Thiol Compounds Interact with Nitric Oxide in Regulating Heme Oxygenase-1 Induction in Endothelial Cells

INVolvEMENT OF SUPEROXIDE AND PEROXYNITRITE ANIONS

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Thiols are very important antioxidants that protect cells against oxidative insults. Recently, a different and new physiological role has been defined for these compounds because of their involvement in nitric oxide (NO) binding and transport in biological systems. In view of these characteristics, we examined the effect of thiols and NO on the expression of the inducible form of heme oxygenase (HO-1), a stress protein that degrades heme to carbon monoxide and biliverdin. Cultured bovine aortic endothelial cells exposed to the NO donors sodium nitroprusside (SNP) and S-nitroso-N-acetylpenicillamine (SNAP) resulted in increased heme oxygenase activity and HO-1 expression. Co-incubation with N-acetylcysteine, a precursor of glutathione synthesis, significantly attenuated heme oxygenase induction by SNP and SNAP, and a reduction in heme oxygenase activity was also observed when cells were preincubated with N-acetylcysteine for 16 h prior to exposure to NO donors. This effect appears to be associated with NO stabilization by thiols through the formation of S-nitrosothiols. Hydroxocobalamin, a specific NO scavenger, significantly decreased endothelial heme oxygenase activity, indicating a direct involvement of NO released by NO donors to regulate the expression of this stress protein. Moreover, superoxide anion (O$_2^-$) and its reaction product with NO, peroxynitrite (ONOO$^-$), were found to partially contribute to the observed NO-mediated activation of endothelial heme oxygenase. Thus, we suggest the existence of a dynamic equilibrium among free NO, O$_2^-$, and endogenous glutathione, which might constitute an interactive signaling mechanism modulating stress and adaptive responses in tissues.

Heme oxygenase is a widely distributed enzyme in mammalian tissues, and its main function is associated with the degradation of heme to biliverdin, iron, and carbon monoxide (CO).$^1$ Two distinct isoforms of the protein have been characterized revealing that one of the isozymes is constitutive (HO-1), whereas the other is inducible (HO-1) (1). Thus, if the first enzyme is constitutively expressed and is part of the normal cellular metabolism, the second is regarded as a heat shock protein, and its expression is elicited by many conditions and factors that produce an imbalance in the cellular functions. Various agents, including heavy metal ions (2), oxidative stress (3), endotoxins (4), and hemoglobin (5) are capable of inducing HO-1 in different tissues, and recent findings showed that nitric oxide (NO) donors increase HO-1 mRNA in the brain (6) and in cultured hepatocytes (7). Accordingly, we have reported the ability of diverse NO releasing agents to modulate heme oxygenase activity in aortic endothelial cells (8).

The physiological importance of HO-1 induction following stress situations is not fully understood, although it has been hypothesized that the expression of this gene is part of the defensive mechanism that cells and tissues are capable of mounting against different stress stimuli. To sustain this idea are the findings that biliverdin and bilirubin, end products of heme catabolism, possess antioxidant properties (9) and that CO seems to mimic many NO functions. Like NO, CO activates soluble guanylate cyclase and inhibits platelet aggregation (10, 11), and its possible role as a neurotransmitter has been postulated (12, 13). Recent findings also show the participation of CO in the regulation of vascular tone in hepatic sinusoidal cells, suggesting that NO and CO could share the control of relaxation processes (14).

NO is a free radical species with multiple biological functions and has been identified as endothelium-derived relaxing factor (15–17). It reacts rapidly with superoxide anion (O$_2^-$) to form the stable peroxynitrite anion (ONOO$^-$), which decomposes once protonated to yield a strong oxidant with reactivity similar to hydroxyl radical (OH•) (18). In addition, NO has a high affinity for heme-iron molecules and interacts readily with thiol groups of proteins and glutathione producing S-nitrosoproteins and S-nitrosothiols, respectively (19, 20). It has been suggested that S-nitrosylation of proteins could mediate signaling functions similar to those of protein phosphorylation (21) and that thiols may be involved in NO stabilization and metabolism (22). S-Nitrosothiols indeed possess endothelium-derived relaxing factor-like characteristics and, in virtue of their NO releasing capacities, may invoke many and possibly all NO actions (23). There is at present a commonly accepted concept that NO diffuses freely in tissues where it exerts its biological activities. A recent report, however, considers the perspective that NO, to reach its pharmacological targets, has to diffuse through the intracellular environment where glutathione levels are in the range of 5–10 mM (24).

