 Resistance to Nitric Oxide-induced Necrosis in Heme Oxygenase-1 Overexpressing Pulmonary Epithelial Cells Associated with Decreased Lipid Peroxidation

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Increased expression of heme oxygenase-1 (HO-1) increases NO resistance in several cell types, although the biochemical mechanism for this protection is unknown. To address this issue, we have measured different molecular markers of nitrosative stress in three stably transfected cell lines derived from the human lung epithelial line A549: two lines that overexpress rat HO-1 (L1 and A4), and a control line with the empty vector (Neo). Compared with the control Neo cells, L1 and A4 cells had, respectively, 5.8- and 3.8-fold greater HO activity accompanied by increased resistance to NO-induced necrosis. Compared with the Neo control, the HO-1-overexpressing cells also showed significantly less lipid peroxide formation and decreased perturbation of transition metal oxidation and coordination states following a cytotoxic NO exposure. These effects were blocked by the HO-1 inhibitors Zn- and Sn-protoporphyrin IX. In contrast, HO-1 overexpression did not significantly affect total reactive oxygen or nitrogen species, the levels of the nucleobase deamination products in DNA (xanthine, inosine, and uracil) following NO exposure, or NO-induced protein nitration. While increased HO-1 activity prevented NO-induced fluctuations in transition metal homeostasis, addition of an iron chelator decreased NO toxicity only slightly. Our results indicate that transition metal homeostasis, addition of an iron chelator and increased HO-1 activity prevented NO-induced fluctuations in DNA damage by NO involving nitrosative base deamination (18, 19). In these reactions, the secondary product, N2O3, directly attacks the heterocyclic amines of DNA bases to form diazo-génics, and tumor cells during an inflammatory response (5). In contrast to its beneficial role in cell signaling and immune function, NO production has been implicated for a role in several different disease states including cancer (6), atherosclerosis (7), arthritis (8), and the neuropathologies amyotrophic lateral sclerosis, multiple sclerosis, Alzheimer’s disease, and Parkinson disease (9). The contribution of NO to these disease states is thought to depend on its ability to form highly reactive secondary species such as peroxynitrite (ONOO−) and nitrous anhydride (N2O3), following reaction with superoxide (O2−) and molecular oxygen, respectively (10).

These secondary products of NO can react with a variety of cellular structures including proteins (10, 11), lipids (12), and DNA (10). NO also directly binds protein heme and Fe-S centers to modulate or inhibit enzyme activity (13, 14). Because of these reactions, NO is hypothesized to have a detrimental effect on cellular metal homeostasis. Support for this hypothesis comes from whole cell spectroscopy studies, which indicate the formation of nitrosylated-heme and dinitrosyl-iron (DNIC) complexes in NO-treated cells (15). Cellular exposure to NO also leads to protein tyrosine nitration mediated by the secondary product nitrogen dioxide (NO2) (10, 11) and perhaps other mechanisms (16). In addition, cysteine nitrosylation generates 3-nitrosothiols in as many as 115 proteins upon NO treatment (17).

Exposure of cells to NO damages membranes through lipid peroxidation and nitration, which yield lipid hydroperoxides, nitro-epoxy lipids, and nitrated lipids (12). One mechanism of DNA damage by NO involves nitrosative base deamination (18, 19). In these reactions, the secondary product, N2O3, directly attacks the heterocyclic amines of DNA bases to form diazo-netic resonance; H2DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; HO-1, heme oxygenase-1; ICP-ES, inductively coupled plasma emission spectroscopy; MF-PBS, metal-free phosphate-buffered saline; NO2−, nitrogen dioxide; N2O3, nitrous anhydride; O2•−, superoxide; ONOO−, peroxynitrite; PARP, poly(ADP-ribose) polymerase; PBS, phosphate-buffered saline; RNS, reactive nitrogen species; ROS, reactive oxygen species; SnPPIX, tin protoporphyrin IX; SPERNO, spermine-NONOate; Y-NO2, 3-nitrotyrosine; ZnPPIX, zinc protoporphyrin IX.

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1 Supported by National Institutes of Health Training Grant T32 CA09078, and by a development grant from the Muscular Dystrophy Association (MDA3934).

2 The abbreviations used are: NOS, nitric-oxide synthase; DETA-NO, (2-aminoethyl)amino)diazen-1-ium-1,2-diolate; dI, deoxyinosine; dO, deoxyoxanine; dX, deoxyxanthine; EPR, electron paramagnetic resonance; H2DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; HO-1, heme oxygenase-1; ICP-ES, inductively coupled plasma emission spectroscopy; MF-PBS, metal-free phosphate-buffered saline; NO2−, nitrogen dioxide; N2O3, nitrous anhydride; O2•−, superoxide; ONOO−, peroxynitrite; PARP, poly(ADP-ribose) polymerase; PBS, phosphate-buffered saline; RNS, reactive nitrogen species; ROS, reactive oxygen species; SnPPIX, tin protoporphyrin IX; SPERNO, spermine-NONOate; Y-NO2, 3-nitrotyrosine; ZnPPIX, zinc protoporphyrin IX.

