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Endothelial Heme Oxygenase-1 Induction by Hypoxia

MODULATION BY INDUCIBLE NITRIC-OXIDE SYNTHASE AND *S*-NITROSOTHIOLS*

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The stress protein heme oxygenase-1 (HO-1) is induced in endothelial cells exposed to nitric oxide (NO)-releasing agents, and this process is finely modulated by thiols (Foresti, R., Clark, J. E., Green, C. J., and Motterlini R. (1997) *J. Biol. Chem.* 272, 18411–18417). Here, we report that up-regulation of HO-1 in aortic endothelial cells by severe hypoxic conditions ($pO_2 \leq 2$ mm Hg) is preceded by increased inducible NO synthase and NO synthase activity. This effect is accompanied by oxidation of intracellular glutathione and formation of *S*-nitrosothiols. Incubation of cells with a selective inhibitor of inducible NO synthase (*S*-(2-aminoethyl)-isothiourea) or a NO scavenger ([2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide]) significantly attenuated the increase in heme oxygenase activity caused by reduced oxygen availability. A series of antioxidant agents did not prevent the elevation in heme oxygenase activity by hypoxia; however, the precursor of glutathione synthesis and thiol donor, *N*-acetylcysteine, completely abolished HO-1 induction. We also found that the hypoxia-mediated increase in endothelial heme oxygenase activity was potentiated by the presence of *S*-nitrosoglutathione. These results indicate that intracellular interaction of thiols with NO is an important determinant in the mechanism leading to HO-1 induction by reduced oxygen levels. We suggest that in addition to oxidative stress, HO-1 gene expression can be regulated by redox reactions involving NO and *S*-nitrosothiols (nitrosative stress), emphasizing a versatile role for the heme oxygenase pathway in the cellular adaptation to a variety of stressful conditions.

Tissue hypoxia is a condition of reduced oxygen levels which characterizes several pathophysiological states including ischemia, atherosclerosis, and cancer. Mammalian organisms have evolved a series of stratagems to counteract the negative effect of intracellular oxygen deficiency and favoring the adaptation of tissues to low oxygen tension. For example, there exist sensitive genes that, in the absence of oxygen, are readily stimulated to encode for modulators of erythropoiesis (erythropoietin), promoters of angiogenesis (vascular endothelial growth factor), and proteins involved in alternative metabolic pathways for ATP generation (glycolytic enzymes) (1). Thus, the ultimate purpose of these inducible systems is to increase the oxygen-carrying capacity of the blood and improve oxygen delivery to hypoxic tissues. The expression of hypoxic-sensitive genes appears to require redox modification and phosphorylation of specific transcription factors such as hypoxia inducible factor-1 (HIF-1)[1] (1). In addition to the above mentioned systems, low oxygen or anoxic conditions affect the protein expression of nitric-oxide synthase (NOS) and heme oxygenase, two enzymatic pathways responsible for the synthesis of signaling molecules that regulate important biological activities (2).

Nitric oxide (NO), generated from the oxidation of L-arginine by NOS enzymes, is a multifunctional interactive molecule involved in the modulation of vascular tone, inhibition of platelet aggregation, and oxygen transport to tissues (3–5). Under most physiological conditions, NO derives mainly from constitutively expressed endothelial NOS (ecNOS); however, the inducible isoform (iNOS) can generate substantial amounts of NO once appropriately stimulated by cytokines or other inflammation-mediated stimuli. Hypoxia has also been shown to affect differentially the expression and activity of the diverse NOS isoforms. Exposure of human umbilical vein endothelial cells to hypoxia results in decreased transcription of the ecNOS protein as well as reduced mRNA stability (6, 7). Similarly, bovine pulmonary artery endothelial cells exposed to low oxygen levels show a marked repression in the ecNOS transcript and decreased NOS activity (8). In contrast, the iNOS gene appears to be up-regulated by conditions of low oxygen availability. Indeed, a functional hypoxia-responsive element has been detected in the promoter region of iNOS in murine macrophages (9), and induction of the iNOS transcript in vascular tissue was found using a chronic hypoxia model of pulmonary hypertension (10).

Heme oxygenase is the rate-limiting step in heme degradation; it catalyzes the oxidation of the *α-meso* carbon of the protoporphyrin ring leading to the formation of carbon monoxide (CO), free iron, and biliverdin (11). The inducible isoform of heme oxygenase, HO-1, is a ubiquitous heat shock protein (HSP32) that is highly induced by diverse stress-related conditions (2, 12). The end products of heme oxygenase may have

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[1] The abbreviations used are: HIF-1, hypoxia inducible factor-1; NO, nitric oxide; NOS, nitric-oxide synthase; iNOS, inducible nitric-oxide synthase; ecNOS, endothelial constitutive nitric-oxide synthase; CO, carbon monoxide; HO-1, heme oxygenase-1; CPTIO, ([2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide]); MnTBAP, manganese (III) tetrakis(4-benzoic acid) porphyrin; GSNO, *S*-nitrosoglutathione; ODQ, [1H-[1,2,4]Oxadiazole[4,3-a]quinoxalin-1-one]; SnPPIX, tin protoporphyrin IX; L-NAME, N^G-nitro-L-arginine methyl ester; ITU, *S*-(2-aminoethyl)isothiourea; L-NIL, [L-N^6-(1-iminoethyl)-lysine dihydrochloride]; RSNO, *S*-nitrosothiols; PBS, phosphate-buffered saline; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid).

crucial biological functions in the cardiovascular system. Bilirubin, which is formed from biliverdin by biliverdin reductase, is a potent antioxidant (13) and has been shown recently to ameliorate postischemic myocardial dysfunction in a model of isolated heart (14). Moreover, enhanced CO production following HO-1 induction in vascular tissue effectively contributes to the suppression of both aortic vasoconstriction *in vitro* (15) and acute hypertensive responses *in vivo* (16). Reports have also demonstrated that the HO-1/CO pathway is markedly up-regulated by hypoxia in vascular smooth muscle cells, cardiomyocytes, and heart tissue (17–19). It has been suggested that aortic vasoconstriction following chronic hypoxia in rats involves the induction of endothelial HO-1 and the enhanced production of CO (20). In view of the vasoregulatory effects of both NO and CO, it appears that the heme oxygenase and NOS pathways could actively participate in the maintenance of local tissue oxygenation; however, how these two systems interact mutually in response to limited availability of oxygen has not been examined previously.

