Expression of Heme Oxygenase in Human Airway Epithelial Cells

Louise E. Donnelly and Peter J. Barnes

Department of Thoracic Medicine, Imperial College School of Science, Technology and Medicine, National Heart and Lung Institute, London, United Kingdom

Elevated levels of carbon monoxide (CO) are found in the exhaled breath of patients with inflammatory diseases such as asthma and cystic fibrosis. Endogenous CO is derived from heme oxygenase (HO) (EC 1.14.99.3), which catabolizes heme-producing CO and biliverdin. There are three isoforms of HO: HO-1 is inducible by inflammatory cytokines and oxidants, including nitric oxide (NO), whereas HO-2 and HO-3 are expressed constitutively. Primary airway epithelial cells were treated with either 50 ng/ml interleukin-1β, tumor necrosis factor-α, and interferon-γ (cytomix), or the NO donor NOC-18 for up to 24 h. Cytomix-induced HO-1 expression peaked at 4 h, returning to baseline by 24 h, whereas HO-2 expression remained unchanged. This increase in HO-1 expression could not be explained by an increase in NO production as inducible NO synthase expression increased between 12 and 24 h. However, the NO donor NOC-18 (500 μM) increased HO-1 expression twofold and HO activity 25-fold, whereas cytomix treatment increased HO activity eightfold. NO induction of HO-1 was not mediated via guanylyl cyclase and was not attenuated by 1 μM dexamethasone, although dexamethasone increased HO-2 protein. Therefore, airway epithelial cells express HO-2 and can express HO-1; thus, the epithelium may be a source of increased CO in airway diseases.

Heme oxygenase (HO) (EC 1.14.99.3) is the enzyme that catabolizes heme to produce carbon monoxide (CO), free iron, and biliverdin. Biliverdin is rapidly converted to the antioxidant bilirubin by the enzyme biliverdin reductase and any free iron is sequestered by ferritin. Three distinct isoforms of HO have been identified, each the product of a separate gene. HO-1 is a 32-kD protein (1, 2) that can be induced in cells after exposure to a variety of agents, including oxidative stress, heavy metals, ultraviolet light, and heme and its derivatives (3). HO-2 is expressed constitutively in tissues involved in heme catabolism, such as the liver and spleen. HO-2 is expressed in neuronal tissue and this may reflect the putative role of CO as a neurotransmitter (4). HO-3 is a 33-kD protein that, so far, has only been expressed constitutively in rat neurones (5).

The role of HO as an antioxidant enzyme has been well documented in several studies. HO-1 is induced in the lung after hyperoxic injury (6) and overexpression of the HO-1 gene in cells has revealed a role for this protein in protection against oxidative stress (7). More recently, overexpression of HO-1 by gene transfer into rat lungs provided protection against hyperoxic injury (8). Furthermore, embryonic fibroblasts from HO-1–deficient mice have a reduced defense against oxidant stress (9). Further evidence of a role for HO-1 in protection against oxidative stress has been demonstrated in hamster fibroblast cells treated with antisense sequences of HO-1, which provided to be hypersensitive to hypoxia (10).

The function of exogenous CO is still unclear, although it activates guanylyl cyclase and stimulates cyclic guanosine monophosphate (cGMP) production in a manner analogous to nitric oxide (NO) (11). Therefore, CO can act as a vasorelaxant (12) and may be involved in the hypoxic regulation of airway tone (13). Recently, CO itself has also been shown to protect against hyperoxic lung injury (14) and hence act as an antioxidant. CO levels are also increased in the exhaled breath of patients with a variety of airway diseases, including asthma (15), cystic fibrosis, and bronchiectasis (16). The precise HO isoform, which is responsible for the elevation of exhaled CO in airway diseases, is not known, although HO-1 may be induced by a variety of inflammatory cytokines that may have a role in airway inflammation (3, 17). The cellular source of CO in airway diseases is unclear, although airway macrophages from asthmatic patients express elevated levels of HO-1 when compared with cells from normal individuals (15).

Exhaled NO is also elevated in asthma (18) and has been associated with an increase in the expression of inducible nitric oxide synthase (iNOS) in the airway. NO stimulates HO-1 expression in endothelial, skeletal muscle, and glial cells (19–21). Although the precise mechanism of NO induction of HO-1 is not fully understood, it has been suggested that the production of the oxidant peroxynitrite might lead to the induction of HO-1 (19). Alternatively, HO-1 induction may be through an NO-mediated elevation of cGMP (22), although cGMP failed to elevate HO-1 expression in rat vascular smooth muscle cells (23). Therefore, the regulation of HO-1 induction by NO may be cell-type specific.

This study examined (*1*) whether the airway epithelium could be a source of elevated exhaled CO observed in airway diseases via the expression of HO and (*2*) whether inflammatory mediators known to be important in airway disease, such as cytokines and NO, could regulate HO activity and expression.

Materials and Methods

Materials

Keratinocyte serum-free medium, epidermal growth factor (EGF), bovine pituitary extract, Dulbecco's modified Eagle's medium, Ham's F12 nutrient medium, and Hanks' balanced salt solution

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Address correspondence to: Dr. L. E. Donnelly, Dept. of Thoracic Medicine, Imperial College School of Science, Technology and Medicine, National Heart and Lung Institute, Dovehouse Street, London SW3 6LY, UK. E-mail: l.donnelly@ic.ac.uk

Abbreviations: cyclic guanosine monophosphate, cGMP; carbon monoxide, CO; deoxynucleotide triphosphate, dNTP; enhanced chemiluminescence, ECL; fetal calf serum, FCS; granulocyte macrophage colony-stimulating factor, GM-CSF; Hanks' balanced salt solution, HBSS; heme oxygenase, HO; interleukin, IL; inducible nitric oxide synthase, iNOS; messenger RNA, mRNA; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT; nitric oxide, NO; nitric oxide synthase, NOS; reverse transcription/polymerase chain reaction, RT-PCR; sodium dodecyl sulfate, SDS; tumor necrosis factor, TNF.

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(HBSS) were purchased form Gibco Ltd. (Paisley, UK). Interleukin (IL)-1β, tumor necrosis factor (TNF)-α, interferon (IFN)-γ, granulocyte macrophage colony-stimulating factor (GM-CSF) were purchased from R&D Systems Europe Ltd. (Abingdon, Oxfordshire, UK). NOC-18, ODQ, and KT5823 were purchased from Alexis Ltd. (Nottingham, UK). Avian myeloblastosis virus (AMV)-reverse transcriptase, RNAsin, and random primers were all purchased from Promega Ltd. (Southampton, UK). Taq polymerase, deoxynucleotide triphosphates (dNTPs), and KCl buffer were purchased from Bioline Ltd. (London, UK). Protein assay reagent was purchased from Bio-Rad Laboratories Ltd. (Hemel Hempsted, Hertfordshire, UK). An RNA extraction kit was purchased from Qiagen Ltd. (Crawley, Sussex, UK). Anti-GM-CSF antibodies were purchased from PharMingen (San Diego, CA). Anti-HO-1 and anti-HO-2 antibodies were purchased from Stressgen Ltd. (Victoria, BC, Canada). Antirabbit immunoglobulin (Ig) G conjugated to horseradish peroxidase (HRP) was purchased from Dako Ltd. (High Wycombe, Berkshire, UK). Hybond-enhanced chemiluminescence (ECL) and ECL reagent were purchased from Amersham Ltd. (Amersham Place, UK). All other reagents were supplied by either Sigma (Poole, UK) or BDH (Poole, UK).