Nitric oxide is a free radical gas produced by three different isoforms of nitric-oxide synthase (NOS)1: neuronal NOS, inducible NOS, and endothelial NOS (1). Low nanomolar concentrations of NO produced by endothelial NOS and neuronal NOS play a role in physiological processes including smooth muscle relaxation (endothelial NOS) (2) and neuronal signaling (endothelial NOS and neuronal NOS implicated) (3, 4). High concentrations of NO produced by inducible NOS in neutrophils and macrophages contribute to killing of bacteria, pathogens, and tumor cells during an inflammatory response (5). In contrast to its beneficial role in cell signaling and immune function, NO production has been implicated for a role in several different disease states including cancer (6), atherosclerosis (7), arthritis (8), and the neuropathologies amyotrophic lateral sclerosis, multiple sclerosis, Alzheimer’s disease, and Parkinson disease (9). The contribution of NO to these disease states is thought to depend on its ability to form highly reactive secondary species such as peroxynitrite (ONOO−) and nitrous anhydride (N2O3), following reaction with superoxide (O2−) and molecular oxygen, respectively (10).
nium ions, which, among other reactions, can hydrolyze to complete the deamination; however, other mechanisms are possible (20). Thus, adenine, cytosine, and 5-methylcytosine are converted to inosine (dI), uracil (dU), and thymine, respectively, while guanine gives rise to two products: xanthine (dX) and oxanine (dO). Lymphoblastoid cells exposed to toxic levels of NO gas showed only a modest (2–4-fold) increase in dX, dI, and dU, while dO was undetected (19). A 6-fold increase in dX formation was also observed in the genomic DNA of stimulated macrophages (21). Other major products of ONOO−-induced DNA damage are 8-nitroguanine, 8-oxoguanine, and nitroimidazole, while deoxyriboside oxidation occurs to a lesser extent (10, 22). The many targets for NO-induced damage, and the diversity of lesions, likely underlie its relation to a wide variety of diseases.

In the face of NO generation, cells may express antioxidant molecules and defense enzymes to prevent or reverse the cellular damage described above. Heme oxygenase-1 (HO-1) was recently implicated in basal (23) and inducible (24) resistance to NO toxicity in mouse motor neurons. In addition, an inhibitor of HO-1 (tin protoporphyrin-IX, SnPPIX) blocks adaptive resistance to NO toxicity in both mouse hepatocytes (25) and L929 cells (26). HO-1 is the first enzyme in heme catabolism, producing CO, Fe2+, and biliverdin, which in turn can be reduced to bilirubin by biliverdin reductase (27). Both biliverdin and bilirubin are antioxidants that can prevent H2O2/O2−-induced lipid peroxidation and cell death (28). CO was identified as a signaling molecule (29) in the prevention of NO toxicity in HeLa cells (30) and L929 fibroblasts (26).

At present it is unknown how HO-1 prevents NO-induced toxicity. HO-1 may provide resistance by preventing or decreasing NO modification of specific cellular targets. In this report, we explore whether and how HO-1 overexpression prevents NO-induced modifications in human pulmonary epithelial cells. For these studies, we primarily utilized stable HO-1-overexpressing cell lines rather than the transient induction of HO-1 by chemical agents, which typically are toxic, elicit oxidative stress, and induce a complex genetic response in the exposed cells. Thus, the overexpressing lines allow the assessment of HO-1 function specifically, without the confounding background of other effects.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Culture Conditions**—Human lung epithelial cells (A549) stably transfected with a neomycin selection plasmid, pcDNA 3-neo (Neo cells), or with both this selection plasmid and a rat HO-1 expression plasmid (L1 and A4 cells) were kind gifts of Prof. Augustine Choi and Prof. Patty Lee (31). The rat HO-1 expression plasmid contains a 4.3-kbp EcoRI/AluI fragment with the human β-actin promoter, 5′-untranslated region, and first intron, a 1-kbp Xhol/HindIII fragment with the rat HO-1 cDNA, and a 1-kbp HindIII/KpnI fragment containing the 3′-untranslated region and polyadenylation sequence of mouse HO-1 (31). The cells were grown in Ham’s F12 medium (Invitrogen, Gaithersburg, MD) supplemented with 10% fetal bovine serum (HyClone, Logan, UT) (Ham’s F12-10% FBS), penicillin at 25 international units/ml, and streptomycin at 25 μg/ml (Mediatech, Herndon, VA). The cells were cultured at 5% CO2 and 37 °C in a humidified incubator.

**HO-1 Overexpression Prevents NO-induced Lipid Peroxidation**

**Experimental Procedures**

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**HO-1 Overexpression Prevents NO-induced Lipid Peroxidation**

The cells were grown to ~80% confluency in Ham’s F12-10% fetal bovine serum in 150-cm2 plates, washed once in 3 ml of cold phosphate-buffered saline (PBS), scraped into 3 ml cold PBS, and centrifuged at 1,400 × g for 5 min at 4 °C. The supernatant was aspirated and the cell pellet lysed according to a published protocol (30). Lysate samples (130 μg of total protein) were electrophoresed on 10% SDS-polyacrylamide gels. Proteins were electrotransferred onto nitrocellulose membranes and immunoblotted for HO-1 and β-actin according to a published protocol (30). Recombinant rat HO-1 lacking its transmembrane domain (Stressgen Biotechnologies, San Diego, CA) was used as a control. The Western blot shown is representative of two independent experiments.

A separate experiment was performed to determine the ability of NaAsO2 to induce HO-1 protein expression in Neo cells. For each sample, ~2.4 × 106 cells were left untreated or treated with up to 25 μM NaAsO2 for 8 h prior to harvest. Lysate samples (30 μg of total protein per lane) were electrophoresed and immunoblotted for HO-1 and β-actin as described above.

**HO Activity Assays**—Two 150-cm2 plates (containing a total of ~36 × 106 cells per sample) were washed twice in PBS; scraped into 2 ml of cold PBS containing 1 mM EDTA and protease inhibitor mixture (Sigma Aldrich); and centrifuged at 150 × g for 5 min at 4 °C. Each cell pellet was resuspended in 0.5 ml of 0.25 M sucrose, 20 mM Tris-Cl, pH 7.4, and centrifuged at 15,000 × g for 20 min, and the supernatants collected. Assays were performed according to the protocol described by Ryter et al. (32), using a final protein concentration of 1.0 mg/ml. Mean HO activities ± 0.5 S.D. are reported as pmol of bilirubin (mg of protein)−1 h−1.