In previous studies, we demonstrated that various NO-releasing compounds induce endothelial HO-1 protein expression and heme oxygenase activity and that thiols are important regulators of this effect (21, 22). In the present study we examined the temporal pattern of ecNOS/iNOS and HO-1 expression in aortic endothelial cells exposed to hypoxia. Having established that iNOS expression precedes the up-regulation of HO-1 protein, we investigated a possible involvement of endogenously generated NO, glutathione, and *S*-nitrosothiols in modulating hypoxia-mediated HO-1 induction.

EXPERIMENTAL PROCEDURES

Reagents—Inhibitors of ecNOS and iNOS, carboxy-PTIO (CPTIO), manganese (III) tetrakis(4-benzoic acid) porphyrin (MnTBAP), *S*-nitrosoglutathione (GSNO), and [1H-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one] (ODQ) were obtained from Alexis Corporation (Bingham, Nottingham, U. K.). Tin protoporphyrin IX (SnPPIX) was from Porphyrin Products Inc. (Logan, UT). *N*-Acetylcysteine, uric acid, 1,3-dimethyl-2-thiourea, and all other chemicals were obtained from Sigma unless otherwise specified.

Cell Culture and Hypoxia—Bovine aortic endothelial cells were purchased from the European Collection of Animal Cell Culture (ECACC, Salisbury, U. K.), 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, 100 units/ml penicillin, and 0.1 mg/ml streptomycin. Endothelial cells at confluence were transferred to an air-tight chamber (Billups-Rothenberg Inc., Del Mar, CA) and flushed with a mixture of 95% N_2 and 5% CO_2. The gas was infused continuously into the air-tight chamber at a flow rate of 5 liters/min for the first 2 h and at 1 liter/min for the following hours of incubation. In preliminary experiments conducted under these conditions, it was found that the pO_2 measured in the media by an oxygen electrode was 2 mm Hg after a 2-h exposure to hypoxia and did not fluctuate from this value throughout the remaining incubation period. Within the hypoxia chamber, cells were maintained in a humidified atmosphere at 37 °C.

Experimental Protocol—A time course (0–24 h) of heme oxygenase activity, HO-1, cNOS, and iNOS protein expression was determined in endothelial cells exposed to hypoxia. At specific time points, NOS activity was also measured. To examine the involvement of endogenously produced NO on heme oxygenase activation, cells were exposed to hypoxia (18 h) in the presence of ecNOS/iNOS inhibitors. N^G-nitro-L-arginine methyl ester (L-NAME, 1 mM) was used as the inhibitor of ecNOS, whereas *S*-(2-aminoethyl)isothiourea (ITU) and [L-N^6-(1-iminoethyl)-lysine dihydrochloride] (L-NIL) at concentrations of 20 and 40 μM were used as selective inhibitors of iNOS. In an additional set of experiments, heme oxygenase activity was measured in endothelial cells 18 h after hypoxic treatment in the presence of 100 μM CPTIO, a NO scavenger. To assess a possible role of intracellular redox changes in the stimulation of the heme oxygenase pathway by hypoxia, total glutathione (GSH + GSSG) and glutathione disulfide (GSSG) were measured at the indicated time points. In addition, heme oxygenase activity was determined in endothelial cells exposed to low oxygen tension in the presence of the following compounds: *N*-acetylcysteine (1 mM), a precursor of glutathione synthesis and permeable reducing agent; uric acid (1 mM), a scavenger of peroxynitrite; 1,3-dimethyl-2-thiourea (1 mM), a specific scavenger of hydroxyl radical; MnTBAP, a cell-permeable superoxide dismutase mimetic and peroxynitrite decomposition catalyst; and the antioxidant enzymes superoxide dismutase and catalase. *S*-Nitrosothiols (RSNO) were also measured during hypoxia at specific time points. To examine the possible role of *S*-nitrosation in the modulation of heme oxygenase activity, cells were exposed to hypoxia for 6 h in the presence of 0.5 mM GSNO and compared with cells treated with the compound in normoxic conditions. In additional experiments, cells were exposed to hypoxia in the presence of SnPPIX (50 μM), an inhibitor of heme oxygenase activity, or ODQ (100 nM–10 μM), a potent inhibitor of guanylate cyclase activity.

Assay for Endothelial Heme Oxygenase Activity—Heme oxygenase activity assay was performed as described previously (23). Briefly, microsomes from harvested cells were added to a reaction mixture containing NADPH, rat liver cytosol as a source of biliverdin reductase, and the substrate hemin. The reaction was conducted at 37 °C in the dark for 1 h, 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 mM^{-1} cm^{-1}).

Western Blot Analysis for HO-1, cNOS, and iNOS—Samples of endothelial cells treated for the heme oxygenase activity assay were also analyzed by Western immunoblot technique as described previously (21). Briefly, an equal amount of proteins (30 μg) for each sample was separated by SDS-polyacrylamide gel electrophoresis, transferred overnight to nitrocellulose membranes, and the nonspecific binding of antibodies was blocked with 3% non-fat dried milk in PBS. Membranes were then probed with a polyclonal rabbit anti-HO-1 antibody (Stressgen, Victoria, Canada) (1:1,000 dilution in Tris-buffered saline, pH 7.4) for 2 h at room temperature. When probed for NOS proteins, membranes were incubated for 2 h with anti-ecNOS and anti-iNOS antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:20,000 dilution in Tris-buffered saline (pH 7.4). 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).

Immunocytochemistry for HO-1—Endothelial cells were seeded onto Lab-Tek® chamber slides (Nunc Inc., Naperville, IL) at a density of 10^6 cells/ml. They were grown to confluence and exposed to hypoxia for 18 h in the presence of various agents. The medium was then discarded, and the upper part of the slide well frame was removed. Slides were washed twice very gently with cold (4 °C) PBS for 5 min. Cells were then fixed in cold 95% v/v ethanol for 10 min and gently washed again in cold PBS for 5 min. Slides were immersed in cold 3% v/v H_2O_2 in PBS for 5 min followed by 5% v/v normal goat serum (Vector Laboratories Ltd., Peterborough, Cambridgeshire, U. K.) in PBS for 20 min at room temperature. Next, slides were covered with rabbit anti-HO-1 antibody (1:1,000 dilution in PBS) and left for 18 h at 4 °C. Slides were then covered with a 1:100 dilution in PBS of biotinylated goat anti-rabbit IgG antibody (Vector Laboratories Ltd.) and left for 60 min at room temperature on an orbital shaker. After incubation with the secondary antibody, slides were washed again with PBS at room temperature, and the tertiary avidin-biotin-antibody complex was applied (Vectastain® ABC Elite Kit, Vector Laboratories Ltd.) for 60 min at room temperature on an orbital shaker. Slides were washed in PBS for 5 min and finally incubated with 3,3′-diaminobenzidine (DAB Substrate Kit, Vector Laboratories Ltd.) until suitable staining developed (about 10 min) and analyzed by an imaging densitometer.

