Nitric oxide increases IL-8 gene transcription and mRNA stability to enhance IL-8 gene expression in lung epithelial cells

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Sparkman, Loretta, and Vijayakumar Boggaram. Nitric oxide increases IL-8 gene transcription and mRNA stability to enhance IL-8 gene expression in lung epithelial cells. Am J Physiol Lung Cell Mol Physiol 287: L764–L773, 2004. First published May 28, 2004; 10.1152/ajplung.00165.2004.—Interleukin (IL)-8, a C-X-C chemokine, is a potent chemoattractant and an activator for neutrophils, T cells, and other immune cells. The airway and respiratory epithelia play important roles in the initiation and modulation of inflammatory responses via production of cytokines and surfactant. The association between elevated levels of nitric oxide (NO) and IL-8 in acute lung injury associated with sepsis, acute respiratory distress syndrome, respiratory syncytial virus infection in infants, and other inflammatory diseases suggested that NO may play important roles in the control of IL-8 gene expression in the lung. We investigated the role of NO in the control of IL-8 gene expression in H441 lung epithelial cells. We found that a variety of NO donors significantly induced IL-8 mRNA levels, and the increase in IL-8 mRNA was associated with an increase in IL-8 protein. NO induction of IL-8 mRNA was due to increases in IL-8 gene transcription and mRNA stability. NO induction of IL-8 mRNA levels was not inhibited by 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one and KT-5823, inhibitors of soluble guanyl cyclase and protein kinase G, respectively, and 8-bromo-cGMP did not increase IL-8 mRNA levels. This indicated that NO induces IL-8 mRNA levels independently of changes in the intracellular cGMP levels. NO induction of IL-8 mRNA was significantly reduced by inhibitors of extracellular regulated kinase and protein kinase C. IL-8 induction by NO was also reduced by hydroxyl radical scavengers such as dimethyl sulfoxide and dimethylthiourea, indicating the involvement of hydroxyl radicals in the induction process. NO induction of IL-8 gene expression could be a significant contributing factor in the initiation and induction of inflammatory response in the respiratory epithelium.

Nitric oxide (NO) plays a central role in a variety of physiological and pathological processes (53). NO synthases (NOS), enzymes that produce NO, are composed of three isoforms, endothelial and neuronal enzymes, which are constitutively expressed, and inducible NOS, which is induced by proinflammatory stimuli (35). NO has been called a double-edged sword, owing to its beneficial and damaging effects on cellular physiology. NO produced by the actions of constitutively expressed NOS serves as a messenger/modulator agent vital for the functions of immune and other cells. On the other hand, induction of inducible NOS results in the production of NO at nanomolar levels that is sustained for extended periods of time ranging from several hours to days (39). Elevated NO is believed to contribute to tissue damage through its actions to induce an inflammatory response. Elevated NO levels are found in a number of inflammatory diseases of the lung, such as adult respiratory distress syndrome (48), murine model of RSV infection (19), and asthma (22). Mice in which inducible NOS had been genetically ablated showed a significant decrease in the inflammatory response when subjected to direct endotoxin (45) or ozone (11) challenge, indicating the involvement of NO in the inflammatory response. The association between elevated NO levels and IL-8 during inflammation suggests that NO levels may play important roles in the control of IL-8 gene expression in the lung.

Although cytokine induction of IL-8 gene expression has been extensively studied, no information is available on the NO regulation of IL-8 gene expression in the respiratory epithelium. Considering that the respiratory epithelium has the capacity to produce high levels of NO, it is important to understand the molecular mechanisms that mediate NO regulation of IL-8 gene expression. In this study, we investigated the effects of NO donors on IL-8 gene expression in lung respiratory epithelia play important roles in the clearance of inhaled particulates, antimicrobial defense, and maintenance of alveolar integrity. In addition, the airway and respiratory epithelial cells are also involved in the induction and modulation of inflammatory responses via production of proinflammatory mediators such as cytokines (33, 50) and surfactant (9). High concentrations of IL-8 are present in the bronchoalveolar lavage fluid and lung edema fluids from patients with adult respiratory distress syndrome (8, 18, 29) and in nasal lavage fluids of children with RSV infection (36), indicating that IL-8 plays a major role in the recruitment of neutrophils into the lung. Neutralizing antibodies against IL-8 prevented lung injury in animal models of lung disease, indicating that IL-8 is an important mediator of lung injury (5, 30).