**NO Toxicity Assays**—On the day prior to the assays, the cells (230,000 per ml) were plated into individual wells of 6-well plates (2 ml/well) and allowed to adhere overnight. The following day, the cells were either left untreated, or treated with 1–4 mM spermine-NONOate (SPERNO, Alexis Biochemicals, Lausen, Switzerland), an NO donor with a decay half-life of 39 min at pH 7.4, according to the supplier. A 2 mM SPERNO challenge provides a steady state concentration of ~9 μM NO at pH 7.4 for 2 h. This concentration of NO corresponds to ~1 μM NO measured at sights of inflammation in vivo (reference in Ref. 18). Following a 6-h challenge, the medium was removed by aspiration, the cells trypsinized (300 μl of trypsin at 2.5 g/liter), followed by a 4-fold dilution into trypan blue (0.4%, Sigma Aldrich). A hemacytometer was used to count both viable (Trypan Blue-excluding) and nonviable cells. The mean percentage of viable cells (n = 4) ± 0.5 S.D. based on two independent experiments is reported. Student’s paired t tests were performed to compare L1 and A4 samples to like-challenged Neo samples.

Additional NO toxicity experiments were performed on Neo cells following pretreatment with NaAsO2. Neo cells were plated as described above and left untreated or treated with up to 25 μM NaAsO2 (8 h) prior to a 6-h challenge with 2 mM SPERNO. Following the challenge, cell viability was determined...
using trypan blue staining as described above. The mean percentage of viable cells ($n = 4$) ± 0.5 S.D. is reported. Student’s paired $t$ tests were performed to compare untreated and treated Neo samples for 25 μM NaAsO₂ pretreatment, like-challenged samples.

Separate experiments were performed to test the effect of SnPPIX (Frontier Scientific Porphyrin Products, Logan, UT), bilirubin (Frontier Scientific), and desferroxamine mesylate (DFO, Sigma Aldrich) on NO toxicity. These experiments were performed as described above, except the cells were left untreated or pretreated with either 50 μM SnPPIX (2 h), 15 μM bilirubin (10 min), or 100 μM DFO (2 h), prior to a 6-h challenge with 3 mM SPERNO. Cell viability was determined using trypan blue staining as described above. The mean percentage of viable cells ($n = 4$) ± 0.5 S.D. is reported. Student’s paired $t$ tests were performed on: Neo cells ± bilirubin pretreatment; L1 cells ± SnPPIX pretreatment; and A4 cells ± SnPPIX pretreatment.

**Lipid Peroxide Assays**—For each sample, ~36 × 10⁶ cells grown to ~80% confluency on two 150-cm² plates were challenged with 2 mM SPERNO for 12 h at 37 °C. The cells were harvested at 4 °C by collecting in the same tube: the cell medium; a 3-ml metal free-PBS wash (MF-PBS); 3 ml of cell suspension (following scraping in MF-PBS); and a 3-ml of MF-PBS wash of the scraped plates. These samples were then centrifuged at 320 × g for 5 min at 4 °C, washed once in 5 ml of MF-PBS, and resuspended in 550 μl of cold MF-PBS. The samples were then sonicated three times for 5-s bursts, and the lysate was used directly for both protein assays (using Coomasie Blue) and lipid peroxide assays using the Lipid Hydroperoxide (LPO) Assay Kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer’s instructions. Briefly, the lipid hydroperoxides were extracted from lysates in deoxygenated chloroform and added to the kit chromophore for detection at 500 nm. A standard curve was prepared from serial dilutions of 50 μM 13-hydroperoxy octadecadienoic acid. The lipid peroxide measurements (nmol per sample) were normalized to a protein concentration of 3 mg/ml. The mean lipid peroxide values (in nmol) ± 0.5 S.D. are reported with an $n$ of 7, 8, and 6 for Neo, L1, and A4 cells, respectively. Student’s paired $t$ tests were performed to compare the L1 and A4 samples to the Neo samples.

Additional experiments were performed to determine the effect of NaAsO₂ pretreatment on NO-induced lipid peroxide formation in Neo cells. These experiments were performed as described above, except the samples received the following treatments: 1) 12-h challenge with 2 mM SPERNO; 2) 8-h pretreatment with 25 μM NaAsO₂ and 12-h recovery; 3) 8-h pretreatment with 25 μM NaAsO₂ and 12-h challenge with 2 mM SPERNO; 4) the same treatment as (3) except for the addition of 50 μM SnPPIX both during the last 2 h of NaAsO₂ pretreatment and during the SPERNO challenge; 5) the same treatment with NaAsO₂ and SnPPIX as (4), except lacking SPERNO challenge. Lipid peroxide values were normalized to a protein concentration of 3.0 mg/ml. The mean nmol of lipid peroxide ($n$ of 7, 5, 7, and 5 for (1) through (5), respectively) is reported for each treatment ± 0.5 S.D. Student’s $t$ tests were performed to compare: NO-challenged cells ± NaAsO₂ pretreatment; NaAsO₂ pretreated, NO-challenged cells ± SnPPIX treatment; and NaAsO₂ and SnPPIX-treated cells ± SPERNO challenge.

Separate experiments were performed to test the effect of bilirubin and SnPPIX on NO-induced lipid peroxidation in Neo and L1 cells. These experiments were performed as described above, except that the samples were left untreated or pretreated with 15 μM bilirubin (15 min, Neo cells) or 50 μM SnPPIX (2 h, L1 cells) prior to the NO challenge. The mean lipid peroxide values ($n = 6$) ± 0.5 S.D. are shown. Student’s paired $t$ tests were used to compare NO-challenged Neo cells ± bilirubin pretreatment and NO-challenged L1 cells ± SnPPIX pretreatment.