RNA Extraction and Northern Blot Analysis—Total RNA was isolated by phenol-chloroform using the method described by Chomczynski and Sacchi (24). Total RNA was run on a 1.3% denaturing agarose gel containing 2.2 M formaldehyde and transferred onto a nylon membrane. The membrane was hybridized using [α-^{32}P]dCTP-labeled cDNA probes to rat HO-1 gene as described previously (25, 26), and staining of the 18 S rRNA band was used to confirm integrity and equal loading of RNA. The hybridized membrane was exposed to radiographic film and bands analyzed using an imaging densitometer.

Determination of Reduced Glutathione (GSH) and Glutathione Disulfide (GSSG) in Endothelial Cells during Hypoxia—Total glutathione (GSH + GSSG) was measured in endothelial cells at various times of hypoxia using a modification of a method described previously (27). Cells were harvested in ice-cold PBS, centrifuged at 10,000 × *g* for 5 min, and the pellet was resuspended in 100 mM potassium phosphate buffer (pH 7.5), containing 5 mM EDTA (buffer 1). The cell suspension was freeze-thawed three times, and an aliquot (60 μl) was added to an equal volume of 100 mM potassium phosphate buffer (pH 7.5), containing 12 mM EDTA and 10 mM 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB). The sample was mixed by tilting and centrifuged at 12,000 ×

g for 2 min. The supernatant was added to a cuvette containing 0.5 unit of glutathione reductase in buffer 1, equilibrated for 1 min, and the reaction initiated by adding NADPH (220 nmol). The change in absorbance at 412 nm was recorded over a period of 5 min using a reference cuvette containing equal concentrations of NADPH, DTNB, and enzyme. To assay GSSG, an aliquot of the cell suspension (400 μl) was added to an equal volume of 100 mM potassium phosphate buffer (pH 6.8), containing 17.5 mM EDTA and 10 mM *N*-ethylmaleimide. The sample was mixed, centrifuged and the supernatant passed through a C_{18} Sep-Pak cartridge (Waters, Watford, U. K.) to remove the excess *N*-ethylmaleimide and washed with buffer 1. The sample was added to a cuvette containing DTNB and glutathione reductase and the assay performed as for the measurement of total GSH. The intracellular glutathione content, expressed as nmol/mg of protein, was determined by comparison with a standard curve obtained with GSH and GSSG solutions.

Determination of NOS Activity and Detection of S-Nitrosothiols—NOS activity was determined using the hemoglobin assay as described previously (28). The assay is based on the oxidation of oxyhemoglobin with NO and is performed spectrophotometrically by measuring the formation of methemoglobin under initial rate conditions. Briefly, cells were washed with PBS, gently scraped using a rubber policeman, and the cellular pellet separated by centrifugation. The pellet was resuspended in a solution containing 0.32 M sucrose, 10 mM Tris (pH 7.4), 0.5 mM phenylmethylsulfonyl fluoride, and homogenized. The supernatant obtained after centrifugation at 12,000 × *g* for 30 min was used for NOS activity measurements. The reaction mixture contained (in a final volume of 1 ml): 1 mM L-arginine, 1 mM $CaCl_2$, 0.1 mM NADPH, 12 μM tetrahydro-L-biopterin, 5 μM oxyhemoglobin, 4 μM FAD, 100 mM Hepes (pH 7.5), and 0.5 mg of protein sample. The formation of methemoglobin was monitored by the change in absorbance which occurred over time between 411 and 401 nm using a double beam spectrophotometer (Perkin-Elmer 559). Detection of *S*-nitrosothiols in cell extracts was performed after cleavage of the *S*-nitroso bond by measuring the NO released using the chemiluminescence reaction between NO and the luminol-hydrogen peroxide system (29, 30).

Statistical Analysis—Differences in the data among the groups were analyzed by using one-way analysis of variance combined with the Bonferroni test. Values were expressed as a mean ± S.E. and differences between groups were considered to be significant at $p < 0.05$.

RESULTS

Effect of Hypoxia on Heme Oxygenase Activity, HO-1 Gene, and Protein Expression in Bovine Aortic Endothelial Cells—Exposure of aortic endothelial cells to severe hypoxia ($pO_2 \leq 2$ mm Hg) resulted in a time-dependent increase of heme oxygenase activity and HO-1 protein expression (Figs. 1*A* and 2*B*). The increase was evident at 12 h, and both HO-1 protein and heme oxygenase activity continued to rise in the following hours of incubation. Northern blot analysis (Fig. 1*B*) indicated that up-regulation of the HO-1 gene by hypoxia occurs at the transcriptional level and precedes protein expression, as HO-1 mRNA was markedly visible at 6 h and continued to increase for the entire period of the hypoxic treatment.

Time Course of Endothelial iNOS and ecNOS Protein Expression during Hypoxia: Comparison with Hypoxia-mediated HO-1 Expression—A time-dependent increase in the expression of endothelial iNOS protein was also observed during hypoxia (Fig. 2*A*); this was accompanied by enhanced NOS activity (see Fig. 7). However, iNOS protein was significantly up-regulated at 6 and 12 h hypoxia and started to decrease at 18 and 24 h. It is important to emphasize that at 6 h hypoxia, iNOS expression was already markedly elevated, whereas no changes in heme oxygenase activity and HO-1 protein expression were yet to be detected (Figs. 1, 2*A*, and 2*B*). In contrast, endothelial ecNOS protein, which is highly expressed under control conditions (normoxia), was down-regulated by hypoxia in a time-dependent manner (Fig. 2*A*). These data reveal that stimulation of iNOS protein by hypoxia occurred very rapidly and preceded both the increased expression of HO-1 protein and heme oxygenase activity.