Bronchoscopy

Normal subjects (mean age, 26 ± 0.5 yr: mean FEV_1 % predicted, 102 ± 3.5) underwent fiberoptic bronchoscopy. All subjects were nonsmokers. Subjects attended the bronchoscopy suite after having fasted for 12 h and were pretreated intravenously with atropine (0.6 mg) and midazolam (5 to 10 mg). Oxygen (3 liters/min) was administered via nasal prongs throughout the procedure and oxygen saturation was monitored with a digital oximeter. Local anesthesia with lidocaine (2% wt/vol) was applied to the upper airways and larynx, and a fiberoptic bronchoscope (model BF10; Olympus, Key-Med, Essex, UK) was passed through the nasal passages into the trachea. Brushing of the airway was performed essentially as reported by Kelsen and coworkers (24). Briefly, a 2-mm channel cytology brush (1.8 mm insertion diameter; model BC-16C; Olympus) was inserted via the sampling channel of the bronchoscope and rubbed against the epithelial surface. The brush was retracted and cells dissociated by vortexing in ice-cold Ham's F12 nutrient medium. This brushing procedure was repeated four to six times. The Royal Brompton Hospital Ethics Committee approved the experimental protocol for fiberoptic bronchoscopy, and all subjects gave their informed consent to participate.

Isolation and Culture of Human Primary Epithelial Cells

The cell suspension was treated for 20 min with 50 μg/ml DNase at room temperature and then filtered through a 100-μM cell strainer. The cells were centrifuged for 5 min at 200 × *g* and washed once with HBSS. Cells were suspended in Ham's F12 nutrient medium containing 5% (vol/vol) fetal calf serum (FCS), 1 μM hydrocortisone, 5 ng/ml EGF, 10 μg/ml insulin, 10 nM retinoic acid, 0.5 μg/ml transferrin, 2 μg/ml triiodothyronine, 1.5 mg/ml $NaHCO_3$, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B, and seeded onto 24-well plates coated with 1% (wt/vol) collagen at a density of 5×10^5 cells/well. The cells were incubated at 37°C in a humidified atmosphere containing 95% (vol/vol) air/5% (vol/vol) CO_2. The cells were cultured until confluence (approximately 5 d) and then cultured for 24 h in additive-free medium before experimental treatments.

Cell Viability

Cells were treated with 1 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in HBSS for 30 min at 37°C. The MTT solution was removed from the surface of the cells and dimethyl sulfoximine added. The absorbance of the resultant solution was measured at 550 nm and treated cells were compared with control cells.

Measurement of Nitrite in Cell Culture Media

The amount of nitrite in cell culture media was measured using a modification of the method of Misko and colleagues (25). Briefly, 200 μl of medium or nitrite standard solution was mixed with 100 μl of 2% (wt/vol) charcoal in 0.2% (wt/vol) dextran. The suspension was centrifuged at 10,000 × *g* for 10 min, and the cleared supernatant was mixed with 10 μl of 0.05 mg/ml 2,3-diaminonaphthalene in 0.625 M HCl and incubated in the dark for 10 min. The reaction was stopped by the addition of 10 μl of 1.4 M NaOH, and fluorescence was measured using a Biolite F1 plate fluorimeter (Labtech, Uckfield, UK) with the excitation wavelength set at 360 nm and the emission wavelength set at 460 nm, with the sensitivity of the fluorimeter set between 40 and 50%. The amount of nitrite in the sample was calculated using a standard curve of known nitrite concentrations, and the assay was sensitive to 0.1 μM.

Measurement of GM-CSF in Cell Culture Media

GM-CSF was measured by sandwich enzyme-linked immunosorbent assay. Some 96-well plates were coated overnight at 4°C with rat monoclonal capture antibody against GM-CSF diluted 1:500 in 0.1 M $NaHCO_3$ containing 15 mM NaN_3. Plates were washed in wash buffer (145 mM NaCl, 4 mM KCl, 10 mM NaH_2PO_4, 0.05% [vol/vol] Tween-20, pH 7.4) then blocked for 2 h at room temperature with 10% (vol/vol) FCS. The plates were washed, and samples and standards were added to the wells and incubated at 4°C overnight. The plates were washed extensively and then incubated for 45 min with a biotinylated rat antihuman monoclonal GM-CSF antibody diluted 1:1,000 in 10% (vol/vol) FCS in wash buffer. Plates were then incubated for 30 min with a 1:400 dilution of avidin-peroxidase in 10% (vol/vol) FCS in wash buffer. The plates were then developed with ABTS substrate solution (0.547 mM 2,2′azino-bis(3-ethylbenzthiazoline-6-sulphonic acid), 0.1 M citric acid, pH 4.35, 0.03% [vol/vol] H_2O_2). Absorbance was measured spectrophotometrically at 405 nm and the amount of GM-CSF in each sample was calculated from a standard curve. The detection limit of this assay is 32 pg GM-CSF/ml. Data is expressed as a percentage of cytomix-stimulated cells.

RNA Isolation

RNA was isolated from primary epithelial cells using the Qiagen RNeasy mini kit according to the manufacturer's instructions.

Reverse Transcription/Polymerase Chain Reaction

Reverse transcription (RT) was performed on 0.5 μg of RNA. RNA was heated to 70°C for 5 min and then mixed with 0.01 μg/μl random primers, 1.0 mM dNTP, 1 μg/μl RNAsin, 0.25 μg/μl AMV-reverse transcriptase, in 1× reverse transcriptase buffer and incubated at 42°C for 1 h followed by denaturation at 90°C for 4 min. The resultant complementary DNA (cDNA) was then diluted by the addition of 80 μl of water.