Chemokines constitute a superfamily of small (8–10 kDa) proinflammatory cytokines that are involved in a variety of immune and inflammatory responses, acting primarily as chemotactants and activators of specific types of leukocytes (25). Interleukin (IL)-8, a chemokine that belongs to the C-X-C family of chemokines, is a potent chemoattractant and an activator for neutrophils, T cells, and other immune cells (31). Acute lung injury associated with sepsis (37), adult respiratory distress syndrome (57), and respiratory syncytial virus (RSV) infection in infants (10) is characterized by the accumulation of increased numbers of neutrophils in the lungs and a pulmonary inflammatory response. Activated neutrophils release degradative enzymes such as elastase, collagenase, and reactive oxygen and nitrogen radicals to cause tissue injury. The airway and
epithelial cells. We found that NO donors increased IL-8 gene expression by increasing gene transcription and mRNA stability. Furthermore, we found that the NO induction of IL-8 mRNA levels occurred independently of alterations in the intracellular cGMP levels but was inhibited by mitogen-activated protein kinase (MAPK) and protein kinase C inhibitors. NO induction of IL-8 mRNA levels was also inhibited by curcumin and pyrroldineldithiocarbamate (PDTC), inhibitors of nuclear factor (NF)-κB and activator protein (AP)-1 transcription factors. Hydroxyl radical scavengers, such as dimethyl sulfoxide (DMSO) and dimethylthiourea (DMTU), blocked the NO induction of IL-8, indicating the involvement of hydroxyl radicals in the induction process.

METHODS

Cell culture. NCI-H441 cells [American Type Culture Collection (ATCC) HTB-174], a human lung adenocarcinoma cell line of bronchial (Clara) cell lineage, and BEAS-2B cells (ATCC CRL-9609), an SV40 transformed human bronchial epithelial cell line, were grown on plastic tissue culture dishes in RPMI 1640 medium containing 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and amphotericin B (0.25 μg/ml). AS49 cells (ATCC CCL-185), a human lung carcinoma cell line of certain characteristics of alveolar type II cells, were grown on plastic tissue culture dishes in F-12K medium containing 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and amphotericin B (0.25 μg/ml).

Chemicals. S-nitro-N-acetylpenicillamine (SNAP) (Sigma), diethylenetriamine/NO adduct (DETA/NO) (Sigma), and spermine NONOate (SP/NO) (Calbiochem) served as NO donors (12). These donors undergo spontaneous decomposition in aqueous medium at physiological pH to release NO. Cells were exposed to DETA/NO and SP/NO in serum-containing medium. Because SNAP can react with proteins present in serum via transnitrosation reaction, cells were exposed to SNAP in serum-free medium. Due to its long half-life (20 h) and first-order kinetics of decomposition, DETA/NO was preferred as the NO donor for the majority of the experiments.

Hemoglobin from bovine erythrocytes was from Calbiochem.

RNA isolation and Northern blot analysis. Experimental procedures for the isolation of RNA and Northern blotting analysis are as described previously (27). Total RNA from cells was isolated by the acid-guanidinium thiocyanate-phenol method using TRI Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer’s instructions. Cytosolic RNA was extracted according to published protocol (14). RNA concentration was determined by measuring absorbance at 260 nm. Equal amounts of total RNA (10–15 μg) were separated by electrophoresis on agarose gels (1%) containing 0.32 M sucrose, 2 mM magnesium acetate, 0.1 mM EDTA, 1 mM DTT, and 0.5% (vol/vol) Nonidet P-40 for 5 min on ice. Nuclei were collected by centrifugation at 500 0 g for 5 min at 4°C and washed once in 4 ml of ice-cold sucrose buffer I. Supernatant was saved for the isolation of cytoplasmic RNA. Nuclei were suspended in 100 μl of glycerol storage buffer [50 mM Tris·Cl, pH 8.3, containing 40% (vol/vol) glycerol, 5 mM MgCl2, and 0.1 mM EDTA] and counted with a hemacytometer. Nuclei (100 μl containing 7–15 × 106 nuclei) were labeled by incubating with an equal volume of 2× reaction buffer (10 mM Tris·Cl, pH 8.0, containing 5 mM MgCl2, 0.3 M KCl, 1 mM each of ATP, CTP, and GTP, and 5 mM DTT) and 100 μCi [32P]UTP (3,000 Ci/mmol) for 30 min at 30°C. Reaction was terminated by adding 1.4 ml of TRI Reagent, and RNA was isolated. Methods for RNA isolation and hybridization have been described previously (3). Equal amounts (10–30 × 106 counts/min) of radioactive RNA were hybridized to nitrocellulose membranes containing immobilized plasmid DNAs containing human IL-8 and GAPDH cDNAs. After washing, radioactivity bound to the filters was quantified with a PhosphorImager.