**Whole Cell Electron Paramagnetic Resonance (EPR) Spectroscopy**—Neo and L1 cells were grown to ~80% confluency on three 150-cm² plates. The cells in each plate were trypsinized in 2 ml trypsin (2.5 g/liter) and combined to provide a cell stock. Each cell stock was diluted 20-fold and counted using a hemacytometer. The cells were washed twice with 5 ml of Krebs-Ringer buffer (Sigma Aldrich). Each cell pellet was resuspended in Krebs-Ringer buffer to provide a final stock concentration of 40 × 10⁶ cells/ml. Samples were aliquoted into individual EPR tubes (250 μl per tube) and were either left untreated; challenged with 2 mM SPERNO for 45 min; or pretreated for 15 min with 50 μM zinc protoporphyrin IX (ZnPPIX, Frontier Scientific) prior to SPERNO challenge. All sample treatments were performed at 37 °C and stopped by rapid freezing in liquid nitrogen. EPR measurements were recorded at the Massachusetts Institute of Technology Department of Chemistry Instrumentation Facility. Whole cell EPR spectra were recorded on a Bruker Model ESP-300 equipped with a flow cryostat. Spectra were recorded under the following conditions: microwave frequency, 9.38 GHz; modulation amplitude and frequency, 5.7 G and 100 kHz; and microwave power, 1.0 milliwatt. The spectra shown, offset for clarity, are examples of three independent experiments.

**Protein Nitration Assays**—For each sample, two 60-mm plates (~1.4 × 10⁶ cells, ~80% confluency) were left untreated or treated with 2 mM SPERNO for up to 4 h. Cells were harvested and lysates prepared as described above. Protein lysate aliquots (containing 100 μg of total protein) from each sample were electrophoresed on denaturing 10% polyacrylamide gels and electrotransferred to nitrocellulose membranes. The membranes were probed sequentially with rabbit anti-3-nitro-tyrosine polyclonal (Stressgen) and mouse anti-β-actin monoclonal antibodies (Sigma Aldrich). Lysates from Neo and L1 cells, and Neo and A4 cells were used for individual experiments. Quantiﬁcation of the Y-NO₂ signal intensity included a correction for protein loading by normalization to β-actin. The increase in the Y-NO₂ signal in lysates from the treated L1 and A4 cells is reported relative to the intensity of the signal in matched Neo samples from the same experiment. The results shown are representative of two experiments.

**DNA Base Deamination Assays**—Deoxynucleoside deamination products from Neo and L1 cells treated with NO were quantitatively evaluated by LC/MS/MS immediately after exposure by a previously reported method (18). Neo and L1 (~2 × 10⁷) were grown in 175-cm² flasks, treated with 0–2 mM (z)-1-[2-(2-aminoethyl)-N-(2-ammonioethyl)diamino]diazene-1,2-diolate (DETA-NO, Alexis Biochemicals) for 3 h, and harvested for genomic DNA purification using a genomic-tip 100/G kit (Qiagen, Valencia, CA). DETA-NO is a NO donor
with a decay half-life of 20 h at pH 7.4, according to the supplier. A 2 mM DETA-NO challenge is estimated to produce a steady state concentration of ~13 μM NO at pH 7.4 for 18 h. Following cell treatment and harvest, genomic DNA was purified following the manufacturer’s instructions except for the addition of deaminase inhibitors coformycin (National Cancer Institute) and tetrahydrouridine (Calbiochem, San Diego, CA) to the cell lysis buffer at final concentrations of 5 μg/ml and 125 μg/ml, respectively. Genomic DNA (50 μg) was hydrolyzed by the combination of three enzymes (nuclease P1, phosphodiesterase I, and alkaline phosphatase) in the presence of appropriate amounts of isotope-labeled internal standards and deaminase inhibitors. Nuclease P1 and bovine alkaline phosphatase were purchased from Sigma Aldrich and phosphodiesterase I from USB Corporation (Cleveland, OH). The resulting deaminated nucleosides were resolved by HPLC on a Hewlett Packard model 1100, equipped with a 1040 diode array detector. The fractions containing dx, do, dl, and du with 15N-labeled internal standards were collected for subsequent LC/MS/MS quantification in an API 3000 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA) with MRM (multiple reactions monitoring) positive mode.

RESULTS

In view of the implied role of HO-1 in basal (23) and inducible (24–26) resistance to NO toxicity, we tested whether specific biochemical or cellular indicators could be associated with these effects. We used three stably transfected human lung epithelial cell lines derived from A549: one line was derived by transfection with a control vector (Neo line), and two clones expressing rat HO-1 (L1 and A4 lines). We first verified the HO-1 protein expression and activity levels in the transfected cell lines. A ~35-kDa band representing HO-1 was identified in cell lysates (Fig. 1A). This assignment was verified by the ability of the recombinant protein to block the immunoreactivity of the protein during development (supplementary Fig. S1). As measured by immunoblotting, compared with Neo cells, the L1 and A4 cells clearly overexpressed the HO-1 polypeptide (6.0- and 3.2-fold compared with Neo cells, respectively) (Fig. 1A). The increased HO-1 protein levels were reasonably consistent with the 5.8- and 3.8-fold higher HO activity in lysates of L1 and A4 cells, respectively, compared with lysates from Neo cells (Fig. 1B). The combined results suggest that the induced HO-1 in the L1 and A4 cells is active.

Confirming the role of HO-1 in cellular NO resistance, L1 and A4 cells were significantly more resistant to a 6-h SPERNO exposure than were Neo cells (Fig. 2). The rescuing effect of elevated HO-1 protein was also seen at 48 h post-exposure (supplemental Fig. S2). Because PARP cleavage in these cells does not occur before 16 h of exposure (supplemental Fig. S3), increased HO-1 levels seem to protect against both necrosis (observed at 6 h) and apoptotic cell death (48 h).