For polymerase chain reaction (PCR), 5 μl of cDNA were incubated in a final volume of 25 μl containing 1× KCl buffer, 2 mM dNTP, 5 ng/μl specific primers, and 0.02 μg/μl Taq polymerase. Specific primers for iNOS PCR gave a PCR product of 312 bp: forward primer, 5′-GAGCTTCTACCTCAAGCTATC-3′; reverse primer, 5′-CCTGATGTTGCCATTGTTGGT-3′. The cycles used were 94°C for 45 s, 56°C for 45 s, 72°C for 60 s for 32 cycles followed by 72°C for 10 min. Specific primers for HO-1 gave a specific product of 616 bp: forward primer, 5′-CAGCATGCCCCAGGATTTG-3′; reverse primer, 5′-AGCTGGATGTTGAGCAGGA-3′. The cycles used were 94°C for 45 s, 58°C for 45 s, 72°C for 60 s for 30 cycles followed by 72°C for 10 min. Specific primers for HO-2 gave a specific product of 829 bp: forward primer, 5′-GGGACCAAGGAAGCACA-3′; reverse primer, 5′-ATGTAGTACCAGGCCAAGA-3′. The cycles used were 94°C for 45 s, 58°C for 45 s, 72°C for 60 s for 30 cycles followed by 72°C for 10 min. RT-PCR of glyceraldehyde-3-phosphate dehydroge-

nase (GAPDH) was performed to act as an internal control and the specific primers used gave a PCR product of 571 bp: forward primer, 5′-ATTCCATGGCACCGTCAAGGCT-3′; reverse primer, 5′-TCAGGTCCACCACTGACACGT-3′. The cycles used were 94°C for 45 s, 56°C for 45 s, 72°C for 60 s for 26 cycles followed by 72°C for 10 min. PCR products were identified on 2% (wt/vol) agarose gels. Samples that did not contain reverse transcriptase were used as negative controls.

Western Blot Analysis

Cells were lysed in 50 mM Tris/HCl, pH 7.4, containing 0.25 mM ethylene diaminetetraacetic acid, 0.5 mM phenylmethylsulphonyl fluoride, 5 μg/ml antipain, 5 μg/ml leupeptin, and 5 μg/ml benzaminidine. Protein concentration was determined using the Bio-Rad protein assay kit according to the manufacturer's instructions. Cell proteins were solubilized by boiling in sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) sample buffer (0.0625 mM Tris/HCl, pH 6.8, containing 10% [vol/vol] glycerol, 1% [wt/vol] SDS, 1% [wt/vol] β-mercaptoethanol, and 0.01% [wt/vol] bromophenol blue). The proteins (10 μg per lane) were resolved by electrophoresis in 12% (wt/vol) SDS polyacrylamide gels and transferred to Hybond-ECL nitrocellulose membranes. Equal protein loading was determined by staining the blot with 0.1% (wt/vol) Ponceau S in 5% (vol/vol) acetic acid. The nitrocellulose was blocked overnight at 4°C in 10% (wt/vol) dried milk protein in phosphate-buffered saline (PBS) containing 0.05% (vol/vol) Tween-20. The blots were washed in PBS containing 0.05% (vol/vol) Tween-20 and incubated for 1 h in the presence of primary antibody (1:2,000). The blots were washed extensively and then incubated for 1 h with antirabbit IgG conjugated to HRP (1:4,000). The blots were washed extensively again and the bands were visualized using ECL.

Assay of HO Activity

HO activity was assayed by measuring the production of CO from cells. Hemoglobin was added to the cell culture medium at a final concentration of 10 μM for 1 h. The medium was removed and the amount of carboxyhemoglobin was measured using dual wavelength spectroscopy according to the method of Sakata and Haga (26).

Analysis of Data

The intensity of bands for PCR was measured densitometrically using UVP Gel works documentation system GDS800 (UVP, Cambridge, UK).

Statistical Analysis

Statistical analysis was performed using one-way analysis of variance or Mann-Whitney *t* test as appropriate. Data is presented as