Plasmid DNA isolation. Reporter plasmids containing −546/+44 bp and −133/+44 bp sequences of human IL-8 gene (32) linked to luciferase reporter were kindly provided by Dr. Naofumi Mukaida, Cancer Research Institute, Kanazawa University (Kanazawa, Japan). Plasmid DNAs were amplified in Escherichia coli top 10 strain (Invitrogen, Carlsbad, CA) and purified by anion exchange chromatography with a QIA filter placid purification kit (Qiagen, Valencia, CA). The quality of the plasmid DNAs was verified by agarose gel electrophoresis, and at least two independent plasmid preparations were used in transfection assays.

Transient transfection and reporter gene assays. Plasmid DNAs were transiently transfected into cells by liposome-mediated DNA transfer with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. β-Galactosidase and luciferase reporter activities in cell extracts were determined using β-galactosidase (Tropix, Bedford, MA) and luciferase (Promega) chemiluminescent assay kits, respectively.

Statistical analysis. Data are shown as means ± SE. In experiments where IL-8 mRNA level/promoter activity in control cells was arbitrarily set at 100%, statistical significance was analyzed by one-sample t-test. For other samples, unpaired t-test was used to analyze statistical significance. One-tailed P values of <0.05 were considered significant.

RESULTS

NO donors increase IL-8 mRNA levels in H441 lung epithelial cells. The role of NO in the regulation of IL-8 gene expression in lung epithelial cells is not known. Increased production of NO during inflammatory response is associated with increased recruitment and infiltration of neutrophils into airways and alveoli. Because IL-8 serves as an important chemoattractant for neutrophils, the enhanced recruitment and infiltration of neutrophils may be due to NO-mediated induction of IL-8 expression. To determine whether NO influences IL-8 gene expression, we analyzed the effects of NO donors on IL-8 mRNA levels in H441 lung epithelial cells. Semiconfluent H441 cells were exposed to varying concentrations (0.1–1 mM) of the following NO donors, DETA/NO, SP/NO, and SNAP for 12 h, and their effects on IL-8 mRNA levels were analyzed by Northern blotting. Results (Fig. 1) showed that all the NO donors tested acted in a dose-dependent manner to increase IL-8 mRNA levels. At the highest concentration used, DETA/NO, SP/NO, and SNAP increased IL-8 mRNA levels by 13-, 10-, and 4-fold, respectively, compared with control. The inductive effects of DETA/NO were time dependent; the
Inductive effect was evident after 6 h and attained peak levels after 12 h (Fig. 2). Treatment of other cells such as A549, a cell line with characteristics of alveolar type II cells, and BEAS-2B, a bronchial epithelial line, with DETA/NO (0.1–1 mM) increased IL-8 mRNA levels in a dose-dependent manner, indicating that NO has similar inductive effects on IL-8 gene expression in different epithelial cells of the lung (data not shown).

At the concentrations used, the NO donors did not have significant cytotoxic effects on the cells as assessed by light microscopy, total RNA yields, and differential gene expression. Light microscopic examination did not reveal any significant detachment of cells, and the total RNA yields from control and treated cells were similar, indicating a lack of significant cytotoxic effects of NO donors. This was further indicated by the differential effects of the NO donors on gene expression in H441 cells; although NO donors increased IL-8 mRNA levels, they inhibited SP-B (42) and had no effect on GAPDH mRNA levels.

NO donor increase of IL-8 mRNA levels is associated with increase of IL-8 protein levels. To determine whether NO donor induction of IL-8 mRNA levels is associated with increases in IL-8 protein levels, we determined the levels of IL-8 in media from control cells and cells treated with DETA/NO (1 mM) by ELISA. Results (Fig. 3) showed that, after 24 h of incubation, DETA/NO increased immunoreactive IL-8 levels in the media by nearly fourfold compared with control cells. These data indicate that NO increase in IL-8 mRNA levels is associated with increases in IL-8 protein levels, albeit to a lesser extent.

NO donor induction of IL-8 mRNA levels is due to NO. The NO donors used in this study are pharmacological agents that serve as sources of NO with the understanding that their effects are primarily due to the released NO. However, this may not always be the case as metabolites of NO and decomposed products of NO donors can exert similar effects as NO (12).
IL-8 mRNA levels were quantified and normalized to 18S rRNA levels. IL-8 and GAPDH mRNAs were analyzed by Northern blotting. A representative autoradiogram showing the effects of NO donors, decomposed NO donors, and hemoglobin on IL-8 mRNA levels. H441 cells were incubated with DETA/NO, decomposed DETA/NO, SNAP, or N-acetyl-penicillamine at a concentration of 1 mM for 12 h, and IL-8 mRNA levels were determined by Northern blotting. Furthermore, cells were also incubated with DETA/NO in the presence or absence of hemoglobin (20 μM), an effective scavenger of NO (12). Results showed that, whereas DETA/NO and SNAP increased IL-8 mRNA levels several fold, decomposed DETA/NO and N-acetyl-penicillamine did not have any inductive effects on IL-8 mRNA levels. Furthermore, the NO scavenger hemoglobin significantly antagonized the inductive effects of DETA/NO. These data clearly indicated that the inductive effects of NO donors are indeed due to NO, and the byproducts of decomposition of NO donors do not influence IL-8 mRNA levels. The induction of IL-8 by DETA/NO was reversible and required the continuous presence of the NO donor (data not shown).