Low NO concentrations can terminate lipid peroxidation (33), while high concentrations favor formation of ONOO−, which mediates oxidation of unsaturated fatty acids (34). Because of the NO resistance mediated by HO-1 and the potential toxicity of lipid peroxidation, we examined NO-induced lipid peroxidation in the transfected cell lines (Fig. 3). Following a 12-h challenge with 2 mM SPERNO, both HO-1 overexpressing cell lines had accumulated less (about 2-fold) lipid peroxide than did Neo cells (p values of 0.007 and 0.04 for L1 and A4 cells, respectively, compared with Neo cells). Thus, HO-1 suppresses an important molecular marker of the oxidative damage exerted by high levels of NO.

In addition to the increased expression of HO-1 from a transgene, we performed experiments to determine whether induction of endogenous HO-1 by NaAsO2 would also decrease NO
HO-1 Overexpression Prevents NO-induced Lipid Peroxidation

Effect of HO-1 overexpression on NO-induced lipid peroxide formation. Neo, L1, and A4 cells (~36 × 10⁶) were treated with 2 mM SPERNO for 12 h at 37 °C and were subsequently harvested for lipid peroxide assays according to the manufacturer's instructions. Sample lipid peroxide values (nmol) were normalized for equal protein concentration (3 mg/ml) in each sample. The mean lipid peroxide values ± 0.5 S.D. are shown with an n of 7, 8, and 6 for Neo, L1, and A4 cells, respectively. The values for L1 and A4 cells were statistically different from the Neo cell values (p = 0.007 and **, p = 0.04).

Effect of NaAsO₂ on HO-1 expression, resistance to NO toxicity, and NO-induced lipid peroxide formation. A, Neo cells were left untreated or were treated with up to 25 µM NaAsO₂ for 8 h. A sample of lysate protein (30 µg) for each treatment was electrophoresed on a denaturing 10% polyacrylamide gel and electrotransferred to a nitrocellulose membrane. The resulting membrane was serially developed using rabbit anti-HO-1 polyclonal and mouse anti-β-actin monoclonal antibodies. Recombinant rat HO-1 lacking its transmembrane domain was used as a standard control (rHO-1). The bold numbers represent the fold-increase in HO-1 protein following correction for β-actin. B, Neo cells were left untreated or treated with up to 25 µM NaAsO₂ prior to a 6-h SPERNO challenge (2 mM). Cell viability was determined using trypan blue staining and the average percentage of viable cells (n = 4) is reported for each treatment ± 0.5 S.D. The values for untreated and 25 µM NaAsO₂ pretreated cells were statistically different (*, p = 0.003), C, lipid peroxide assays were performed on Neo cells receiving the following treatments: 1) 12-h challenge with 2 mM SPERNO; 2) 8-h pretreatment with 25 µM NaAsO₂ and 12-h recovery; 3) 8-h pretreatment with 25 µM NaAsO₂ and 12-h challenge with 2 mM SPERNO; 4) the same treatment as (3) except for the addition of 50 µM SnPPPIX both during the last 2 h of NaAsO₂ pretreatment and during the SPERNO challenge; 5) the same treatment with NaAsO₂ and SnPPPIX as (4), except lacking SPERNO challenge. Lipid peroxide values are normalized for equal protein concentration (3.0 mg/ml) in each sample. Mean nmol lipid peroxide (n of 7, 7, 5, 7, and 5 for (1) through (5), respectively) is reported for each treatment ± 0.5 S.D. The values of NO-challenged cells ± NaAsO₂ pretreatment; NaAsO₂ pretreated, NO-challenged cells ± SnPPPIX treatment; and NaAsO₂ and SnPPPIX-treated cells ± HO-1 challenge are statistically different (*, p < 0.01 for the first two and **, p = 0.065 for the later, respectively). NO products (Fig. 5). Neo cells treated with NO exhibited unique resonances compared with untreated controls with g values of 10.7 and 7.3 (compare traces A and C in Fig. 5). The amplitude of these NO-induced resonances was decreased in the L1 cells compared with Neo (Fig. 5A), while there was much less difference between Neo and L1 for the untreated cells (Fig. 5C). The differences between Neo and L1 for the NO-treated cells evidently were dependent on HO-1 activity: a 15-min pre-treatment with the HO inhibitor (ZnPPIX, 50 µM) blocked the relative difference in the formation of NO-induced species between the two cell lines (Fig. 5B, arrows). The differences associated with HO-1 expression did not seem to be due to large changes in the total Fe levels of the L1 line compared with Neo (supplemental Fig. S4). While the specific molecules responsible for the NO-induced signals remain unknown, the calculated g-factors suggest transition metal species with an S = 5/2 spin state.

With the observation that HO-1 overexpression protects from lipid peroxidation, we examined other molecular indica-

FIGURE 3. Effect of HO-1 overexpression on NO-induced lipid peroxide formation. Neo, L1, and A4 cells (~36 × 10⁶) were treated with 2 mM SPERNO for 12 h at 37 °C and were subsequently harvested for lipid peroxide assays according to the manufacturer's instructions. Sample lipid peroxide values (nmol) were normalized for equal protein concentration (3 mg/ml) in each sample. The mean lipid peroxide values ± 0.5 S.D. are shown with an n of 7, 8, and 6 for Neo, L1, and A4 cells, respectively. The values for L1 and A4 cells were statistically different from the Neo cell values (p = 0.007 and **, p = 0.04).