Effect of ecNOS/iNOS Inhibitors and the NO Scavenger, CPTIO, on Hypoxia-stimulated Increase in Heme Oxygenase Activity—Our group has demonstrated the ability of various NO-releasing agents to increase heme oxygenase activity and HO-1 protein expression in bovine aortic endothelial cells, aortic tissue, and other cell types (15, 21, 25, 31, 32). Because a transient increase in iNOS protein and NOS activity preceded the induction of heme oxygenase during hypoxia, we wanted to verify whether iNOS-derived NO is required for triggering the expression of HO-1. As shown in Fig. 3, exposure of endothelial cells to hypoxia in the presence of a selective inhibitor of iNOS (ITU) resulted in a concentration-dependent attenuation of heme oxygenase activity ($p < 0.05$). We could not use higher concentrations of ITU because in preliminary experiments we found that concentrations above 40 μM can directly affect heme oxygenase activity in an *in vitro* assay. L-NIL, another inhibitor of iNOS activity, had a less pronounced effect; however, the presence of the NO scavenger CPTIO (100 μM) during hypoxia decreased endothelial heme oxygenase activity from 2,231 ± 140 to 969 ± 60 pmol of bilirubin/mg of protein/h ($p < 0.05$, Fig. 3). In contrast, L-NAME (1 mM), an inhibitor of cNOS, had no effect on heme oxygenase activation by hypoxia (Fig. 3). These data suggest that augmented NO production following stimulation of iNOS contributes to increased HO-1 expression and heme oxygenase activity under conditions of reduced oxygen tension. This effect appears to be cGMP-independent because ODQ, a selective inhibitor of guanylate cyclase activity, did not prevent

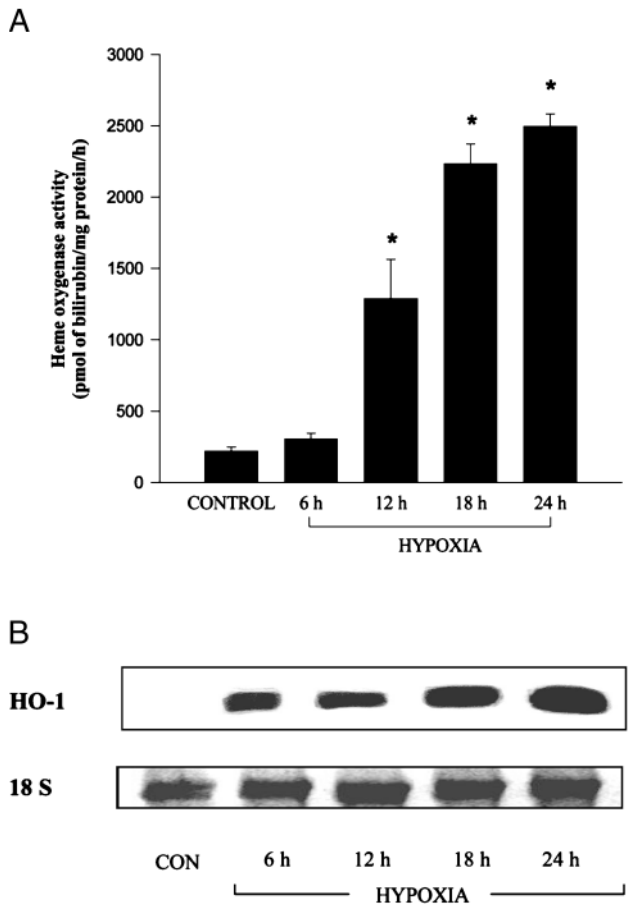

FIG. 1. **Effect of hypoxia on heme oxygenase activity and HO-1 mRNA expression in bovine aortic endothelial cells.** *Panel A*, heme oxygenase activity was measured at different time points after incubation of cells under severe hypoxic conditions (see "Experimental Procedures") and compared with untreated cells (*CONTROL*). Each *bar* represents the mean (± S.E.) of five or six experiments performed independently. * $p < 0.05$ *versus* control. *Panel B*, endothelial cells were exposed to hypoxia, and total RNA was extracted at the times indicated. HO-1 mRNA was analyzed by Northern blot; the 18 S band is shown to confirm integrity and equal loading of RNA. *CON*, control.

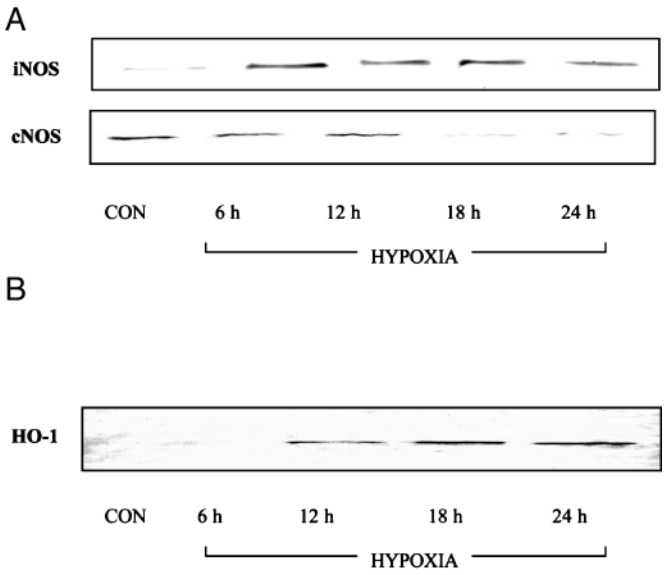

FIG. 2. **Time course of endothelial iNOS, ecNOS, and HO-1 protein expression during hypoxia.** Western blot analysis of cells exposed to hypoxia for different times using specific antibodies for iNOS and ecNOS (*panel A*) and HO-1 (*panel B*) is shown. Samples were processed as described under "Experimental Procedures." Each blot is representative of three independent experiments.

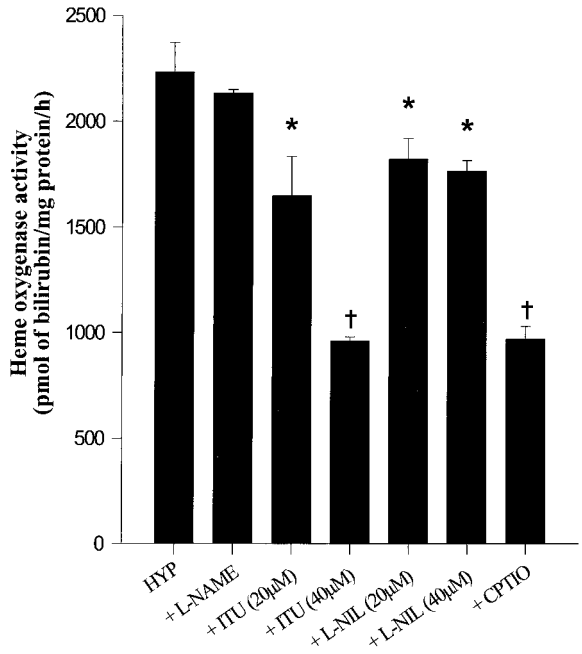

FIG. 3. **Effect of ecNOS/iNOS inhibitors and the NO scavenger carboxy-PTIO on hypoxia-stimulated increase in heme oxygenase activity.** Endothelial cells were exposed to hypoxia for 18 h in the presence of 1 mM L-NAME, 20 and 40 μM ITU, 20 and 40 μM L-NIL, or 100 μM CPTIO. Heme oxygenase activity was measured and compared with cells exposed to hypoxia for 18 h in the presence of medium alone (*HYP*). Each *bar* represents the mean (±S.E.) of five or six experiments performed independently. * $p < 0.05$ *versus* HYP; † $p < 0.01$ *versus* HYP.

hypoxia-mediated heme oxygenase activation (data not shown).