NO induction of IL-8 mRNA levels is not mediated via tumor necrosis factor-α and IL-1β. NO is known to induce the synthesis of tumor necrosis factor (TNF-α) in other cells, such as peripheral blood mononuclear cells (56). The effects of NO on IL-1β levels are not known. Because TNF-α and IL-1β are known inducers of IL-8, we determined whether the effects of NO donors to induce IL-8 mRNA levels are primary or secondary to changes in the levels of TNF-α and IL-1β. H441 cells were exposed to DETA/NO for 12 h in the absence or presence of neutralizing antibodies to TNF-α (0.5 μg/ml) (R & D Systems), and IL-1β (0.5 μg/ml) (R & D Systems) and IL-8 mRNA levels were analyzed by Northern blotting. Results showed that TNF-α and IL-1β antibodies did not block the inductive effects of DETA/NO, indicating that the NO effects are not mediated through the elevation of TNF-α and IL-1β levels. In separate experiments (data not shown), we found that the TNF-α neutralizing antibodies at 1 μg/ml completely blocked the inductive effects of DETA/NO (1 ng/ml and 10 ng/ml) on IL-8 mRNA levels, validating the efficacy of the antibodies. In several independent experiments (data not shown), IL-1β failed to increase IL-8 mRNA levels in H441 cells, indicating the lack of positive effects on IL-8 gene expression. These data indicate that the inductive effects of NO donors on IL-8 mRNA levels are not the result of secondary changes in the levels of TNF-α and IL-1β.

NO donor stimulates IL-8 promoter activity. NO induction of IL-8 mRNA levels could be due to an increase in the IL-8 gene transcription rate or an increase in the stability of IL-8 mRNA or a combination of both mechanisms. Transcriptional inhibitors 5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole (DRB) and actinomycin D significantly reduced DETA/NO treatment effects on DETA/NO induction of IL-8 mRNA. H441 cells were incubated with DETA/NO (1 mM) in the absence or presence of IL-1α, IL-1β neutralizing antibodies (0.5 μg/ml) (R & D Systems) for 12 h. IL-8 mRNA levels were determined by Northern blotting and normalized to 18S rRNA levels. IL-8 mRNA levels in control cells were arbitrarily set at 100, and the levels in treated cells are shown relative to control levels. Data are means ± SE of 3 independent experiments. **P < 0.01 and ***P < 0.001 for DETA/NO compared with control.
induction of IL-8 mRNA levels (data not shown), indicating that continued transcription is necessary for NO induction of IL-8 gene expression. We assessed the role of transcription in the NO induction of IL-8 mRNA levels by determining the effects of DETA/NO on IL-8 promoter activity. H441 cells were transiently transfected with promoter plasmids containing −546/+44 and −133/+44 bp fragments of human IL-8 gene linked to luciferase reporter gene and then treated with DETA/NO (1 mM). Results (Fig. 6) showed that DETA/NO increased luciferase activity from −546/+44 and −133/+44 bp IL-8 promoters to a similar degree. The stimulatory effects of DETA/NO were apparent after 6 h and increased thereafter. These data indicate that transcription contributes to DETA/NO induction of IL-8 gene expression.

Transcriptional and posttranscriptional mechanisms mediate NO induction of IL-8 mRNA levels. NO induction of IL-8 mRNA levels was inhibited by transcriptional inhibitors, DRB, and actinomycin D, indicating that continued transcription and transcriptional mechanisms are necessary for induction. DETA/NO increased IL-8 promoter activity in transient transfection studies, indicating that transcription contributes to induction of IL-8 mRNA levels. However, these studies do not rule out the contribution by posttranscriptional mechanisms, such as mRNA stability. We analyzed the effects of DETA/NO on IL-8 gene transcription rate and steady-state cytosolic IL-8 mRNA levels to determine the relative contributions of transcriptional and posttranscriptional mechanisms in the NO induction of IL-8 mRNA levels. H441 cells were treated with control medium or medium containing DETA/NO (1 mM) for 12 h, and nuclei and cytosolic RNA were isolated. The effects of DETA/NO on IL-8 gene transcription and IL-8 mRNA levels were determined by transcription run-on assay and Northern blotting, respectively. Results (Fig. 7) showed that DETA/NO-mediated increases in IL-8 mRNA levels were not associated with proportional increases in IL-8 gene transcription rate. In three independent experiments, we found that, although DETA/NO increased IL-8 mRNA levels by 20- to 30-fold, it increased IL-8 gene transcription rate by ~5-fold.