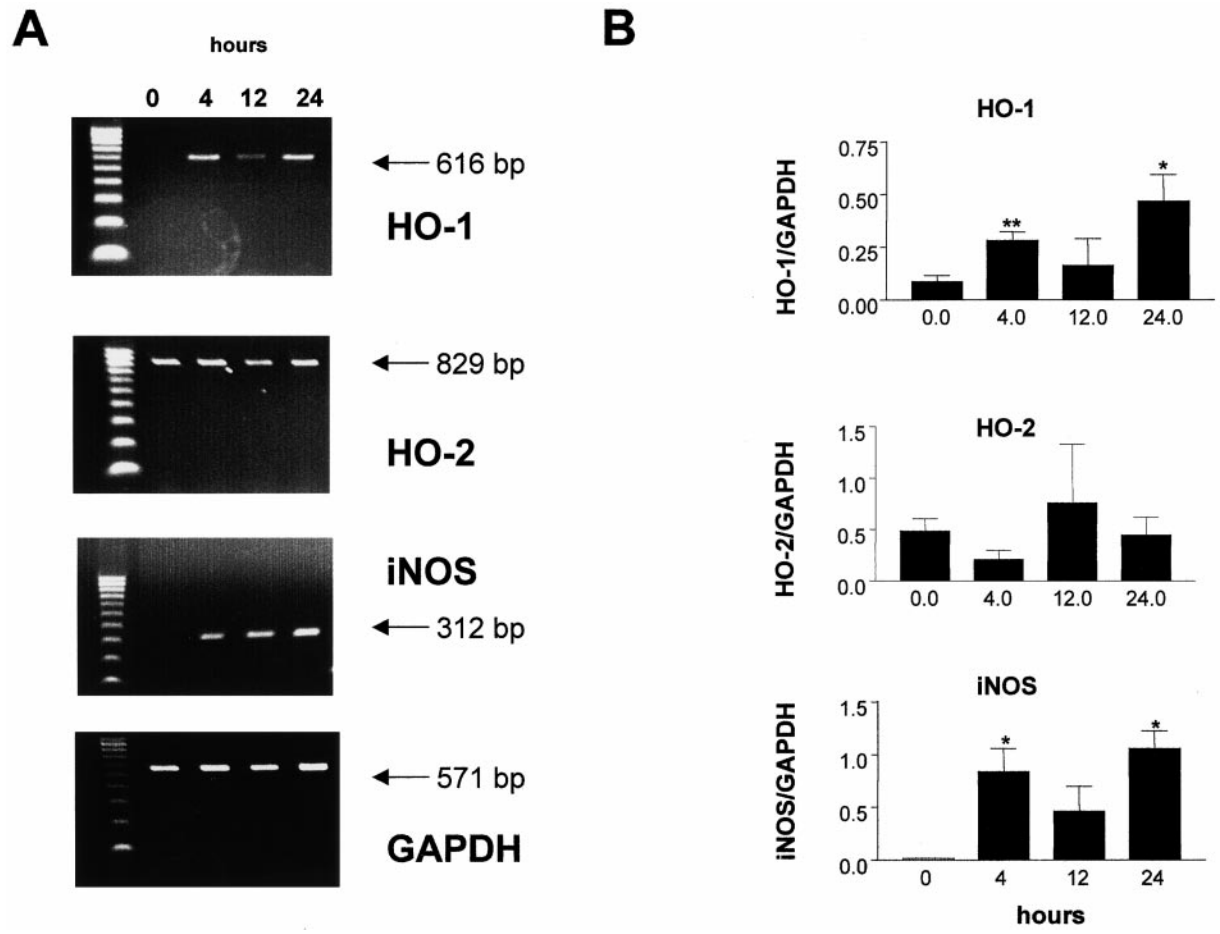

Figure 1. Time course of expression of HO-1, HO-2, and iNOS mRNA in human primary epithelial cells stimulated with 50 ng/ml cytomix. Human primary epithelial cells were cultured in the presence of 50 ng/ml cytomix for the time indicated. RNA was extracted from the cells and RT-PCR performed. (*A*) Representative PCR gels for HO-1, HO-2, iNOS, and GAPDH are shown. (*B*) Densiometric analysis of gels expressed as a ratio of GAPDH for $n = 7$ separate experiments, where $^{**}P < 0.01$ and $^{*}P < 0.05$, is shown.

mean of separate experiments performed using cells from *n* different subjects ± standard error of the mean (SEM).

Results

Expression of HO Messenger RNA in Human Airway Epithelial Cells Exposed to Cytomix

There was baseline expression of HO-1 messenger RNA (mRNA) in human primary epithelial cells; however, culture of the cells in the presence of 50 ng/ml cytomix (IL-1β, TNF-α, and IFN-γ) showed that HO-1 expression increased approximately fivefold after 4 h of exposure (Figure 1). This decreased at 12 h but was significantly greater than basal levels at 24 h. In contrast, HO-2 expression in these cells did not change over 24 h. Cytomix exposure also increased the expression of iNOS in these cells, but this followed a different time course than did HO-1 with maximum expression seen after 24 h of exposure to cytomix. The effect of dexamethasone on cytomix-induced HO-1 and HO-2 expression was also examined. However, the treatment of primary epithelial cells with 1 μM dexamethasone did not inhibit the expression of cytomix-induced HO-1 mRNA expression. There was also no effect on HO-2 mRNA expression (data not shown).

Effect of Dexamethasone on Cytomix-Induced Nitrite and GM-CSF Accumulation from Human Primary Epithelial Cells

Culture of primary human epithelial cells in the presence of dexamethasone failed to inhibit cytomix-induced nitrite accumulation in these cells (Figure 2a). Cytomix stimulated cells to produce nitrite when compared with unstimulated cells (1.77 ± 0.29 versus 3.46 ± 0.52 μM nitrite); however, incubation of cells in the presence of 1 μM dexamethasone and cytomix had no effect on nitrite accumulation (3.34 ± 0.79 μM nitrite). Dexamethasone did inhibit cytomix-stimulated GM-CSF release from these cells (Figure 2b). Cytomix stimulated GM-CSF release from these cells (0.2 ± 0.15 versus 0.8 ± 0.4 ng/ml, $n = 8$); however, 1 μM dexamethasone inhibited release of GM-CSF by 50%. Therefore, human primary epithelial cells were not steroid insensitive.

Effect of 1400W and L-NAME on Cytomix-Induced Expression of HO-1 in Primary Human Epithelial Cells

In an attempt to separate the possible effect of NO generated from the induction of iNOS and the induction of HO-1 by cytomix, primary epithelial cells were cultured in the absence or presence of cytomix with or without the specific iNOS inhibitor 1400W (5 μM). The 1400W inhibited cytomix-induced nitrite accumulation in epithelial cells (3.1 ± 0.5 versus 2 ± 0.3 μM nitrite, $n = 12$) (Figure 3C); however, there was no effect on the cytomix induction of iNOS in these cells. Culture of epithelial cells in the presence of 1400W attenuated cytomix induction of HO-1 mRNA (Figure 3). The possibility that 1400W may also be acting as an oxidant scavenger was addressed by using the less specific NOS inhibitor N^G-nitro-L-arginine-methylester (L-NAME) (1 mM). L-NAME inhibited cytomix-induced nitrite production (3.1 ± 0.5 versus 1.7 ± 0.2 μM nitrite, $n = 12$) and reduced the induction of HO-1 significantly in these cells (Figure 3). Neither 1400W nor L-NAME had any effect on iNOS transcription (Figure 3). This would suggest that iNOS-derived NO may play a role in the induction of HO-1 mRNA expression.

Effect of an NO donor on HO mRNA Expression

The effect of NO directly on induction of HO-1 was examined by the incubation of primary epithelial cells in the presence of an NO donor, NOC-18. Exposure of cells to 500 μM NOC-18 for 4 h led to an induction of HO-1 expression, but there was no change in HO-2 expression in these cells (Figure 4). There was no effect of lower concentrations (50 to 100 μM) of NOC-18 on HO-1 expression (Figure 4). NOC-18 treatment of cells led to increased nitrite accumulation in cell culture media of approximately 20, 40, and 50 μM nitrite for 50, 100, and 500 μM NOC-18, respectively. These concentrations of NOC-18 did not effect cell viability as measured by MTT assay at either 4 or 24 h.

Expression of HO mRNA in Human Airway Epithelial Cells Exposed to NOC-18

Culture of human primary epithelial cells in the presence of 500 μM NOC-18 showed that HO-1 expression increased twofold after 4 h of exposure (Figure 5). There was no effect of NOC-18 on HO-2 expression in these cells.

Effect of Dexamethasone on NO-Induced HO-1 Expression in Human Primary Epithelial Cells

The effect of dexamethasone on NOC-18–induced HO-1 and HO-2 mRNA expression was examined. Treatment of primary epithelial cells with 1 μM dexamethasone did not inhibit the expression of NOC-18–induced HO-1 expression. There was also no effect on HO-2 mRNA expression (Figure 6). To determine whether NO induction of HO-1 was mediated through increased levels of cGMP, human primary epithelial cells were exposed to NOC-18 in the presence of the guanylyl cyclase inhibitor ODQ or the protein kinase G inhibitor KT5823. Neither ODQ nor KT5823 had any effect on the NO-induced induction of HO-1 mRNA (data not shown).

