numerous studies have characterized the kinetics and magnitude of expression of iNOS as well as the production of NO-derived nitrite and nitrate. However, little is known regarding the ability of iNOS-derived NO to interact with physiological substrates such as thiols to yield biologically active *S*-nitrosothiols during endotoxemia. It has been hypothesized that these relatively stable, vaso-active compounds may serve as a storage system for NO and they may thus play an important role in the pathophysiology associated with endotoxemia. In the present study, we demonstrate that 5 h after i.p. administration of LPS in rats, circulating *S*-nitrosoalbumin was increased by ~3.4-fold over control. *S*-nitrosohemoglobin was increased by ~25-fold over controls and by threefold over *S*-nitrosoalbumin. No increase in low molecular weight *S*-nitrosothiols (i.e., *S*-nitrosoglutathione and *S*-nitrosocysteine) could be detected under our experimental conditions. Taken together these data demonstrate that endotoxemia dramatically enhances circulating *S*-nitrosothiol formation.

***Key Words:* *S*-nitrosothiol; nitric oxide; *S*-nitrosoalbumin; *S*-nitrosohemoglobin; *S*-nitrosoglutathione; endotoxemia; septic shock.**

A major feature of endotoxemia is the up-regulation of the inducible isoform of nitric oxide synthase (iNOS) in a wide variety of tissues with the resulting production of large quantities of the free radical nitric oxide (NO) (1). The overproduction of NO has been proposed to contribute to the circulatory failure, myocardial dysfunction, organ injury, and multiple organ dysfunction syndrome associated with endotoxic shock (2, 3). While NO is rapidly cleared from the circulation by reaction with oxyhemoglobin to form nitrate (4, 5), it also binds to the heme centers of hemoglobin to yield nitrosylated hemes which may maintain the NO group in a bioactive form (6). In addition, reactive nitrogen species derived from NO may mediate the S-nitrosation of thiol residues in proteins and peptides to yield a variety of *S*-nitroso derivatives with important physiological activities including vasodilation (7, 8). Although it has been demonstrated that a fraction of albumin, hemoglobin, and cysteine are S-nitrosated in the systemic circulation (8–12), little is known regarding the effect of endotoxemia on plasma and erythrocyte levels of these metabolites *in vivo.* The objective of the present study was to characterize and to quantify plasma and erythrocyte *S*-nitrosothiols in response to LPS-challenge in the rat.

MATERIALS AND METHODS

Chemicals and reagents. Aspergillus nitrate reductase was purchased from Boeringer-Mannhein (Manheim, Germany). All other chemicals were obtained from Sigma Chemicals Co. (St. Louis, MO).

Preparation of S-nitrosocysteine. *S*-nitrosocysteine was prepared by incubating 1 volume of 200 mM cysteine in 1 N HCl with 1 volume of an equimolar concentration of sodium nitrite in distilled water for 30 min at room temperature in the dark. The solution was then neutralized with 2 volumes of 1 M K_2HPO_4 pH 7.4 containing 200 μM DTPA. The yields of CySNO formation were between 85 and 100% as calculated using an extinction coefficient of 900 $M^{-1} \cdot cm^{-1}$ at 338 nm (13). The presence of S—NO bonds was also confirmed by using the Saville assay as described below.

Nitrosation of oxyhemoglobin. *S*-nitroso-hemoglobin was prepared by exposing 50 μM oxyhemoglobin to 500 μM CySNO for 30 min at room temperature in the dark. The solution was then desalted over a column of Sephadex G-25 equilibrated with PBS pH 7.5 containing 100 μM DTPA.

Animals. Male Sprague-Dawley rats (250–350 g) were obtained from Charles River Laboratories (Wilmington, MA). The animals were allowed to acclimate to their surroundings for at least 1 week before use. LPS (serotype 055:B5, Sigma Chemical Co., St. Louis, MO) was dissolved in sterile saline and administered i.p at a dose of 5 mg/kg. Blood was obtained from the jugular vein of anaesthetized

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rats 5 h after LPS administration using heparin as an anticoagulant. Animals were then sacrificed by either CO_2 inhalation or pentobarbital overdose.