toxicity and NO-induced lipid peroxide formation. Sodium arsenite has been shown to induce HO-1 expression in human airway epithelial cells (35). Treatment of Neo cells with 25 µM NaAsO₂ for 8 h resulted in a 4.3-fold increase in HO-1 (Fig. 4A), which was intermediate between the levels observed for the untreated L1 and A4 cells (Fig. 1). Most notably, Neo cells pretreated with NaAsO₂ (25 µM, 8 h) were significantly more resistant to a 6-h SPERNO challenge (p = 0.003) (Fig. 4B). In addition, Neo cells pretreated with 25 µM NaAsO₂ showed a significant decrease (~80%) in NO-induced lipid peroxide formation compared with cells treated with NO alone (p = 0.007) (Fig. 4C). This effect of NaAsO₂ pretreatment was significantly reversed by the addition of the HO-1 inhibitor, SnPPPIX (p < 0.001) (Fig. 4C). However, as NaAsO₂ can induce several genes in exposed cells, the increased cellular resistance to NO and the decreased NO-induced lipid peroxide formation cannot be attributed to the increased expression of HO-1 alone. It should also be noted that NaAsO₂ alone induces oxidative stress in cells: ~40% the amount of lipid peroxide formation as NO exposure (Fig. 4C). The amount of lipid oxidation induced by NaAsO₂ is exacerbated by co-treatment with SnPPPIX (Fig. 4C), which suggests that cells receiving both treatments generate increased levels of oxygen radical species and/or are deficient in their antioxidant defenses. The data are consistent with the ability of NaAsO₂ to induce cellular oxidative stress.

As noted in the Introduction, NO exposure can disrupt cellular iron homeostasis. To follow these effects and determine whether HO-1 overexpression prevents such alterations, we developed a whole cell EPR spectroscopy approach. L1 and Neo cells were challenged with NO (2 mM SPERNO, 45 min) and analyzed by EPR under conditions to detect DNIC and other
HO-1 Overexpression Prevents NO-induced Lipid Peroxidation

![Graph](image_url)

**FIGURE 5. Effect of HO-1 overexpression on maintenance of transition metal homeostasis following NO exposure.** Whole cell EPR spectra were recorded at 4 K for Neo and L1 cells (1 × 10⁶) that were either left untreated (C) or were treated with 2 mM SPERNO for 45 min at 37 °C with (B) or without (A) a 15 min pretreatment with ZnPPIX (50 μM). Spectra were recorded under the following conditions: microwave frequency, 9.38 GHz; modulation amplitude and frequency, 5.7 G and 100 kHz; microwave power, 1.0 milliwatt. The spectra are offset for clarity. The arrows indicate the loss of specific resonance features upon addition of ZnPPIX (B). The bold numbers indicate the g values for specific resonance features in the spectra.

Tors of nitrosative and oxidative damage. The SPERNO-challenged cell lines were assessed for Y-NO₂ formation using immunoblotting, which actually indicated a modest increase in tyrosine nitration for the HO-1 overexpressing cells compared with the Neo line: 1.4–1.8-fold in L1, and 1.4–1.7-fold in A4 (Fig. 6). Immunoreactivity was blocked by co-incubation of the primary antibody with 10 mM free Y-NO₂, which supports the interpretation that these signals are due to tyrosine nitration (supplemental Fig. S5B). Thus, HO-1 overexpression in human lung epithelial cells does not prevent tyrosine nitration, and may even augment it.

The observed lipid peroxidation accompanying NO exposure suggested that the cells might be under oxidative stress. Because HO-1 overexpression affected NO-dependent changes in the coordination state and oxidation state of cellular transition metals (Fig. 5), we hypothesized that NO could also induce ROS/RNS formation that is modulated by HO-1 activity. The general redox status of the cells was tested using the dye 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA), which becomes fluorescent following oxidation. However, while a clear NO-dependent signal was detected with this probe, there was no difference between Neo and L1 cells (supplemental Fig. S6).

One mode of NO-mediated DNA damage is base deamination, and the deaminated base xanthine has been detected in the chromosomal DNA of NO-exposed lymphoblastoid cells (19) and macrophages (21). We assayed NO-induced DNA base deamination using an LC/MS/MS approach adapted from a published LC/MS method (18). For these experiments the cells were treated for 12 h with 0.01 to 2 mM DETA-NO. Following DETA-NO treatment of Neo and L1 cells, genomic DNA samples were harvested for determination of dX, dO, dl, and dU content. The NO-challenged cells indeed showed a dose-dependent increase in all three base deamination products except dO, which was not detectable above a limit of 5 per 10⁸ nucleotides (data not shown) (Fig. 7). The increase in base deamination products corresponds to the increasing toxicity of the DETA-NO exposure: 2 mM DETA-NO was sufficient to mediate significant Neo cell death at both 12 h (~30% toxicity) (supplemental Fig. S7A) and at 48-h postexposure (~20% of the cells surviving compared with untreated controls) (supplemental Fig. S7B). Most importantly, however, there was no detectable difference in the DNA deamination products between the Neo and L1 cells upon toxic exposure to DETA-NO.

The foregoing studies indicated that HO-1 overexpression prevents both NO-induced lipid peroxidation and NO-dependent alterations in transition metal homeostasis. To test whether HO-1 activity per se, as opposed to specific HO-1-generated products, is important for cellular resistance to NO, cytotoxicity assays were performed using SnPPIX (50 μM), bilirubin (15 μM), and the iron chelator DFO (100 μM) (Fig. 8). Bilirubin, a secondary product of HO-1, was used because it is able to prevent H₂O₂-induced lipid peroxidation (28). Pretreatment of the L1 and A4 cells with SnPPIX significantly sensitized both lines to NO toxicity compared with the controls treated with NO alone (p < 0.001 for both L1 (control versus SnPPIX) and A4 (control versus SnPPIX)). In contrast, treatment of the Neo line with SnPPIX did not alter its NO sensitivity (Fig. 8). Pretreat-