Effect of Hypoxia on Intracellular Glutathione Levels—Several lines of evidence suggest that glutathione is a crucial intracellular modulator of HO-1 gene expression both *in vitro* and *in vivo* (21, 33–36). Therefore, we examined glutathione levels after exposure of endothelial cells to low oxygen tension. Fig. 4 shows the changes in total glutathione (GSH + GSSG), oxidized glutathione (GSSG), and the ratio between reduced

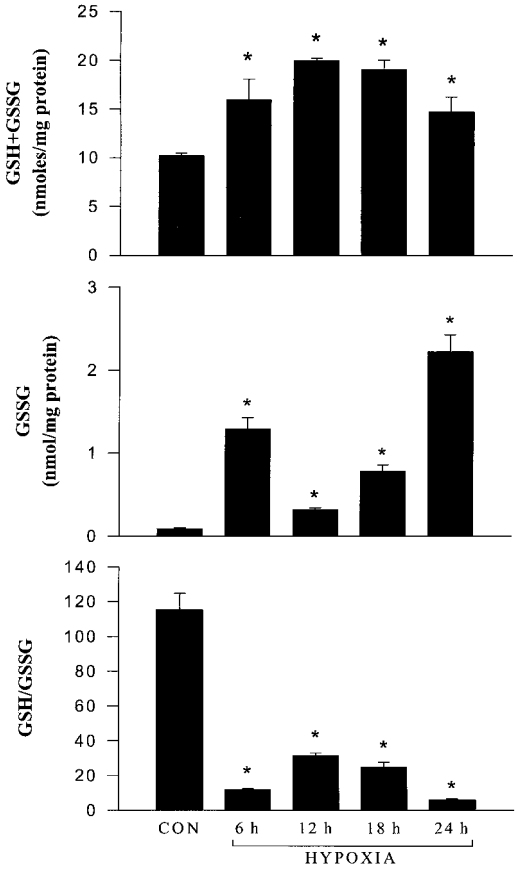

FIG. 4. **Effect of hypoxia on intracellular glutathione levels.** Total glutathione (GSH + GSSG) and oxidized glutathione (GSSG) were measured after exposure of endothelial cells to hypoxia for the times indicated. The ratio between reduced and oxidized glutathione (GSH:GSSG) is also shown. Each *bar* represents the mean (±S.E.) of five experiments performed independently. * $p < 0.05$ *versus* control (*CON*).

and oxidized glutathione (GSH:GSSG) in endothelial cells at various time points of hypoxia. Hypoxia caused a substantial change in GSSG content (Fig. 4, *center*) which was markedly increased at 6 h hypoxia (14-fold, $p < 0.05$ *versus* control), transiently diminished at 12 h, and significantly elevated after 24 h hypoxia (24.5-fold, $p < 0.05$). It can be observed that this effect was associated with a gradual and significant increase in total glutathione levels (Fig. 4, *top*) that were maximal at 12 h hypoxia (95%, $p < 0.05$ *versus* control), suggesting that reduced oxygen tension stimulates the enzymatic activities responsible for glutathione synthesis. However, at all time points examined, hypoxia caused a substantial change in the GSH:GSSG ratio, an index of the redox status of the cell. As shown in Fig. 4 (*bottom*), the GSH:GSSG ratio decreased significantly from 115 ± 10 (control) to 11.3 ± 0.9 after 6 h hypoxia ($p < 0.05$); although this index transiently increased at 12 h hypoxia, a further decrease of this parameter was observed at 24 h hypoxia (5.8 ± 0.8, $p < 0.05$). The fact that the transient recovery of the GSH:GSSG ratio at 12 h hypoxia correlated with a rise in total glutathione is indicative of the inherent ability of cells to produce new glutathione in response to augmented GSSG. Taken together, these data reveal that exposure of cells to severe and prolonged hypoxic conditions results in the rapid formation of GSSG, leading to a marked alteration in the redox status of the intracellular milieu.

Effect of N-Acetylcysteine and Various Antioxidants on Endothelial HO-1 Protein Expression and Heme Oxygenase Activity during Hypoxia—Because thiol residues are important tar-

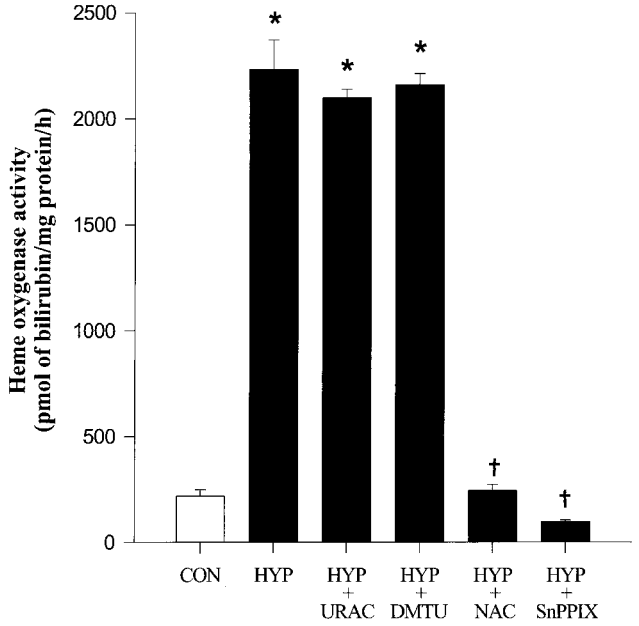

FIG. 5. **Effect of *N*-acetylcysteine and various antioxidants on endothelial HO-1 protein expression and heme oxygenase activity during hypoxia.** Endothelial cells were exposed to hypoxia for 18 h in the presence of *N*-acetylcysteine (*NAC*, 1 mM), uric acid (*URAC*, 1 mM), 1,3-dimethyl-2-thiourea (*DMTU*, 1 mM), or SnPPIX (50 μM), and heme oxygenase activity was determined as reported under "Experimental Procedures." Each *bar* represents the mean (±S.E.) of five experiments performed independently. * $p < 0.05$ *versus* control (*CON*); † $p < 0.01$ *versus* hypoxia alone (*HYP*).