Recovery of plasma and hemoglobin for S-nitrosothiol determination. Plasma and red blood cells were recovered by centrifugation at 1000 g for 10 min. The pellet was washed twice with cold isotonic saline containing 100 μM DTPA. Plasma was immediately tested for *S*-nitrosothiol content or stored at −70°C until assayed for nitrite/nitrate. The red blood cells were lysed by the addition of 1 volume of distilled water containing 100 μM DTPA for 10 min at 4°C. The lysate (500 μl) was then loaded on a G-25 column equilibrated with 0.1 M NaCl containing 100 μM DTPA, pH 7.5. The concentration of oxyhemoglobin was determined using the reported molar absorption coefficient and the samples were immediately used for *S*-nitrosohemoglobin determination.

Determination of S-nitrosohemoglobin concentrations. *S*-nitrosohemoglobin concentrations were determined using $HgCl_2$ to displace NO from thiol residues followed by determination of nitrite content using 2,3-diaminonaphthalene (DAN). Hemoglobin was exposed to a tenfold molar excess of $HgCl_2$ for 10 min at room temperature and in the dark. The sample was then centrifuged for 30 min at 11,000 g and 4°C using a centrifugal filter unit with a molecular weight cut-off of 10,000. Nitrite content was determined by incubating 200 μl of the filtrate with 20 μl of DAN 200 μM in 1 N HCl for 10 min at 37°C. The samples were made alkaline by addition of 35 μl of 10 N NaOH and dilution with 600 μl of 10 mM NaOH (14). The fluorescence of the sample was determined using an excitation and emission wavelength of 406 and 450 nm, respectively.

Determination of S-nitrosothiol concentrations in plasma. Plasma or erythrocyte low-molecular weight *S*-nitrosothiols were analyzed by reversed-phase chromatography using a modification of the method developed by Akaike and co-workers (15, 16). Prior to injection the samples were subjected to ultracentrifugation using a centrifugal unit with a molecular weight cut-off of 10,000 (Millipore, Bedford, MA). The ultrafiltrate was then injected onto a 250 × 4.6 mm 5 μm ODS C_{18} ultrasphere column (Beckman, Fullerton, CA) isocratically running at a flow rate of 0.55 ml/min with 10 mM sodium acetate buffer (pH 5.5) containing 0.5 mM DTPA. The eluate from the HPLC column was mixed with a solution (flow rate 0.25 ml/min) containing 1.75 mM mercuric chloride, 0.1% naphthylethylenediamine, 1% sulfanilamide, and 2% phosphoric acid. The diazo compound formed from the interaction of the nitrosating agent liberated by the mercury-induced decomposition of the *S*-nitrosothiol with the Griess reagents was detected at 540 nm. Typical retention times under these conditions were 9, 14 and 17 min for CySNO, CG-SNO, and GSNO, respectively. High molecular weight nitrosothiols were analyzed in a similar fashion using a 300 × 7.5 mm 12–15 μm SigmaChrom GFC-1300 column (Supelco Inc., Bellefonte, PA) with 10 mM sodium acetate (pH 5.5) containing 0.15 mM sodium chloride and 0.5 mM DTPA. The retention time for *S*-nitrosoalbumin was 18 min. The detection limit for *S*-nitrosothiols with either method was 20 nM.