**FIGURE 6. Effect of HO-1 overexpression on cellular protein nitration following NO exposure.** Neo, L1, and A4 cells (1.4–10⁶) were treated with 2 mM SPERNO for up to 4 h at 37 °C. The cells were harvested at various times of exposure for immunoblotting. Protein lysate sample (100 μg) from each cell type was electrophoresed on a denaturing 10% polyacrylamide gel and electrotransferred to a nitrocellulose membrane. The resulting membrane was serially developed using rabbit anti-Y-NO₂ polyclonal and mouse anti-β-actin monoclonal antibodies. Lysates from Neo and L1 (A) or A4 (B) cells were used for the individual experiments. Quantitation of the intensity of the Y-NO₂ signal was corrected for protein loading by normalization to β-actin. The relative fold-increase in the Y-NO₂ signal in lysates from treated L1 (A) and A4 (B) cells is reported relative to the intensity of the signal in matched Neo samples from the same experiment (numbers shown below each panel). The results shown are representative of two experiments.
ment of the Neo cells with DFO did not significantly enhance their survival following an NO challenge (Fig. 8). However, a 15-min pretreatment with bilirubin (15 μM) conferred on the Neo cells significant protection against NO, with no effect on the L1 and A4 lines (Fig. 8). Further toxicity experiments indicate that 1-h pretreatment of Neo cells with 15 μM bilirubin was sufficient to confer significant protection against subsequent challenges with 3 mM SPERNO (supplemental Fig. S8A) or 500 μM H2O2 (supplemental Fig. S8B). The results show an inverse relationship between NO-induced lipid peroxidation and NO survival. A potential explanation for this correlation is that HO-1 overexpression may prevent NO-induced lipid peroxidation via its secondary product, bilirubin.

Lipid peroxide assays were performed to determine whether bilirubin prevents NO-induced lipid peroxidation in A549 cells. In these experiments, Neo cells were pretreated for 15 min with bilirubin (15 μM) prior to a 12-h exposure to 2 mM SPERNO. Compared with cells not preincubated with bilirubin, Neo cells pretreated with bilirubin had significantly decreased lipid peroxidation following NO treatment (p = 0.04) (Fig. 9). In addition, pretreatment of L1 cells for 2 h with SnPPIX (50 μM) increased the level of lipid peroxidation ~5-fold compared with non-pretreated controls (p = 0.001). The results indicate that the endogenous bilirubin produced in L1 cells (by the overexpressed rat HO-1) mediates protection against NO-induced lipid peroxidation. This conclusion was supported by quantitative bilirubin measurements, which showed that the L1 cells had ~1.5-fold more bilirubin than Neo cells (supplemental Fig.)

After RNA and protein removal, the purified DNA was digested by nuclease P1, phosphodiesterase 1, and alkaline phosphatase in the presence of appropriate amounts of isotope-labeled internal standards and deaminase inhibitors. The resulting nucleoside mixture was separated using an HPLC reversed-phase column. Fractions containing each nucleoside deamination product were collected for subsequent LC/MS/MS quantification. Data represent mean ± S.D. for four independent experiments.
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FIGURE 9. Effect of bilirubin and SnPPIX on NO-induced lipid peroxide formation in Neo and L1 cells. Neo and L1 cells (36 × 10⁶) were either left untreated or pretreated with 15 μM bilirubin (15 min, Neo) or 50 μM SnPPIX (2 h, L1) prior to challenge with 2 mM SPERNO for 12 h at 37 °C. The cells were subsequently harvested for lipid peroxide assays as described for Fig. 3. Sample lipid peroxide values (nmol) were normalized for equal protein concentration (3 mg/ml) in each sample. The mean lipid peroxide values (n = 6) ± 0.5 S.D. are shown. The data with n = 5 are indicated (†). The data for NO-challenged Neo cells + bilirubin pretreatment and for NO-challenged L1 cells + SnPPIX pretreatment were statistically different (*, p = 0.04 and **, p = 0.001).

S9). Given the ability of bilirubin to redox cycle (36) and the efficiency of NO/ONOO− scavenging by bilirubin (37, 38), a relatively small fold-increase in cellular bilirubin levels may greatly enhance cellular survival of an NO challenge.

DISCUSSION

HO-1 is transcriptionally regulated by a wide variety of stress stimuli including: heme, sodium arsenite, cadmium chloride, UVA (320–380 nm) irradiation, hyperoxia, hypoxia, H₂O₂, glutathione depletion, and NO (39). Corresponding to the increased HO-1 expression described above, HO-1 provides protection against different toxic stimuli in various cell types including heme toxicity in coronary endothelial cells, hyperoxia in pulmonary epithelial cells and UVA-induced damage in fibroblasts (references in Ref. 40). Alternatively, a deficiency of HO-1 in humans and mice leads to enhanced endothelial cell injury and hypersensitivity of fibroblasts to heme and H₂O₂, respectively (40). The protective role of HO-1 in each example may be attributed to removal of its substrate, prooxidant heme, from the cytosol (41). HO-1 may also contribute to cellular survival through generation of its products CO, biliverdin (converted to bilirubin), and Fe²⁺.

Both biliverdin and bilirubin are antioxidant molecules that may confer protection via their ability to quench peroxyl radicals and singlet oxygen, thus interfering with lipid peroxidation (28). CO has been recently recognized as a signaling molecule (29), which may provide additional resistance mechanisms. The beneficial effects of CO may include its ability to mediate a shift from pro-inflammatory (tumor necrosis factor-α, interleukin-1β, macrophage inflammatory protein-1β) to anti-inflammatory cytokine gene expression (interleukin-10) and the prevention of apoptosis triggered by tumor necrosis factor-α (reviewed in Ref. 42), and protection against NO-induced toxicity (26, 30).

As mentioned above, NO and its secondary products, are able to modify (damage) a variety of important cellular molecules including proteins (10, 11), lipids (12), and DNA (10). However, prior to the present study, the ability of HO-1 to prevent specific NO-induced modifications had not been assessed. Our studies indicate that HO-1 specifically prevents both NO-induced lipid peroxidation and alterations in transition metal homeostasis caused by NO exposure.