Several important roles distinguish glutathione as the most important cellular nonprotein thiol. It is a cofactor for many glycolytic enzymes, participates in the transport of amino acids, and constitutes the major cellular antioxidant, being pres-
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Ent at high concentrations in most animal cells (25). It plays a key function in reacting with harmful free radicals produced during the metabolism contributing to cellular detoxification. However, depletion of intracellular levels of glutathione may occur in conditions of severe and intense oxidant stress leading to increased susceptibility to cytotoxicity. A correlation between heme oxygenase and glutathione has been documented (4, 26) where HO-1 levels are augmented in conditions of decreased intracellular glutathione. Whether decreased glutathione levels affect heme oxygenase via accumulation of reactive oxygen species or by modulating a signaling mechanism is unknown.

The purpose of the present study was to examine the role of exogenous and endogenous thiol-containing compounds in the regulation of heme oxygenase induction by NO and other radical species in bovine aortic endothelial cells. We report that increased thiols levels reduce heme oxygenase expression and activity mediated by NO donors and that both O$_2^-$ and ONOO$^-$ appear to partially contribute to the stimulation of this stress response; the physiological relevance of these findings will be discussed.

**EXPERIMENTAL PROCEDURES**

**Materials**—Bovine aortic endothelial cells and human umbilical vein endothelial cells were purchased from the European Collection of Animal Cell Culture (Salisbury, UK). Hemin was obtained from Porphyridium Products Inc. (Logan, UT). Sodium nitroprusside (SNP), S-nitroso-N-acetylpenicillamine (SNAP), N-acetylcysteine, N-acetyl-DL-penicillamine (AP) sulfoximine (BSO), S-nitrosoglutathione (GSNO), hydroxocobalamin, and superoxide dismutase (SOD, bovine liver) were all purchased from Sigma. 3-Morpholinosydnonimine (SIN-1) and sodium peroxynitrite were obtained from Alexis Corp. (Bingham, Nottingham, UK). Peroxynitrite was prepared fresh in sodium hydroxide (0.5 M), shipped via dry ice and immediately stored at $-80\, ^\circ\text{C}$. The concentration of peroxynitrite was monitored each day before use by measuring the absorbance at 302 nm (extinction coefficient $= 1670\, \text{M}^{-1}\text{cm}^{-1}$) after addition of 25 (stock solution) to 975 μl of ice-cold 0.3 M sodium hydroxide. All the experiments with peroxynitrite were carried out within 10 days after receiving the drug, and the concentration was verified to be >95% of the stipulated one. Only small volumes of peroxynitrite were added to the culture media, and no significant changes in pH were observed.

**Culture of Endothelial Cells**—Bovine aortic endothelial cells were cultured in 75-cm$^2$ flasks and grown in Iscove's modified Dulbecco's medium supplemented with 8% fetal bovine serum, 2 mM l-glutamine, penicillin (100 units/ml), and streptomycin (0.1 mg/ml). Cells used for the heme oxygenase assay were cultured to reach confluency in 75-cm$^2$ flasks; the glutathione determination method was performed in confluent cells grown in 6-well plates, and for the nitrite and NO measurements cells were subcultured in 24-well tissue culture plates. Human umbilical vein endothelial cells were cultured in 75-cm$^2$ flasks in Medium 199 supplemented with 10% fetal bovine serum, 2 mM l-glutamine, and antibiotics.