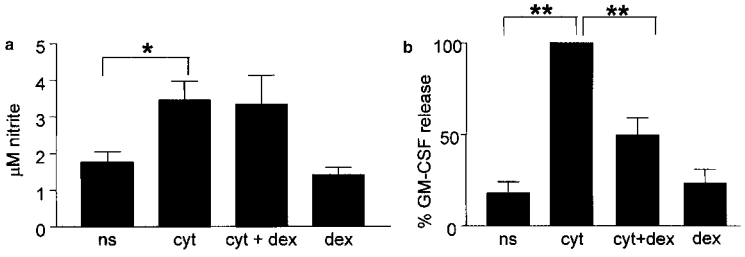

Figure 2. Effect of dexamethasone on cytomix-induced nitrite and GM-CSF accumulation in human primary epithelial cells. Human primary epithelial cells were cultured for 24 h in the absence or presence of 50 ng/ml cytomix with or without 1 μM dexamethasone. Medium was harvested and nitrite levels (*a*) and GM-CSF levels (*b*) measured as described in MATERIALS AND METHODS. Data is expressed as mean ± SEM, $n = 4$, where $^{**}P < 0.01$ and $^{*}P < 0.05$.

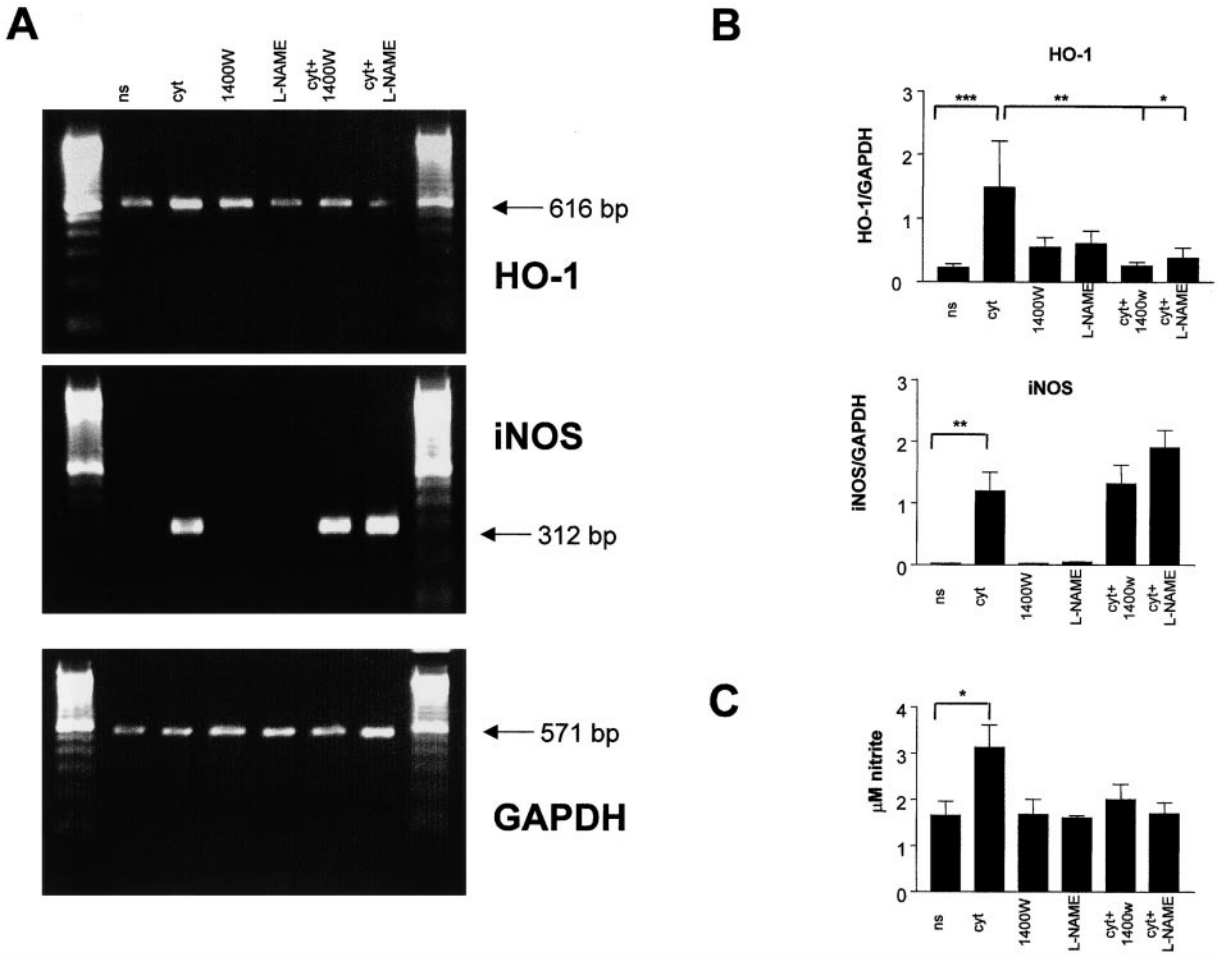

Figure 3. Effect of the iNOS inhibitors 1400W and L-NAME on HO-1 and iNOS mRNA expression in human primary epithelial cells. Human primary epithelial cells were cultured for 24 h in the absence or presence of 50 ng/ml cytomix with or without 5 μM 1400W or 1 mM L-NAME. RNA was extracted from the cells and RT-PCR performed for HO-1, iNOS, and GAPDH, and the medium was assayed for nitrite. (*A*) Representative PCR gels for HO-1, iNOS, and GAPDH are shown. (*B*) Densiometric analysis of gels expressed as a ratio of GAPDH is shown. (*C*) Nitrite accumulation in the cell medium for $n = 11$ separate experiments, where $^{***}P < 0.001$, $^{**}P < 0.01$, and $^{*}P < 0.05$, is shown.

Expression of HO-1 and HO-2 Protein in Human Primary Epithelial Cells

Examination of the level of HO expression in human primary epithelial cells by Western blot analysis showed that HO-2 is constitutively expressed in human primary epithelial cells (Figure 7) and its expression was not altered by incubation with cytomix. HO-1 protein was not expressed in these cells but could be induced after incubation for 24 h in the presence of 50 ng/ml cytomix (Figure 7). Dexamethasone treatment of cytomix-stimulated cells had no effect on the expression of HO-1; however, dexamethasone enhanced the protein expression of HO-2 in both the absence and presence of cytomix (Figure 7).

HO Activity in Human Primary Epithelial Cells

HO activity was measured using the production of CO as a marker of enzyme activity. CO binds rapidly to hemoglobin, and the resultant carboxyhemglobin was measured spectrophotometrically. Primary epithelial cells exposed to 50 ng/ml cytomix for 24 h increased CO production by eightfold (Figure 8). Exposure of the cells to NOC-18 led to a dose-dependent increase in HO activity with the largest increase in activity following exposure to 500 μM NOC-18 (25-fold) (Figure 8).