Determination of nitrite/nitrate concentrations. Plasma nitrite and nitrate concentrations was determined using a modification of a previously described method (17). The samples were subjected to ultracentrifugation using a centrifugal unit with a molecular weight cut-off of 10,000 (Millipore, Bedford, MA). Forty microliters of filtered sample was mixed with 20 μl of 0.31 M phosphate buffer and 10 μl each of 0.86 mM NADPH, 0.11 mM FAD and 1.0 U/ml of nitrate reductase in individual 96-well plate. Samples were allowed to incubate for 1 h in the dark. Two hundred microlliters of Griess reagent [1:1 mixture of 1% sulfanilamide in 5% phosphoric acid and 0.1% *N*-(1-naphthyl)ethylenediamine] was added to each well and the plates were incubated for an additional 10 min at room temperature. Absorbances were measured at 540 nm using a microplate reader and converted to nitrite/nitrate concentrations by using a standard curve of nitrate.

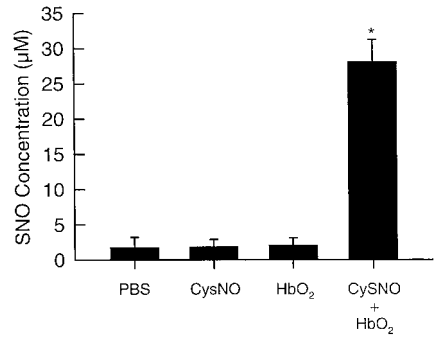

FIG. 1. Synthesis of *S*-nitrosohemoglobin. Oxyhemoglobin 50 μM was incubated in the presence or the absence of tenfold excess of *S*-nitrosocysteine (CySNO) for 30 min at 37°C. The samples were then eluted through a G25 column and *S*-nitrosohemoglobin concentrations were determined via the addtion of $HgCl_2$ as described under Materials and Methods. PBS, phosphate buffer saline; CysNO, *S*-nitrosocysteine; HbO_2, oxyhemoglobin. Each point represents the mean ± SD (n = 3). *$P < 0.001$ compared to PBS.

RESULTS AND DISCUSSION

Validation of the fluorescent assay for the detection of S-nitrosohemoglobin. *S*-nitrosohemoglobin was quantified using a modification of the Saville reaction, in which the nitrosating agent produced from the Hg^{2+}-catalyzed decomposition of the *S*-nitrosothiol is trapped by 2,3-diaminonaphthalene (DAN) to yield the highly fluorescent derivative 2,3 napthotriazole (NAT) (18). Incubation of 50 μM oxyhemogobin (HbO_2) with a tenfold excess of CysNO at 37°C for 30 min resulted in the formation of 28 ± 3.2 μM nitrosated hemoglobin (Fig. 1). Omission of either HbO_2, CySNO or $HgCl_2$ resulted in no detectable S-nitrosohemoglobin. These results indicated that elution through a G-25 column effectively cleared the samples from low molecular weight *S*-nitrosothiols and that addition of mercury was a necessary step for the decomposition of the *S*-nitrosothiol.

The performance of the assay was evaluated by comparing the concentrations of *S*-nitrosohemoglobin obtained using the standard Saville reaction (19) to those obtained using the NAT assay. As shown in Table 1, the concentration of *S*-nitrosohemoglobin determined with either technique was not statistically different from each other. In a subsequent set of experiments, we added nitrate reductase to the incubation mixture after treatment of the samples with $HgCl_2$ to examine for the possibility that hemoglobin may convert some of the nitrite to nitrate during the incubation period (4). To minimize $HgCl_2$-induced inhibition of nitrate reductase, an equimolar amount of DTPA was added to the sample after incubation with $HgCl_2$. Standard curves of sodium nitrate obtained in the presence of $HgCl_2$ and DTPA were identical to those obtained in the absence of these compounds (data not shown). As depicted in Table 1, pretreatment of *S*-nitrosohemoglobin with nitrate reductase before the addition of the Griess re-

TABLE 1

Concentrations of Nitrite Detected with or without Treatment with Nitrate Reductase Using either the Saville Assay or the Fluorometric Assay

	S-nitrosohemoglobin Concentration (μM)	
		+ nitrate reductase
Saville Assay (n = 4)	27.2 ± 5.2	29.0 ± 5.6
Fluorometric Assay (n = 4)	28.0 ± 2.3	29.2 ± 4.6