**Experimental Protocol**—To investigate the effect of NO and the role of NO-derived intermediate(s) on endothelial heme oxygenase activation, several common NO donors were examined either alone or in the presence of various scavengers and agents. In the first group of experiments, cells were incubated for 6 h in complete medium (control) or in medium supplemented with SNP (0.5 and 1 mM), SNAP (0.5 mM), or GSNO (0.5 mM). In a different set of experiments cells were exposed to SNP or SNAP in the presence of the glutathione precursor N-acetylcysteine (0.5, 1, 2, 5, and 5 mM). Hydroxocobalamin (vitamin B12), known as a NO scavenger (27), was also used at a final concentration of 0.5 mM in the presence of SNP or SNAP. A third group of experiments consisted of pre-exposure of cells for 16 h to 2.5 mM N-acetylcysteine, subsequently followed by 6 h incubation with 1 mM SNP or 0.5 mM SNAP. To study the possible interaction between superoxide anion (O$_2^-$) and NO in the modulation of heme oxygenase activity, endothelial cells were exposed for 6 h to the NO releasing agent SIN-1, which is known to generate stoichiometric amounts of NO and O$_2^-$ leading to the formation of the potent oxidant peroxynitrite (ONOO$^-$) (28). The direct involvement of ONOO$^-$ (150 and 300 μM) was also investigated, and the contribution of O$_2^-$ in the induction of heme oxygenase was examined in cell cultures to which the various NO donors (SNP, SNAP, or SIN-1) were added in the presence of SOD (50 units/ml).

**Assay for Endothelial Heme Oxygenase Activity**—Heme oxygenase activity assay was performed as described previously by Motterlini et al. (29). Briefly, micromoles from harvested cells were added to a reaction mixture containing NADPH (0.8 mM), glucose 6-phosphate (2 mM), glucose 6-phosphate dehydrogenase (0.2 units/ml), NADP, and the cofactors. The mixture was prepared from a 105,000 × g supernatant fraction as a source of bilirubin reductase, potassium phosphate buffer (PBS, 100 mM, pH 7.4), MgCl$_2$ (0.2 mM), and hemin (20 μM). The reaction was conducted at 37 °C in the dark for 1 h and terminated by the addition of 1 ml of chloroform, and the extracted bilirubin was calculated by the difference in absorbance between 464 and 530 nm (ε = 40 m$^{-1}$ cm$^{-1}$). Heme oxygenase activity was expressed as picomoles of bilirubin/mg of cell protein/h. The total protein content of confluent cells was determined using a Bio-Rad DC protein assay (Bio-Rad, Herts, UK) by comparison with a standard curve obtained with bovine serum albumin.

**Western Blot Analysis for Heme Oxygenase-1 (HO-1)**—Samples of endothelial cells treated for the heme oxygenase activity assay were also analyzed by Western immunoblot technique. Cells were lysed in cold phosphate-buffered saline containing 1% Triton X-100. Total protein content was determined as reported above, and an equal protein concentration (30 μg/well) from each sample was then boiled for 5 min in Laemmli buffer (30). Protein separation was carried out by SDS-polyacrylamide gel electrophoresis using a 12% acrylamide resolving gel (M. Protein II System, Bio-Rad, Herts, UK). Separated proteins were then transferred overnight to a nitrocellulose membrane, and the nonspecific binding of antibodies was blocked with 3% non-fat dried milk in PBS, pH 7.4, for 2 h at room temperature. Membranes were then probed with a polyclonal rabbit anti-HO-1 antibody (Stressgen, Victoria, Canada) (1:1000 dilution in Tris-buffered saline, pH 7.4) for 2 h at room temperature. After three washes with PBS containing 0.05% (v/v) Tween 20, blots were visualized using an amplified alkaline phosphatase kit from Sigma (Extra-3A) and the relative density of bands analyzed by an imaging densitometer (model GS-700, Bio-Rad, Herts, UK).

**Determination of Endothelial Cell Glutathione Content**—The 5,5'-dithiobis(2-nitrobenzoic acid) colorimetric assay was used for the measurement of glutathione, the only detectable thiol in endothelial cells. To assess the sensitivity of the method in our experimental conditions, cells were incubated for 6 h with BSO (0.5 and 1 mM), a selective inhibitor of glutathione biosynthesis, or with increasing concentrations of N-acetylcycteine (0.25, 1, and 2.5 mM). In a different set of experiments, cells were incubated for 6 h in the presence of different NO donors with or without N-acetylcycteine. Glutathione levels were also measured after a 25 μM-mixture of cells N-acetylcysteine. At the end of the incubation period, cells were washed with PBS, and 600 μl of a 2% (w/v) solution of 5-sulfosalicylic acid was added for cell lysis and deproteinization. The samples were centrifuged for 5 min at 10,000 × g, and 500-μl aliquots were reacted with 500 μl of 5,5'-dithiobis(2-nitrobenzoic acid) solution (0.3 M sodium phosphate buffer, 10 mM EDTA, and 0.2 mM 5,5'-dithiobis(2-nitrobenzoic acid), freshly prepared), and after 5 min the absorbance was read at 412 nm (extinction coefficient was 14.3 m$^{-1}$ cm$^{-1}$) (31).