NO and its secondary products ONOO− and N₂O₃ have been shown to induce formation of various lipid peroxidation products. These include malondialdehyde, 4-hydroxynonenal, F₂-isoprostane, and lipid peroxide (12). In our studies, HO-1 overexpressing lung epithelial cells were significantly protected (~50%) against NO-induced lipid peroxidation (Fig. 3). In addition, physiological induction of HO-1 by NaAsO₂ conferred a pronounced resistance to an NO challenge that was correlated with a strongly diminished accumulation of lipid peroxides (Fig. 4). Thus we have shown a decrease in NO-induced lipid peroxidation upon increased HO-1 expression using two different methods: overexpression from a transgene and induction of endogenous gene expression. Additional experiments showed that the decrease in NO-induced lipid peroxidation associated with increased HO-1 expression could be blocked by the addition of the inhibitor SnPPIX (Figs. 4C and 9). Moreover, protection against NO could be conferred upon Neo cells by pretreatment with bilirubin (Fig. 8). The results indicate that HO-1 mediates protection against NO-induced lipid peroxidation via the endogenous production of biliverdin/bilirubin. This finding is in accord with in vitro studies showing that biliverdin and bilirubin are able to quench the toxic RNS species NO and ONOO− (37, 38). The exogenously added bilirubin seems to enter the cells, as 1-h pretreatment with bilirubin was sufficient to impart resistance to secondary NO and H₂O₂ challenges (supplemental Fig. S8). In addition, it should be noted that the concentration of bilirubin used to elicit protection against lipid peroxidation in our experiments (15 μM) corresponds to physiological levels (43). This same concentration of bilirubin also prevented the lipid peroxidation elicited in Neo cells by Fe²⁺/ascorbate treatment (data not shown). Quantitative measurements of total bilirubin also indicated an increase in the HO-1-overexpressing cell lines (~1.5-fold difference between L1 and Neo cells) (supplemental Fig. S9). Our results show a positive correlation between lipid peroxidation and NO-induced cell death. Indeed, Neo cells pretreated with bilirubin showed both a decrease in lipid peroxidation and a decrease in NO-induced cell death (Figs. 9 and 8, respectively). Overall, the results indicate that HO-1 attenuates NO-induced cell death at least in part by preventing lipid peroxidation via bilirubin/biliverdin production.

NO or inducible NOS induction increases the formation of both DNIC and nitrosylated heme in several cell types (15). We have found that HO-1 overexpression prevents the formation of two different EPR-detectable metal species in pulmonary epithelial cells following NO exposure: g = 10.7 and 7.3 (Fig. 5). The specific identity of these NO-induced metal species is unknown. However, the calculated g-factors could indicate a
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transition metal species with an S = 5/2 or S = 6 spin state. This effect of HO-1 is dependent on its catalytic activity, as the protection was nullified by the addition of ZnPPIX. HO-1 has recently been implicated in cellular iron efflux (44). Therefore, a potential explanation for the protective effect is that HO-1-overexpressing cells contain less iron, but this did not appear to be the case (supplemental Fig. S4). Consistent with this finding, we found that there was no notable difference between the cell lines in ROS/RNS formation (which depends on intracellular transition metals (45)) (supplemental Fig. S6). Although HO-1 had a protective effect against the formation of the NO-induced metal species, pretreatment of the cells with the Fe chelator DFO mediated only a slight decrease in toxicity of NO in the Neo cells (Fig. 8). Therefore, although HO-1 prevents NO-induced fluctuations in transition metal homeostasis, this activity does not prevent cellular ROS/RNS formation, which may not be critical for cellular resistance to NO. The protective effect of HO-1 on transition metal homeostasis, could potentially be due to a redistribution of Fe in the cells, mediated by an indirect up-regulation of ferritin by HO-1 produced Fe2+ (46).

Contrary to its protective effect on lipid peroxidation and metal homeostasis, HO-1 overexpression did not diminish NO-induced protein nitration or DNA base deamination. In our studies, there was a small increase in protein nitration in the HO-1 overexpressing cells following NO exposure (1.4–1.8-fold for each sample) (Fig. 6). Perhaps protection by HO-1 helps maintain peroxidase activities, which in turn would increase protein tyrosine nitration (16).

We found only a slight increase in dX, dI, and dU formation in all three cell lines following exposure to 2 mM DETA-NO (Fig. 7), a challenge that elicits significant cell death in Neo cells (supplemental Fig. S7). This result is consistent with the findings of Dong and Dedon (19), who measured a modest 2–4-fold increase in dX, dI, and dU in lymphoblastoid cells exposed to lethal concentrations of NO. There was also no difference in the formation of deaminated bases between the L1 and Neo cell lines following NO treatment (Fig. 7). The modest increase in deaminated base formation following cellular NO exposure is in contrast to the 8–10-fold increases in dX, dI, and dU measured in plasmid DNA following treatment with NO (18). An explanation for these differences in deaminated base formation is that RNS generated are quenched by other cytosolic substrates. Another possible explanation is that cellular DNA repair enzymes are able to remove and repair deaminated base lesions following NO exposure. However, Dong and Dedon (19) have shown that 24 h recovery of NO-exposed cells only produced a modest decrease in the number of deaminated bases.

The selective protection by HO-1 against NO-induced lipid peroxidation is attributed to its production of biliverdin/bilirubin, which can quench both NO and ONOO− (37, 38). The ability of HO-1 to prevent cellular toxicity by decreasing NO-induced lipid peroxidation suggests possible therapeutic approaches for diseases associated with NO-dependent lipid peroxidation, such as atherosclerosis (47), glaucoma (48), gastric cancer (49), and macular degeneration (50). Lipid peroxide products have been implicated in a number of cellular signaling pathways (51), including the induction of apoptosis (52). An end product of lipid peroxides, 4-hydroxynonenal, elicits apoptosis in various cell lines (51). Based on our present studies, we hypothesize that NO-induced lipid peroxide formation contributes to NO toxicity in cells.

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