Discussion

The airway epithelium may provide a source of exhaled CO because human primary epithelial cells express both HO-2 mRNA and protein, constitutively. The elevated levels of exhaled CO observed in airway diseases such as asthma (15) may also be derived from the airway epithelium because inflammatory cytokines can increase the expression of HO-1 mRNA and protein in human primary epithelial cells. The induction of HO-1 mRNA in these cells after cytokine exposure occurred after 4 h and then declined. This was different from the time course of iNOS mRNA induction in these cells. This would suggest that the 4-h induction of HO-1 is due directly to the action of

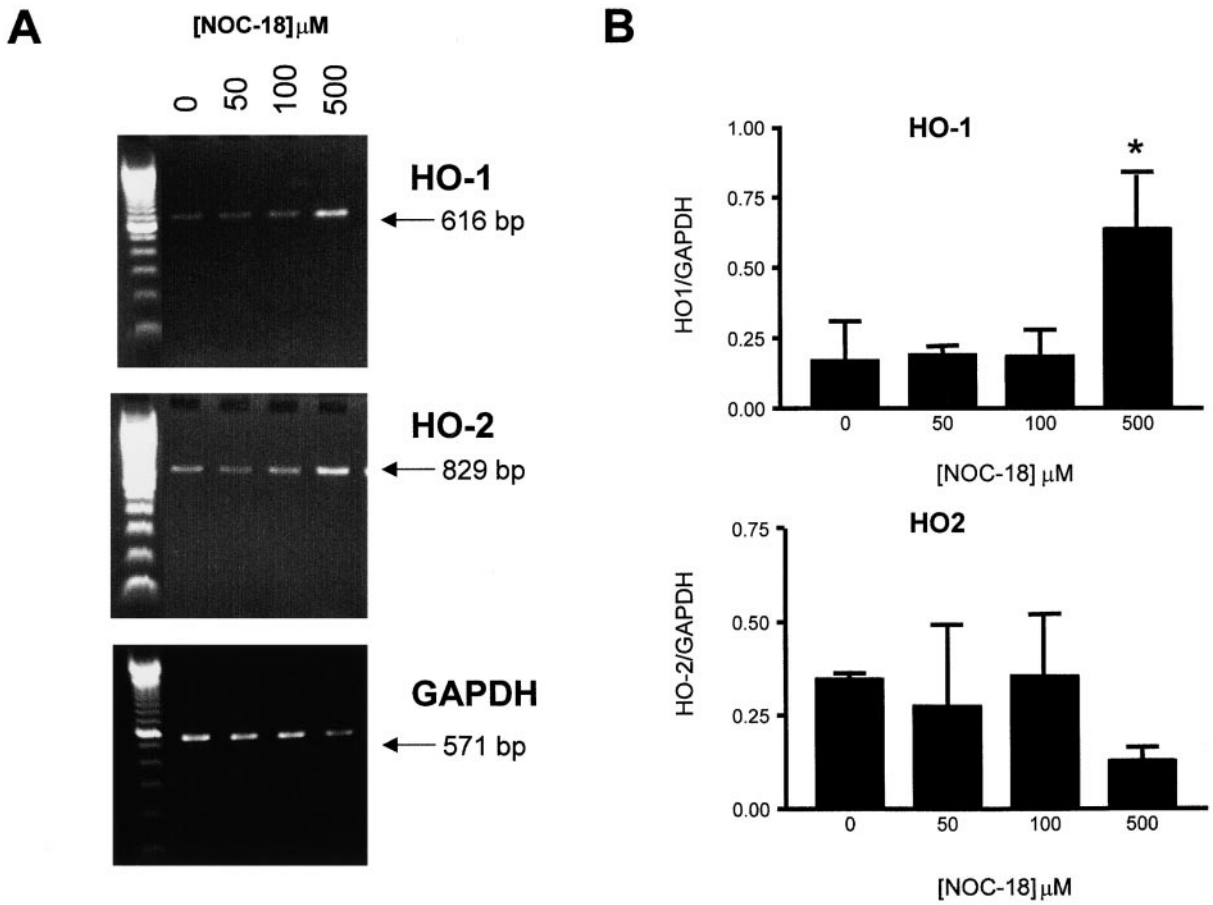

Figure 4. Effect of NOC-18 on the expression of HO-1 and HO-2 mRNA in human primary epithelial cells. Human primary epithelial cells were cultured for 4 h in the presence of NOC-18. RNA was extracted from the cells and RT-PCR performed for HO-1, HO-2, and GAPDH. (*A*) Representative PCR gels for HO-1, HO-2, and GAPDH are shown. (*B*) Densiometric analysis of gels expressed as a ratio of GAPDH for $n = 5$ separate experiments, where $*P < 0.05$, is shown.

inflammatory cytokines rather than to iNOS-derived NO, as 4 h is too short a time for the induction of iNOS protein in human primary epithelial cells (27). HO-1 is induced by inflammatory cytokines in other cell types (3, 17) and human primary epithelium may thus behave in a way similar to other cells.

The observation that the specific iNOS inhibitor 1400W and L-NAME attenuated cytokine-stimulated HO-1 mRNA expression suggests that iNOS-derived NO may play a role in the regulation of HO-1 expression in human primary epithelial cells. The fact that the NO donor NOC-18 could also increase HO-1 mRNA expression further substantiates this. HO-2 expression was not altered in the presence of the NO donor, suggesting a differential effect on HO-1 expression. NO increases HO-1 expression in a number of rat-derived cells at both the mRNA and protein level (20, 21). Human HO-1 has also been induced by NO in HeLa cells (28). This, however, is the first demonstration of HO-1 induction by NO in epithelial cells.

The precise mechanism of induction of HO-1 by NO is unclear. Some reports have suggested that the mechanism may be cGMP independent (19, 23), whereas others have suggested a role for cGMP in HO-1 induction (22). The guanylyl cyclase inhibitor ODQ and the protein kinase G inhibitor KT5823 failed to inhibit NO-induced expression of HO-1. Therefore, it would appear that human primary epithelial cells also exhibit induction of HO-1 by NO via a cGMP-independent mechanism.

Glucocorticosteroids are a commonly used therapy in asthma and other inflammatory diseases; therefore, the effect of the steroid dexamethasone on HO gene expression was examined. However, there was no effect of dexamethasone on HO-1 induction by cytomix or NO, and no effect on HO-2 mRNA induction. Human HO-1 promoter studies have demonstrated a downregulation of HO-1 gene transcription in the presence of dexamethasone (29). It has been suggested that this downregulation of HO-1 transcription was dependent on protein:protein interactions possibly by interacting with the transcription factor nuclear factor-κB. HO-1 expression also requires the transcription factor activator protein-1, which can also be inhibited by steroids through a similar mechanism (30). It is this mechanism that is thought to be responsible for dexamethasone inhibition of hepatic rat HO-1 induction by glutathione depletion (30) and IL-1 and TNF induction of hepatic HO-1 in mice (31). Because dexamethasone did not inhibit HO-1