**Nitrite Production Assay**—Nitrite levels were measured in the culture medium after 2, 4, and 6 h incubation of confluent endothelial cells with various concentrations of NO donors in the presence or absence of N-acetylcycteine. Where indicated, 100 μl of the culture medium were reacted with an equal volume of Griess reagent (0.5% sulfanilamide, 0.05% N-1-naphthylethylenediamine dihydrochloride in 2.5% H$_3$PO$_4$ in 96-well plates at room temperature for 10 min with shaking. The resulting azode product was spectrophotometrically quantitated at 550 nm using a Dynatech MR 7000 microplate reader, and nitrite levels were determined as described previously by comparison with standard curves made from a solution of sodium nitrite (32).

**S-Nitrosothiol Determination**—The Saville method was used for the measurement of S-nitrosothiols (33). Confluent cells were incubated for 0, 0.5, 1, 2, and 4 h with SNAP (1 mM) plus N-acetylcycteine (1 mM) or SNAP (0.5 mM) plus N-acetylcycteine (2.5 mM). After incubation, 50 μl of the culture supernatant was reacted for 5 min with an equivalent volume of solution A (sulfanilamide 1% dissolved in 0.5 M HCl) or solution B (solution A plus 0.2% H$_2$O$_2$), allowing the development of the azodye product. The absorbance of the azodye was obtained reacting the two samples for an additional 5 min with an equal volume of solution C (0.02% of N-1-naphthylethylenediamine dihydrochloride dissolved in 0.5 M HCl), and the absorbance was subsequently read at 550 nm with a Dynatech MR 700 microplate reader. To counteract the high nitrite concentration in the background, an equal volume of 0.5% ammonium sulfamate in water was added to the samples 5 min before the
RESULTS

Influence of N-Acetylcysteine, Hydroxocobalamin, and SOD on HO-1 Induction by SNP and SNAP—N-Acetylcysteine, a well-known glutathione precursor with antioxidant properties, significantly reduced the activation of heme oxygenase by SNP in a dose-dependent manner (Fig. 1A). These results paralleled those obtained with Western blot analysis, showing that the inducible heme oxygenase (HO-1) is the enzymatic form affected by the presence of SNP (Fig. 1B). Similar effects were observed with another NO donor, SNAP. Although higher concentrations of N-acetylcysteine (2.5 and 5 mM) were needed to significantly attenuate HO-1 induction by SNP, the effect was still dose-dependent (Fig. 2). To examine whether an elevated intracellular glutathione concentration could also influence HO-1 induction caused by SNP and SNAP, endothelial cells were pretreated with 2.5 mM N-acetylcysteine for 16 h prior incubation with NO donors. Treatment with N-acetylcysteine produced an increase in intracellular glutathione levels of approximately 100% above the control values, and under this condition, we observed a marked attenuation in heme oxygenase activity mediated by SNP and SNAP (Fig. 3). These findings indicate a possible role of thiol-containing compounds in the modulation of HO-1 induction by NO donors. The interaction between NO released by SNAP or SNAP and O2 produced intracellularly was also considered as a possible mechanism for the increased heme oxygenase activity. As shown in Fig. 3, incubation of cells in the presence of SOD for 6 h resulted in a partial reduction in heme oxygenase activation by SNP (38%) and SNAP (56%), respectively.

There is evidence that hydroxocobalamin could have NO binding properties, as does hemoglobin (34). Therefore, the use of hydroxocobalamin has been an important tool in our experiments to determine the direct involvement of NO in mediating HO-1 induction. As shown in Fig. 3, hydroxocobalamin (0.5 mM) produced a significant decrease in heme oxygenase activation by NO donors (p < 0.05). Although these data do not allow us to distinguish if NO per se or an intermediary molecule(s) is the signal for heme oxygenase induction, they do, however, indicate that NO is the initial element in the cascade of events that leads to up-regulation of the enzyme.