Note. Nitrosated hemoglobin was prepared by incubation of 50 μM oxyhemoglobin with 500 μM CySNO for 30 min in PBS pH 7.5 containing 100 μM DTPA. The reaction mixture was eluted with a G-25 column equilibrated with PBS pH 7.5. The concentration of *S*-nitrosohemoglobin was determined as described under Materials and Methods.

agents did not affect the amount of nitrite recovered (Table 1). This suggested that the oxyhemoglobin-mediated oxidation of nitrite did not result in the formation of nitrate that may have undermined the amount of *S*-nitrosohemoglobin detected. In support to these results, the ~58% conversion rate from HbO_2 to *S*-nitrosohemoglobin obtained in the present study was also in agreement with previous studies (20). Finally, we examined the effects of excess hemoglobin (50 μM) over *S*-nitrosohemoglobin (50–1000 nM) on the determination of *S*-nitrosohemoglobin concentration. As illustrated in Fig. 2, the linear relationship between the amount of *S*-nitrosohemoglobin and the amount of NAT was not different from the one obtained for nitrite and GSNO (in the absence of hemoglobin). In summary, we found that the utilization of acidified solution of 2,3-diaminonaphthalene may represent a simple approach for the determination of nanomolar concentrations of *S*-nitrosohemoglobin in biological samples.

Detection of circulating S-nitrosothiols in LPS-treated rats. There is increasing evidence to suggest that *S*-nitrosothiols formed from the NO-mediated S-nitrosation of thiol-containing peptides and proteins represent important physiological metabolites for the transport, storage and metabolism of NO in the vasculature (21). Recent work from Stamler and co-workers suggests that a specific cysteine located in the β chain of oxyhemoglobin is S-nitrosated in the lung and that the nitroso group once released in arterioles modulates blood flow in response to changes in oxygen tension (22). According to this hypothesis, the nitrosonium ion (NO^+) is transferred from *S*-nitrosohemoglobin to thiols such as cysteine and glutathione during the arterial-venous transit of erythrocyte hemoglobin. The intracellular low-molecular weight *S*-nitrosothiols thus formed are then exported into the blood and subsequently transported to the vascular wall where they mediate smooth muscle relaxation. Thus, the relative distribution of these different *S*-nitrosothiols may be tightly interrelated in the vasculature to insure adequate delivery of NO-like activity to arterioles and distant organs (21, 23). Recent data would suggest that up-regulation of iNOS is associated with increased S-nitrosation of cellular thiols suggesting that increased NO production *in vivo* may result in the alteration of steady-state levels of these derivatives during conditions associated with immune activation and/or inflammation (24). In support to this hypothesis, Gaston and co-workers found that the concentration of *S*-nitrosoglutathione was significantly increased in the bronchoalveolar lavage fluid of patients with pneumonia (25). Similarly, it is possible that the increased NO production that is associated with sepsis may result in an alteration in *S*-nitrosothiol metabolism in the circulation.

In the present study, we found that venous *S*-nitrosoalbumin concentrations (120 nM ± 52 nM) exceeded those of *S*-nirosohemoglobin (51 nM ± 42 nM) under control conditions (Fig. 3) whereas both plasma and erythrocyte-associated low molecular weight *S*-nitrosothiol concentrations fell below the detection limit of our assay (<20 nM, data not shown). These results were in agreement with a recent study where we showed that plasma low-molecular weight *S*-nitrosothiols such as *S*-nitrosoglutathione are unstable and rapidly decompose via transnitrosation with albumin to form *S*-nitrosoalbumin (16). These data are important since they would suggest that albumin may act as a sink for low-molecular weight *S*-nitrosothiols and may limit the S-nitrosation of intraerythrocytic hemoglobin.