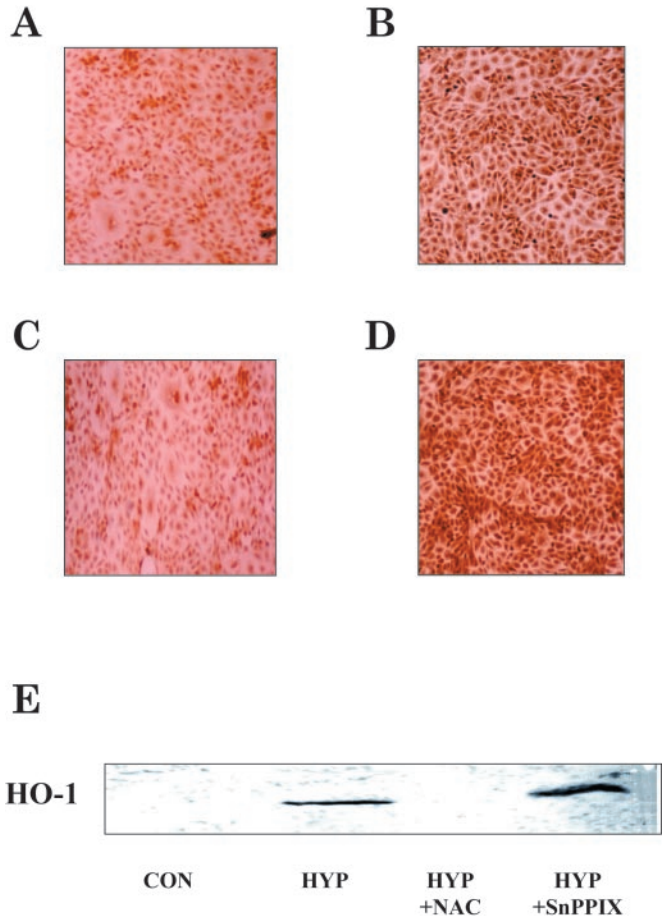

FIG. 6. **Effect of *N*-acetylcysteine and tin protoporphyrin IX on endothelial HO-1 protein expression during hypoxia.** Immunocytochemistry for HO-1 in cells incubated with complete medium (*panel A*, *control*) or exposed to hypoxia for 18 h in the presence of medium alone (*panel B*), 1 mM *N*-acetylcysteine (*panel C*), or 50 μM SnPPIX (*panel D*). *Panel E*, Western blot analysis for HO-1 on cells treated as above. *CON*, control; *HYP*, hypoxia; *HYP+NAC*, hypoxia + *N*-acetylcysteine; *HYP + SnPPIX*, hypoxia + tin protoporphyrin IX.

gets for NO-mediated biological activities, and we observed radical changes in GSH:GSSG ratio during hypoxia, we exposed cells to hypoxic conditions in the presence of *N*-acetylcysteine, a thiol-containing compound used as a precursor of glutathione synthesis. We found that 1 mM *N*-acetylcysteine completely suppressed the increase in HO-1 protein expression and heme oxygenase activity mediated by hypoxia (Figs. 5 and 6). To exclude the possibility that *N*-acetylcysteine suppressed HO-1 expression because of its inherent antioxidant characteristic, cells were exposed to hypoxia (18 h) in the presence of uric acid or 1,3-dimethyl-2-thiourea, scavengers of peroxynitrite and hydroxyl radicals, respectively. These two compounds had no effect on heme oxygenase activation by hypoxia, and Western blot analysis of hypoxic cells also revealed the absence of 3-nitrotyrosine, a marker of peroxynitrite formation (data not shown). Similar results were obtained by exposing cells to hypoxia in the presence of superoxide dismutase, catalase, or MnTBAP, a permeable superoxide dismutase mimetic that also has the ability to scavenge peroxynitrite (data not shown); collectively, these data would exclude a major involvement of superoxide anion and hydrogen peroxide in the observed effect and suggest that classical oxidative stress-mediated reactions might have little or no contribution to hypoxia-mediated HO-1 induction. It is interesting to note that 50 μM SnPPIX, an inhibitor of heme oxygenase, completely abolished the increase in heme oxygenase activity but not HO-1 protein expression mediated by hypoxia (Fig. 5 and Fig. 6, *D* and *E*).

Effect of Hypoxia on NOS Activity and RSNO Formation—Increased iNOS expression was associated with elevated NOS activity during hypoxia (Fig. 7*A*); specifically, NOS activity increased from 0.69 ± 0.03 (control) to 2.61 ± 0.13 and 1.87 ± 0.06 nmol NO/mg of protein/min at 6 and 18 h hypoxia, respectively ($p < 0.05$). Treatment of cells with 40 μM ITU completely suppressed this effect (0.61 ± 0.06 and 0.65 ± 0.12 nmol NO/mg of protein/min at 6 and 18 h, respectively); interestingly, the presence of *N*-acetylcysteine also significantly attenuated the increased NOS activity by hypoxia (Fig. 7*A*). We then measured intracellular RSNO, which may play an important role in the signaling events leading to HO-1 induction by hypoxia. We found that RSNO levels increased markedly at 6 and 18 h hypoxia; however, although this effect was suppressed considerably by *N*-acetylcysteine at 18 h, at 6 h the presence of the thiol compound resulted in an even higher detectable amount of RSNO compared with hypoxia alone.

Effect of S-Nitrosoglutathione on Hypoxia-mediated Increase in Heme Oxygenase Activity—The data presented so far indicate the existence of a strict relationship among increased NOS activity, augmented levels of RSNO, and modulation of the HO-1 gene under hypoxic conditions. Therefore, we hypothesized that NO reacts with susceptible thiol groups that are involved in hypoxia-mediated HO-1 induction. We have indeed demonstrated previously that thiols modulate endothelial HO-1 induction by various NO-releasing agents (21). Using GSNO, we examined the changes in endothelial heme oxygenase activity in normoxic and hypoxic conditions. As shown in Fig. 8, exposure of endothelial cells to 0.5 mM GSNO for 6 h under normoxic conditions produced an increased heme oxygenase activity compared with control (from 350 to 1,078 ± 38 pmol of bilirubin/mg of protein/h, $p < 0.05$); interestingly, this effect was markedly potentiated by hypoxia (1,530 ± 53 pmol of bilirubin/mg of protein/h, $p < 0.05$ *versus* normoxia + GSNO).