Effect of SIN-1 and Peroxynitrite (ONOO⁻) on Heme Oxygenase Activation—Because of its potential ability to generate ONOO⁻ via simultaneous release of NO and O₂, the NO releasing agent SIN-1 was considered separately from SNP and SNAP. As shown in Fig. 4, heme oxygenase activity significantly increased after 6 h exposure to SIN-1 (750 μM) from 214 ± 11 to 2306 ± 104 pmol of bilirubin/mg protein/h (p < 0.05). This effect was almost completely abolished by the presence of SOD in the culture medium (487 ± 18 pmol of bilirubin/mg protein/h). When ONOO⁻ was added directly to the culture medium, a dose-dependent increase in heme oxygenase activity was also observed. The activity increased from 214 ± 11 to 641 ± 17 and 1019 ± 48 pmol of bilirubin/mg protein/h in the presence of 150 and 300 μM ONOO⁻, respectively.

Effect of N-Acetylcysteine on Nitrite Production from NO Donors—Because N-acetylcysteine contains sulphydryl groups that can react with NO, its effect on nitrite production by NO donors was investigated. Pretreatment with N-acetylcysteine significantly attenuated nitrite production in response to SNAP or SNAP and SNAP (Fig. 5). These findings indicate a possible role of thiol-containing compounds in the modulation of NO-induced nitrite production.
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**Fig. 3.** Effect of hydroxocobalamin, preincubation of N-acetylcysteine, and superoxide (O2•−) on endothelial heme oxygenase activity by SNP and SNAP. Heme oxygenase activity was measured after 6 h exposure of cells to SNP (1 mM) and SNAP (0.5 mM) (solid bars); SNP (1 mM) or SNAP (0.5 mM) + 0.5 mM hydroxocobalamin (hatched bars); SNP (1 mM) or SNAP (0.5 mM) following 16 h preincubation with 2.5 mM N-acetylcysteine (wide-hatched bars); SNP (1 mM) or SNAP (0.5 mM) + 50 units/ml superoxide dismutase (shaded bars). Control experiments (CONT) were performed by exposing cells to complete medium alone (open bars). Each bar represents the mean (± S.E.) of 5–6 experiments performed independently. NAC, N-acetylcysteine; HCB, hydroxocobalamin; SOD, superoxide dismutase. *, p < 0.05 compared with control; †, p < 0.05 compared with SNP or SNAP.

Endothelial cells were exposed for 6 h to SIN-1 (750 μM) alone or in the presence of superoxide dismutase (SOD, 50 units/ml). Peroxynitrite, concentration was measured spectrophotometrically at 302 nm before use, was added directly to the culture medium at a final concentration of 150 and 300 μM, respectively. Control experiments (CONT) were represented by cells incubated for 6 h with complete medium alone. Each bar represents the mean (± S.E.) of 5–6 experiments performed independently. *p < 0.05 versus control; †, p < 0.05 versus SIN-1 alone.

**Fig. 4.** Effect of SIN-1 and ONOO− on heme oxygenase activity in aortic endothelial cells. Endothelial cells were exposed for 6 h to SIN-1 (750 μM) alone or in the presence of superoxide dismutase (SOD, 50 units/ml). Peroxynitrite, concentration was measured spectrophotometrically at 302 nm before use, was added directly to the culture medium at a final concentration of 150 and 300 μM, respectively. Control experiments (CONT) were represented by cells incubated for 6 h with complete medium alone. Each bar represents the mean (± S.E.) of 5–6 experiments performed independently. *p < 0.05 versus control; †, p < 0.05 versus SIN-1 alone.