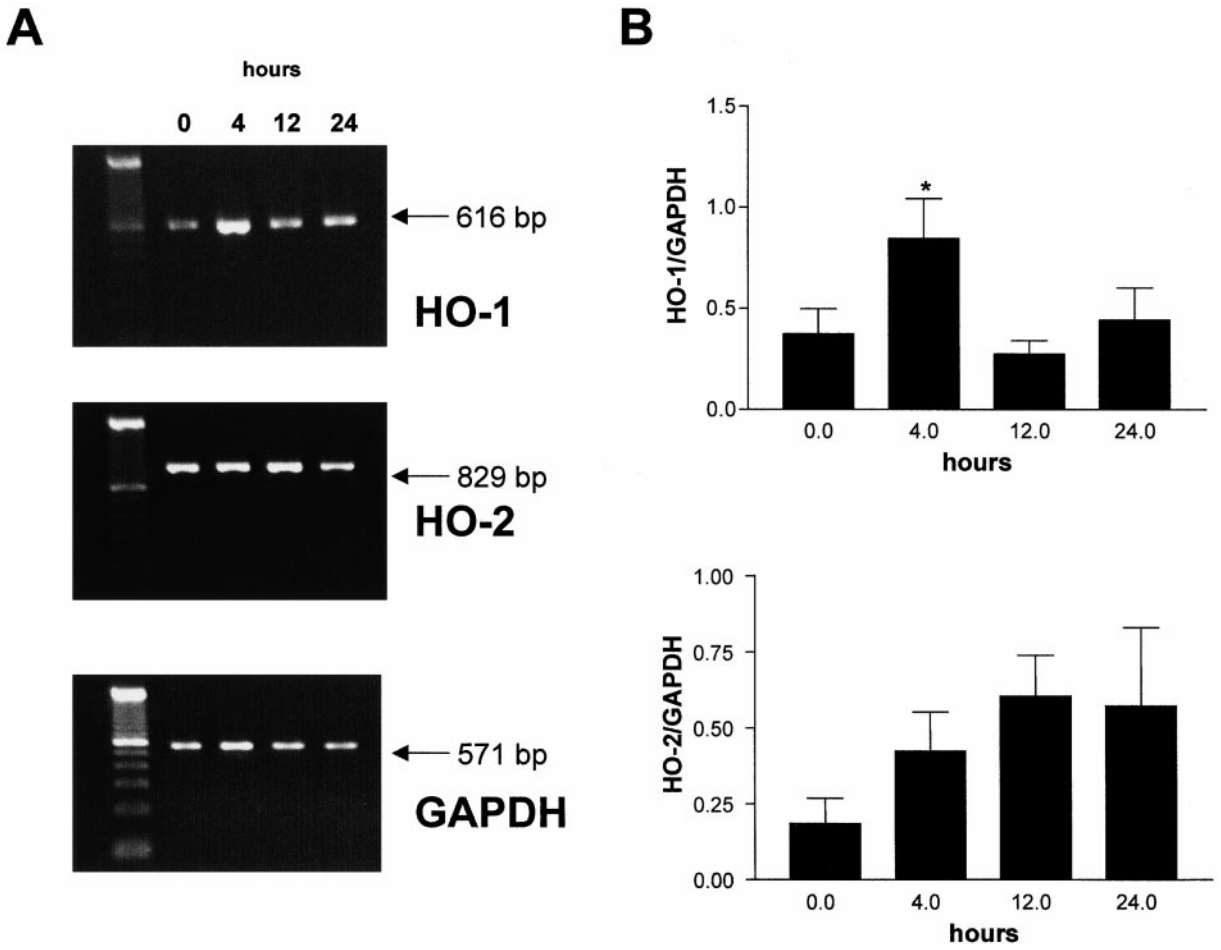

Figure 5. Time course of expression of HO-1 and HO-2 mRNA in human primary epithelial cells stimulated with 500 μM NOC-18. Human primary epithelial cells were cultured in the presence of 500 μM NOC-18 for the time indicated. RNA was extracted and PCR performed. (*A*) Representative PCR gels for HO-1, HO-2, and GAPDH are shown. (*B*) Densiometric analysis of the gels expressed as a ratio of GAPDH for $n = 7$ separate experiments, where $*P < 0.05$, is shown.

mRNA expression in these cells, NO induction of HO-1 may not occur through these mechanisms. The failure of dexamethasone to inhibit cytomix-induced HO-1 mRNA expression may also indicate that endogenously produced NO may have an important role to play in the regulation of HO-1 in the airway.

In contrast, dexamethasone increased the expression of HO-2 protein in human primary epithelial cells. HO-2 has also been shown to be upregulated by dexamethasone in neonatal rat brain (32). This increased expression of HO-2 after exposure with dexamethasone may be important therapeutically by increasing HO activity and hence protection against oxidative stress in the airway. Therefore, this role of steroids to increase an antioxidant may add to the known anti-inflammatory roles of steroids in airway disease.

Increased expression of HO-1 mRNA in primary cells required 500 μM NOC-18. This would generate large amounts of NO and the levels of nitrite generated are far greater than the levels generated by the induction of iNOS in these cells. However, because NO appears to be behaving in an autocrine fashion, it is possible that NO, once synthesized, may not leave the cell before stimulating HO-1 induction. Therefore, the concentration of nitrite in cell culture medium may not be a true reflection of the level of NO produced from human primary epithelial cells.

The observation that NOC-18 was able to increase HO activity at concentrations lower than those required for HO-1 induction would suggest that NO might directly activate HO activity. Whether this increase in activity is due to HO-1 or HO-2 is unknown as there are no selective inhibitors available for either of these isoforms. There are no studies on the stimulation of HO-1 activity, although it has been suggested that HO-2 can be phosphorylated and activated via protein kinase C (33). It is possible that NO may be activating enzyme activity in a similar manner. This would suggest that NO derived from iNOS could not only increase HO-1 expression but also HO activity in human primary epithelial cells. This may be important in inflammatory airway diseases such as asthma, where not only exhaled NO but also exhaled CO is increased (15). This would provide a mechanism for continued production of exhaled CO via induction of HO-1 by inflammatory cytokines followed by continued induction of HO-1 by iNOS-