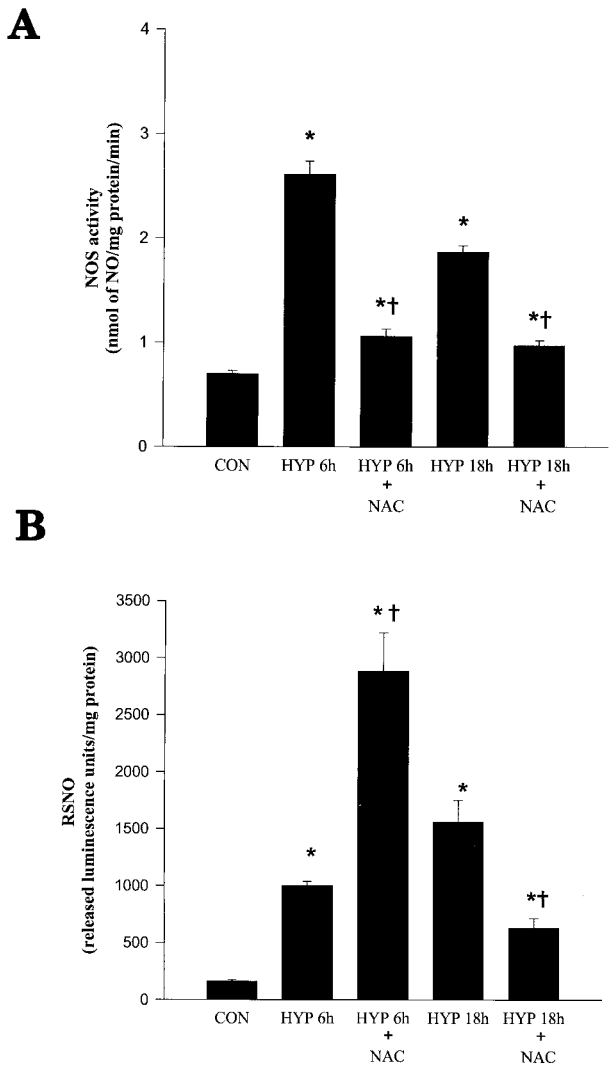

FIG. 7. **Effect of hypoxia on NOS activity and RSNO formation.** Endothelial cells were exposed to hypoxia for 6 or 18 h with or without 1 mM *N*-acetylcysteine (*NAC*). *Panel A*, NOS activity was measured by the hemoglobin assay as reported under "Experimental Procedures." *Panel B*, RSNO was determined by chemiluminescence as reported under "Experimental Procedures." Each *bar* represents the mean (±S.E.) of three or four experiments performed independently. * $p < 0.05$ *versus* control (*CON*); † $p < 0.05$ *versus* hypoxia alone (*HYP*).

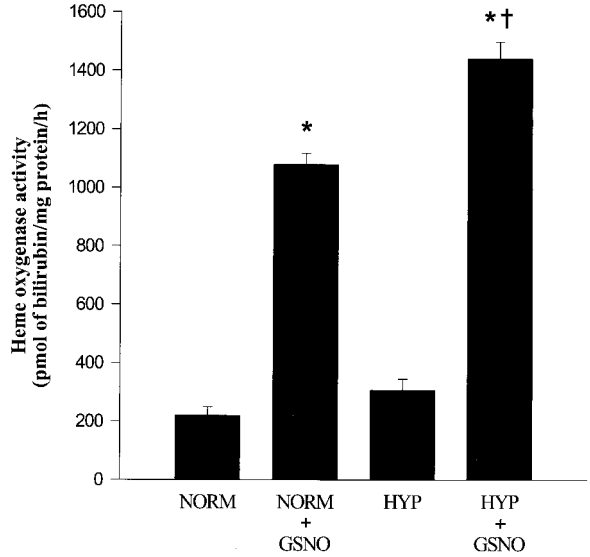

FIG. 8. **Effect of *S*-nitrosoglutathione on hypoxia-mediated increase in heme oxygenase activity.** Heme oxygenase activity was measured in endothelial cells exposed to normoxia or hypoxia (*HYP*) for 6 h in the presence or absence of 0.5 mM (*GSNO*). Each *bar* represents the mean (±S.E.) of four or five experiments performed independently. * $p < 0.05$ *versus* normoxia (*NORM*); † $p < 0.05$ *versus* normoxia + GSNO.

These results suggest that conditions of reduced oxygen tension favor the ability of *S*-nitrosothiols to stimulate HO-1 induction and further support the crucial role for NO-thiols interaction in the activation of the cellular stress response (21, 37).

DISCUSSION

In the present study we report the effect of hypoxia on HO-1, ecNOS, and iNOS expression in aortic endothelial cells. We observed a specific temporal pattern whereby increased iNOS protein and NOS activity precede HO-1 induction; notably, inhibitors of iNOS function significantly attenuated the increase in heme oxygenase activity mediated by low oxygen tension. Oxidation of intracellular glutathione and formation of *S*-nitrosothiols also appear to contribute to the mechanism(s) of hypoxia-mediated HO-1 expression. The importance of thiol groups in the modulation of this process was highlighted by the findings showing a complete suppression of increased heme oxygenase activity by the thiol donor *N*-acetylcysteine.

It has been reported previously that only vascular smooth muscle cells, but not endothelial cells, exposed to reduced oxygen tension express significantly higher levels of HO-1 mRNA than normoxic cells (38). Under those hypoxic conditions, the pO_2 measured in the culture medium was approximately 20 mm Hg; accordingly, by reproducing the same experimental protocol, we found that bovine aortic endothelial cells do not show any change in heme oxygenase activity compared with control (data not shown). However, we observed that incubation of endothelial cells under more severe hypoxic conditions ($pO_2 \leq 2$ mm Hg) resulted in a time-dependent increase in HO-1 mRNA and protein expression which was accompanied by elevation of heme oxygenase activity. These data indicate that expression of certain hypoxic genes is dependent upon a differential sensitivity of vascular cell types to oxygen levels. Although some studies indicate that HO-1 induction under hypoxic conditions is mediated by the transcriptional factor HIF-1 (19), a recent report demonstrates that increased HO-1 mRNA expression in response to low oxygen is strikingly independent of HIF-1 (39); these data reveal that regulation of HO-1 gene during hypoxia is a complex process involving more than one mechanism of activation.

Of major interest in this work is the result showing that up-regulation of iNOS protein and NOS activity occurs prior to HO-1 activation. This finding led us to explore whether NO or NO-related species could participate in the signal transduction events responsible for HO-1 induction by hypoxia. This hypothesis is plausible since we have demonstrated that NO and NO-derivatives are potent stimulators of HO-1 in different cell types and tissues, both in *in vitro* and *in vivo* systems (15, 21, 22, 25, 32, 40, 41). In addition, cells stimulated to produce NO following induction of iNOS with bacterial lipopolysaccharide and cytokines express high levels of HO-1 and heme oxygenase activity, an effect abolished by blockade of iNOS activity (31, 42–44). In the present study, two selective inhibitors of iNOS and a water-soluble NO scavenger considerably attenuated the hypoxia-mediated increase in endothelial heme oxygenase activity, suggesting a direct contribution of endogenously augmented NO in the observed cellular response. In agreement with other reports (6, 8), we also found that ecNOS protein decreased gradually during exposure of cells to hypoxia. The significance of these data is currently unknown; however, it is

possible that ecNOS might represent a suitable source of heme necessary for the activity of increasing HO-1 detected during hypoxia (22).