**Fig. 5.** Effect of N-acetylcysteine on nitrite production by SNP and SNAP. Nitrite levels were measured in the culture medium after 2 (solid bars), 4 (hatched bars), and 6 h (cross-hatched bars) exposure of cells to SNP (1 mM); SNP (1 mM) + N-acetylcysteine (0.25 mM); SNP (1 mM) + N-acetylcysteine (1 mM) (A); SNP (0.5 mM); SNAP (0.5 mM) + N-acetylcysteine (0.25 mM); SNAP (0.5 mM) + N-acetylcysteine (1 mM); SNAP (0.5 mM) + N-acetylcysteine (2.5 mM) (B). Base-line values (BAS) were measured in culture medium of cells exposed to complete medium alone. All values represent the mean (± S.E.) of five experiments performed independently. NAC, N-acetylcysteine.

donors was examined. If N-acetylcysteine stabilizes NO released by NO donors, then a lower nitrite production would be expected in the culture medium supplemented with the sulphydryl agent. In contrast, in the present study a substantial enhancement in nitrite production was observed when N-acetylcysteine was added to the culture medium containing SNP or SNAP (Fig. 5, A and B). This enhancement was dose-dependent with SNP and increasing concentrations of N-acetylcysteine (0.25 and 1 mM); however, in the case of SNAP the significant (p < 0.05) increase in nitrite levels was the same with all the concentrations of N-acetylcysteine used (0.25, 1, and 2.5 mM). Furthermore, at the same concentration of N-acetylcysteine, nitrite accumulation by NO donors remained unchanged at the time points considered in our experimental protocol. These results suggest that the apparent reaction between NO donors and N-acetylcysteine occurs in the early period of co-incubation.

**Formation of S-Nitrosothiols in the Presence of SNP (or SNAP) and N-Acetylcysteine—S-Nitrosothiols (or thionitrites) are one of the products of the reaction between NO and thiol groups. It was therefore deemed necessary to establish whether the conditions of our experiments were favorable for the formation of S-nitrosothiols. The supernatant of cells incubated with SNP (1 mM) plus N-acetylcysteine (1 mM) showed S-nitrosothiol formation only at time 0; at all following time points there was no detection of S-nitrosothiols according to the Saville method (see Table 1). These results suggest that SNP and N-acetylcysteine can react to produce thionitrites; however, the presence of metal traces in the culture medium, the aerobic experimental conditions, and the high concentration of thiols used during the incubation cannot account for the inability to detect S-nitrosothiols after time 0, since the above factors all strongly accelerate thionitrite decomposition. The supernatant of cells incubated with SNAP (0.5 mM) plus N-acetylcysteine (2.5 mM) showed very high amounts of S-nitrosothiols at time 0 and little at the following time points considered. These data...
required a different interpretation since SNAP is an \( \text{S-nitrosothiol} \). At time 0 we likely measured SNAP and the thionitrite produced by the reaction between the NO donor and N-acyethylcysteine. The pronounced decay observed at subsequent times indicated that the half-life of both SNAP and the thionitrite is very short, possibly because of the same factors influencing \( \text{S-nitrosothiol} \) decomposition when SNP and N-acyethylcysteine were used. Moreover, a transnitrosation reaction occurring between SNAP and N-acyethylcysteine could also explain the low amount of thionitrates observed after time 0. In these groups of experiments the concentrations chosen for N-acyethylcysteine were the ones that exhibited a marked reduction in heme oxygenase activity.

**Effect of SNP and SNAP on Intracellular Glutathione Accumulation by N-Acetylcysteine**—The method used for glutathione determination was very sensitive since, as expected, exposure of cells to BSO or N-acyethylcysteine resulted in a dose-dependent depletion or accumulation of detectable soluble thiols, respectively (Fig. 6). The thiol content did not change to any extent when cells were incubated with SNP or SNAP, but interestingly enough, the two compounds were able to lower remarkably glutathione accumulation observed with N-acyethylcysteine alone. A 25% decrease in intracellular thiol levels was measured with N-acyethylcysteine alone (1 mM) plus SNP (1 mM) in comparison with N-acyethylcysteine alone, whereas in the presence of N-acyethylcysteine (2.5 mM) plus SNAP (0.5 mM) the content diminished approximately 35%.