We found that circulating *S*-nitrosothiol concentrations increased in LPS-treated animals such that *S*-nitrosoalbumin and *S*-nitrosohemoglobin concentrations were ~3.4 (410 ± 120 nM) and ~25 (1210 ± 325

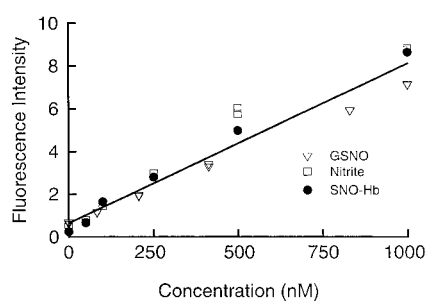

FIG. 2. *S*-nitrosohemoglobin determination in the presence of excess oxyhemoglobin. *S*-nitrosohemoglobin (50–1000 nM) was mixed with oxyhemoglobin 50 μM and the *S*-nitrosohemoglobin concentrations were determined as described under Materials and Methods. The fluorescence intensity obtained from the $HgCl_2$-induced nitrosation of 2,3 diaminonaphathalene by the *S*-nitrosothiol is reported as a function of increasing *S*-nitrosohemoglobin. Standard curves of *S*-nitrosoglutathione (GSNO) and nitrite (in the absence of hemoglobin) are also reported for comparison. Each point represents individual determination for samples ran in duplicates.

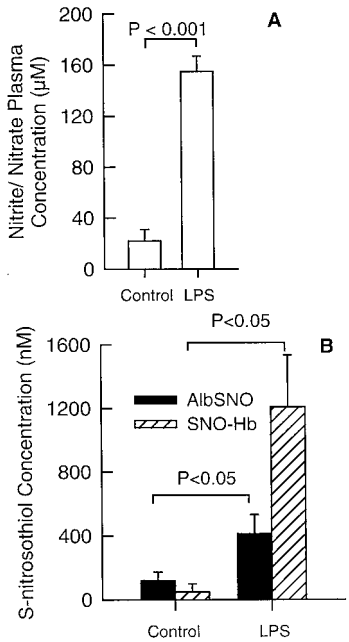

FIG. 3. Effect of LPS treatment on plasma nitrite/nitrate and circulating *S*-nitrosothiols. LPS (5 mg/kg) was administered (i.p.) to rats and the concentrations of plasma nitrite and nitrate (A) and circulating S-nitrosothiols (B) were determined 5 h after the administration of LPS as described under Materials and Methods. SNO-Hb, *S*-nitrosohemoglobin; AlbSNO, *S*-nitrosoalbumin. Each point represents the mean ± SE ($n = 4$).

nM) fold higher than their respective control conditions (Fig. 3). It is interesting to note that LPS administration produced a much larger increase in *S*-nitrosohemoglobin than *S*-nitrosoalbumin. The reasons for these observations are unclear but they further underline the need for a detailed description of the basic determinants of formation, disposition and turnover of circulating S-nitrosothiols. Plasma levels of nitrite and nitrate, the stable decomposition products of NO, increased by approximately tenfold 5 h after i.p. administration of LPS in rats confirming enhanced NO production (17). The cumulative amount of S-nitrosothiols formed under these conditions represented less than 1% of the amount of nitrite/nitrate formed which suggests that alternative pathways may exist for the decomposition of NO-derived nitrosating species. Specific mechanisms may also be activated during inflammation that may result in *S*-nitrosothiol decomposition and lowering of the steady-state concentrations (26). For example, reactive oxygen metabolites such as superoxide that are generated by activated phagocytic leukocytes may contribute to the catabolism of certain *S*-nitrosothiols (27). Taken together, our data demonstrate that LPS administration produces an increase in the concentration of protein associated *S*-nitrosothiols in the circulation suggesting the formation of NO-derived nitrosating agents. Since the increase in *S*-nitrosothiol was well within the concentration range that has been shown to relax arteries *ex vivo* and *in vivo* (9, 20, 28), it is possible that the formation of *S*-nitrosothiols during endotoxemia will participate in the cardiovascular effects associated with shock. The physiological significance of these findings is currently under investigation.

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