It emerges from our results that iNOS-derived NO may not be the only factor involved in HO-1 induction by reduced oxygen tension because the increase in heme oxygenase activity was not totally suppressed by iNOS inhibitors or a NO scavenger. In conditions of physiological oxygen levels, it is known that the HO-1 gene is highly susceptible to up-regulation by oxidative stress-related stimuli (45–47). In our experiments, exposure of cells to hypoxia in the presence of various antioxidant agents (superoxide dismutase, catalase, MnTBAP) and scavengers of oxidant species (uric acid, 1, 3-dimethyl-2-thiourea) did not prevent the rise in heme oxygenase activity, suggesting that oxidative stress-mediated reactions do not play a major role in HO-1 stimulation by oxygen deprivation. Despite this evidence, hypoxia caused a substantial increase in GSSG, leading to a dramatic decrease in the GSH:GSSG ratio. Glutathione is present in virtually all cells at very high concentrations; it constitutes the most important non-enzymatic intracellular antioxidant, and its major function is associated with the maintenance of the cellular redox homeostasis (48). Formation of GSSG is regarded as an index of oxidative stress, and alteration of the thiol redox state is often linked with activation of transcriptional factors and regulation of gene expression (49, 50). Indeed, a decrease in endogenous glutathione and oxidation of sulfhydryl groups have been shown to correlate with induction of HO-1 (33–35) and other heat shock proteins (51). In parallel and in addition to its antioxidant property, another key function for glutathione ought to be considered. Glutathione and thiol groups are critical sites of binding with NO (or NO-related species) leading to the generation of GSNO and RSNO, respectively. In this respect, the inherent ability of thiols to bind or release NO may represent a refined cellular mechanism to control NO bioavailability and function. In fact, RSNO have been implicated in several important physiological processes including oxygen transport to tissues (5), post-translational modifications (52, 53), and modulation of enzymatic activities (54, 55). Under certain conditions, GSNO decomposes to liberate NO and results in the formation of GSSG (56). Our experiments are consistent with the occurrence of such a mechanism because increased GSSG during hypoxia (6 h) directly correlated with RSNO levels. It is interesting to note that formation of GSSG in our system is accompanied by a rise in total glutathione, suggesting that cells adapt to redox imbalances by activating pathways directly involved in the synthesis of new glutathione. The possibility that NO could also contribute to the induction of these pathways is highlighted by recent findings of Moellering and co-workers (57), showing that NO-releasing agents stimulate the production of glutathione through enhanced expression of γ-glutamylcysteine synthetase. In agreement with our results, the same authors found that NO caused an imbalance of the cellular redox status as the GSH:GSSG ratio was decreased markedly after exposure of vascular smooth muscle cells to NO donors (57).

Thus, our data emphasize the importance of NO-thiols interaction in the cellular response and adaptation to stressful conditions such as hypoxia. This concept is corroborated by the finding that, among the various antioxidants used in our study, *N*-acetylcysteine was the only compound capable of completely suppressing hypoxia-mediated HO-1 induction. *N*-Acetylcysteine is a well known precursor of glutathione synthesis and, in virtue of its sulfhydryl group, may serve *per se* as an effective target for NO binding. In fact, we have already shown that increased intra- and extracellular thiols following incubation of cultured endothelial cells with *N*-acetylcysteine markedly reduce HO-1 induction by NO donors; this effect appears to be associated with NO stabilization by sulfhydryl groups through the formation of RSNO (21). In support of this hypothesis, RSNO levels were augmented further in cells exposed to hypoxia (6 h) in the presence of *N*-acetylcysteine. These results indicate that *N*-acetylcysteine may function as an NO antagonist by providing an excess of thiol groups that redirect NO away from other important cellular targets involved in the up-regulation of HO-1 by hypoxia. Although these targets remain to be identified, there exist at least two transcriptional factors (NF-kB and AP-1) that regulate HO-1 gene expression (12) and are known to contain cysteine residues susceptible to functional modulation by NO (58). Pertinent to this post-translational modifications, *S*-nitrosation of the transcriptional activator OxyR by *S*-nitrosocysteine in bacteria appears to be an oxygen-independent type of stress (nitrosative stress) which results in the expression of specific resistance genes (53). Notably, we found that the hypoxia-mediated increase in endothelial heme oxygenase activity was potentiated by the presence of GSNO, indicating that in conditions of impaired availability of oxygen, *S*-nitrosothiols can function as modulators of the cellular stress response.

Also of interest is the finding that *N*-acetylcysteine considerably attenuated the increase in NOS activity caused by hypoxia, indicating a possible role of this thiol compound in directly regulating the expression of the iNOS gene. This suggests that reduction of NOS activity by *N*-acetylcysteine leads to suppression of HO-1 stimulation. It is also conceivable that *N*-acetylcysteine might control two independent redox-sensitive processes affecting iNOS and HO-1 expression by low oxygen levels. However, the contribution of iNOS-derived NO in the induction of HO-1 in our model is still sustained, as discussed above, by the facts that up-regulation of iNOS protein temporally precedes HO-1 induction and that both iNOS inhibitors and a NO scavenger reduced the hypoxia-mediated rise in heme oxygenase activity.

It is widely accepted that oxidative reactions are common denominators of several stressful stimuli and conditions (UVA radiations, heavy metals, hydrogen peroxide, ischemia-reperfusion, septic shock) leading to up-regulation of HO-1 in biological systems (12). Our previous work (21) and the findings presented here point to an alternative signal transduction mechanism involved in the induction of the HO-1 gene. Specifically, control of the HO-1 gene may occur also through nitrosative stress in a manner independent from oxidant mediators. HO-1 and other inducible genes sensitive to NO could be part of the cellular response and adaptation to the threat caused by increased NO and NO-related species (22, 41, 55). It is interesting to report that recent evidence from our laboratory shows that the antioxidants bilirubin and biliverdin, products of heme degradation by heme oxygenase, interact directly with RSNO and NO-related species (59). These data provide a basis to the hypothesis that the HO-1 pathway, in addition to its established role in cytoprotection against oxidant-mediated damage, could function as a system to counteract nitrosative stress.

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Endothelial Heme Oxygenase-1 Induction by Hypoxia: MODULATION BY INDUCIBLE NITRIC-OXIDE SYNTHASE ANDS-NITROSOTHIOLS

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