**Effect of Various NO Donors on Endothelial Heme Oxygenase Activity**—In a recent paper we have demonstrated that NO donors increase heme oxygenase activity in porcine aortic endothelial cells (8). The following experiments were carried out to examine how the stability of different NO donors and their capability in generating NO could affect the activation of heme oxygenase. SNAP and GSNO, two compounds belonging to the same class of NO donors (thionitrites or \( \text{S-nitrosothiols} \)), and SNAP, an iron nitrosyl substance, were tested at the concentration of 0.5 mM. Although SNAP and GSNO are both thionitrites, we observed that SNAP was more effective in inducing heme oxygenase activity compared with GSNO (1644 ± 74 and 862 ± 35 pmol of bilirubin/mg of protein/h, respectively); in addition, SNAP produced a slightly lower heme oxygenase activation compared with GSNO (769 ± 24 and 862 ± 35 pmol of bilirubin/mg of protein/h, respectively). These results suggest that heme oxygenase induction is dependent upon the rate of decomposition of the NO releasing agent and consequently on the amount of NO delivered into the medium. In fact, the data correlate with published observations showing that GSNO is more stable than SNAP and that SNP releases less NO when compared with SNAP or GSNO (35, 36).

**DISCUSSION**

Previous reports demonstrated a clear link between glutathione levels and the enzyme heme oxygenase. Only the inducible form of heme oxygenase (HO-1), but not the constitutive (HO-2), appears to be affected by the intracellular pool of low molecular weight thiols. Studies in vitro have shown that oxidant factors, such as UVA and \( \text{H}_2\text{O}_2 \), lower the endogenous glutathione levels, and this effect is concomitant with the induction of HO-1 (37, 38). Furthermore, in the absence of additional stimulation, depletion of intracellular glutathione by pharmacological means (BSO) is alone sufficient to regulate the expression of the enzyme (26). Low doses of endotoxin produced an increase in hepatic heme oxygenase in vivo and, under conditions of decreased glutathione levels, an enhancement of HO-1 mRNA accumulation mediated by endotoxin was observed (4). Increased intracellular reactive species, derived either by cellular metabolism and/or oxidative stress-inducing treatments, could account for the activation of the stress protein HO-1 when glutathione is depleted (37). However, the possibility that glutathione may also be involved in signal transduction mechanisms that mediate the tissue stress response cannot be excluded.

The essential observation described in the present work is that increased intra- and extracellular thiol concentrations modulate heme oxygenase induction by NO donors in cultured endothelial cells. We have previously shown that NO donors considerably increased heme oxygenase activity in cultured vascular endothelial cells resulting in a higher resistance to oxidant damage mediated by hydrogen peroxide (8). In this study we report that co-exposure of SNAP or SNP with various concentrations of N-acyethylcysteine, a precursor of glutathione with antioxidant properties, resulted in a dose-dependent decrease of HO-1 activity and expression. In addition, augmented intracellular glutathione levels by preincubation with N-acyethylcysteine significantly (\( p < 0.05 \)) attenuated endothelial

**TABLE I**

| TABLE I | Formation of S-nitrosothiols in the presence of SNAP or SNAP and N-acetylcysteine |
|---------|-----------------------------------------------------------------------------|
|         | 0 min | 30 min | 60 min | 120 min | 240 min |
| SNAP (0.5 mM) + NAC\textsuperscript{a} (2.5 mM) | 351 ± 37 | 16.9 ± 2.0 | 33.3 ± 5.7 | 71.6 ± 6.0 | 18.1 ± 0.6 |
| SNP (1 mM) + NAC\textsuperscript{b} (1 mM) | 9.7 ± 2.7 | ND\textsuperscript{c} | ND\textsuperscript{c} | ND\textsuperscript{c} | ND\textsuperscript{c} |

\textsuperscript{a} S-Nitrosothiol determination was carried out in the culture medium of treated cells at the times indicated. All values are the mean (±S.E.) of five experiments performed independently.

\textsuperscript{b} NAC, N-acetylcysteine.

\textsuperscript{c} Not detectable.
heme oxygenase activity by both NO donors. These findings suggest that thiols may have crucial buffering capacities in respect to NO, thereby influencing the ability of this gaseous molecule to affect HO-1 induction.