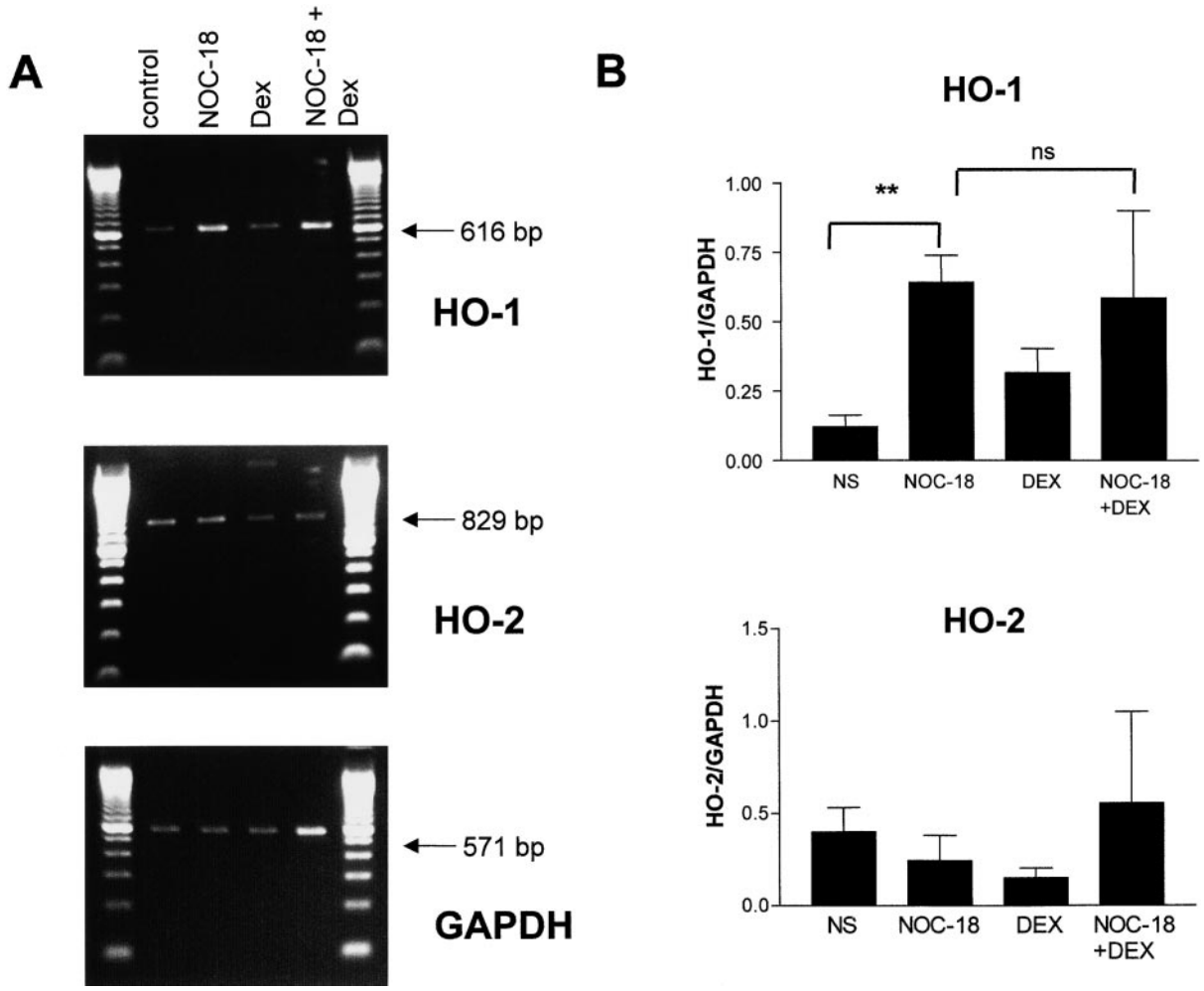

Figure 6. Effect of 1 μM dexamethasone on NOC-18 induced HO-1 mRNA expression in human primary epithelial cells. Human primary epithelial cells were cultured for 4 h in the absence or presence of 500 μM NOC-18 in the absence or presence of 1 μM dexamethasone. RNA was extracted from the cells and RT-PCR performed for HO-1, HO-2, and GAPDH. (*A*) Representative PCR gels for HO-1, HO-2, and GAPDH are shown. (*B*) Densiometric analysis of gels expressed as a ratio of GAPDH for $n = 5$ separate experiments, where $^{**}P < 0.01$, is shown.

derived NO. NO could then also increase HO activity and thus lead to increased levels of exhaled CO. The role of the increased exhaled CO in disease is unclear; however, it may provide an antioxidant function to combat the pro-oxidant effects of NO and other inflammatory mediators.

The relationship between CO and NO may be more complex as NO can also inhibit HO activity by binding to the heme molecule, which is the substrate of HO (34). The mechanism of NO inhibition of HO may be more complex than a simple competition for binding to heme as it has been demonstrated that NO can nitrosylate intracellular heme, thus reducing its availability as a substrate for HO (34). Similarly, as heme is the substrate for HO, its catabolism decreases its availability for NOS synthesis and thus inhibits NOS activity (35). CO can also inhibit NOS activity via binding to the heme molecule contained within the NOS complex. However, the data presented here shows that although the amount of NO generated via NOC-18 may be high, CO production continues to increase. Thus,

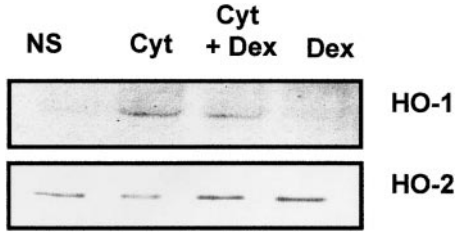

Figure 7. Expression of HO-1 and HO-2 protein in human primary epithelial cells. Human airway epithelial cells were cultured for 24 h in the absence or presence of 50 ng/ml cytomix in the presence or absence of 1 μM dexamethasone. Cells were harvested and 10 μg of total cell protein resolved on 12% (wt/vol) SDS-PAGE. Proteins were transferred onto nitrocellulose and Western blotted for HO-1 and HO-2 protein. The picture is representative of five separate experiments.

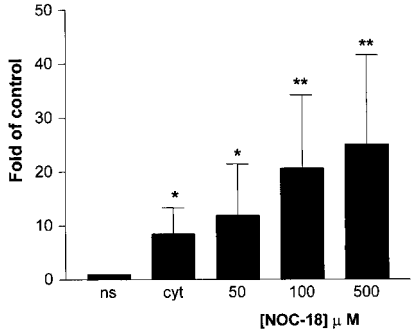

Figure 8. HO activity in human airway epithelial cells. Human airway epithelial cells were cultured for 24 h in the absence or presence of 50 ng/ml cytomix or NOC-18. For the final hour of incubation, hemoglobin was added to the medium at a final concentration of 10 μM. The medium was removed and the difference between the absorbance at 530.1 and 583 nm was measured. Data is expressed as fold increase above untreated cells. The data is $n = 7$ separate experiments, where $*P < 0.05$ and $**P < 0.01$.

sufficient substrate must be available to HO activity under these conditions. This may be similar to the case in asthmatic patients, where both exhaled CO and NO are increased.

This study demonstrates that human airway epithelium could contribute to exhaled CO via the action of HO. Furthermore, mediators that are increased in inflammatory diseases such as asthma also increase HO-1 expression and HO activity *in vitro*, thus providing a mechanism for the increased exhalation of CO observed in airway inflammatory disease.

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