We also observed a higher nitrite production with SNP and SNAP in the presence of N-acetylcysteine compared with that measured when cells were exposed to NO donors alone. These data are in agreement with other published observations showing that thiols enhance NO generation from NO releasing agents (39, 40). Based on our present findings demonstrating that NO donors stimulate heme oxygenase activity, we expected an even greater increase in the expression of this protein when NO released from SNP or SNAP is enhanced by the presence of thiols; however, that assumption is not borne out by the present evidence that heme oxygenase induction by NO donors is dose-dependently inhibited by N-acetylcysteine. One explanation for this apparent controversy could be that relatively low concentrations of NO stimulate the endothelial stress response until a threshold is reached, above which the amount of NO delivered to the tissue is excessive and its signaling effect is abolished by its potential cytotoxic action. Alternatively, a question arises on the significance of the nitrile assay as to whether this parameter has to be taken merely in vivo.

The results of this study showing that SNP and SNAP did not produce any change in the intracellular thiol content support the evidence that NO and/or its reaction products modulate endothelial heme oxygenase for their intrinsic properties as signaling molecules and not as a consequence of glutathione depletion caused by oxidative stress. In agreement with this concept, we have previously reported that at the concentrations of SNP, SNAP, and SIN-1 used in our protocol (0.5–1 mM) no relevant cytotoxic effects to endothelial cells were detected (8). In addition, we observed that in vivo administration of a precursor of the NO releasing agent SIN-1 caused a rapid increase in hepatic HO-1 mRNA expression and activity, and this effect was not associated with overt damage to liver tissue (32). Other authors have reported that the cytotoxic activity of SIN-1, which mediates the formation of ONOO·, occurred in human epithelial cancer cells at high concentrations (5 mM) and only 48 h after incubation (47).

We wanted to explore further what effect the specific blockade of NO release by NO donors could have on endothelial heme oxygenase. Hemoglobin, a conventional and widely utilized NO scavenger, was not appropriate for this type of study since, per se, it induces HO-1 (5, 8). EPR spectroscopy studies have laterly revealed the capacity of cobalamin species to reversibly bind NO (34), and the same compounds have been shown to inhibit relaxation of mouse anococcygeus muscle produced by exogenous NO (48). Based on these pieces of evidence,
we incubated cells with SNP or SNAP in medium supplemented with hydroxocobalamin; this treatment, indeed, resulted in a reduction of heme oxygenase activity, reinforcing the hypothesis that NO is required for the activation of HO-1. In addition, we observed that SNAP was more potent at inducing heme oxygenase than equivalent concentrations of GSNO and SNP; these findings are consistent with previous reports showing that SNAP decomposes more rapidly than GSNO (36) and releases more NO than SNP (35).

In summary, we have shown that (a) thiols, both exogenous and endogenous, are capable of modulating endothelial heme oxygenase activation by NO donors; (b) this modulation is possibly attributable to NO interaction and stabilization by thiols; (c) free NO released by NO donors and $\text{O}_2^-$ are essential messengers for the stimulation of HO-1 expression; and (d) ONOO$^-$, which is generated from the interaction of $\text{O}_2^-$ and NO, increases heme oxygenase activity in vascular endothelial cells.

We propose here a mechanism for a dynamic equilibrium between NO, glutathione, and $\text{O}_2^-$ in their ability to regulate the expression of the stress protein heme oxygenase. Under physiological conditions a balance exists between NO produced, its intracellular association with glutathione, and its target proteins and enzymes. Stress situations, such as oxidative stress and endotoxic shock, may alter this balance leading to glutathione depletion, enhanced $\text{O}_2^-$ formation, and up-regulation of the inducible form of NO synthase. This effect generates NO and NO derivatives which may act as intracellular signals to mediate the expression of heme oxygenase. The induction of this protein would increase endogenous CO, biliverdin, and bilirubin, all catabolites ultimately implicated in protective mechanisms against injury (49). Interestingly, a recent study demonstrated the involvement of NO in the synthesis of heat-induced HSP70 in rat organs, suggesting that the system of NO generation plays an important role in both stress and adaptive responses of the organism (50). Although a full understanding of NO regulation of oxygen radical-dependent reactions is required to unravel the exact mechanism underlying heme oxygenase induction, the data reported herein indicate that glutathione may be regarded as a fine sensor of the NO-mediated stress response in vascular endothelial cells.

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