These data indicate that, in addition to transcription, posttranscriptional mechanisms also contribute to the NO induction of IL-8 mRNA levels.

DETA/NO enhances the stability of IL-8 mRNA. To determine whether DETA/NO alters the stability of IL-8 mRNA, the half-life of IL-8 mRNA in control cells and cells treated with DETA/NO was determined. H441 cells were first incubated in control medium or medium containing DETA/NO (1 mM) for 12 h, and then incubation continued in the presence of the transcriptional inhibitor actinomycin D (5 μM). Total RNA isolated from cells collected at different times after the addition of transcriptional inhibitors was analyzed for IL-8 and GAPDH mRNA levels by Northern blotting. Results (Fig. 8) showed that the half-life of IL-8 mRNA was ~2 h in control cells, and it increased markedly after treatment with DETA/NO. No appreciable degradation of IL-8 mRNA was observed in DETA/NO-treated cells over a 24-h time period. The half-life of GAPDH was not altered by DETA/NO treatment, indicating that the effect of NO to alter IL-8 mRNA half-life is specific.
Similar results were obtained when DRB was used as a transcriptional inhibitor (data not shown). These data indicate that enhanced mRNA stability significantly contributes to the NO increase of IL-8 mRNA levels.

NO increases IL-8 mRNA levels independently of cGMP signaling pathway. NO exerts many of its biological actions via activation of soluble guanylate cyclase (sGC) (43), which results in elevated intracellular levels of cGMP. We assessed the role of the cGMP signaling pathway in the DETA/NO increase of IL-8 mRNA level by use of inhibitors that block activation of sGC and protein kinase G, a downstream mediator of cGMP. H441 cells were first treated with 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) (5 μM), a sGC inhibitor, or KT-5823 (5 μM), a protein kinase G inhibitor, for 2 h and then exposed to DETA/NO (1 mM) for 12 h. The effects of the inhibitors on DETA/NO induction of IL-8 mRNA levels were analyzed. Results (Fig. 9A) showed that both ODQ and KT-5823 failed to block the inductive effects of DETA/NO on IL-8 mRNA levels, indicating that the cGMP signaling pathway does not play a role in the NO induction of IL-8 mRNA levels. A cell-permeable analog of cGMP, 8-bromo-cGMP, did not alter the expression of IL-8 mRNA (Fig. 9B), further indicating the lack of involvement of the cGMP signaling pathway in the NO induction of IL-8 mRNA.

In other experiments, we found that the cyclic AMP analog dibutyl cyclic AMP (0.1–2 mM) did not alter the levels of IL-8 in control and DETA/NO-treated cells, indicating that the cyclic AMP-mediated protein kinase A pathway is not involved in the NO regulation of IL-8 mRNA levels (data not shown).

Role of protein kinase signaling pathways in the NO induction of IL-8 mRNA. In contrast to the agents that alter intracellular signaling networks by binding to cell surface receptor(s), NO freely diffuses across cell membranes to act on targets at various intracellular locations to alter signaling networks. NO was found to activate all the three MAPK and phosphatidylinositol 3-kinase cascades as well as protein kinase C in response to DETA/NO (44).

Fig. 8. Effect of DETA/NO on the degradation of IL-8 and GAPDH mRNAs. H441 cells were incubated in control medium or medium containing DETA/NO (1 mM) for 12 h, and then incubation continued in the presence of actinomycin D (5 μM). Cells were processed for RNA isolation at indicated times after the addition of actinomycin D and IL-8, and GAPDH mRNA levels were analyzed by Northern blotting. A: autoradiogram of a representative Northern blot showing the effect of DETA/NO on the degradation of IL-8 and GAPDH mRNAs. B and C: levels of IL-8 and GAPDH mRNAs were quantified with a PhosphorImager and normalized to 18S rRNA levels. IL-8 and GAPDH mRNA levels at 0 h were arbitrarily set at 100, and their levels at different times after the addition of actinomycin D were determined relative to that at 0 h. Data are means ± SE of 3 independent experiments.

Fig. 9. A: effects of cGMP pathway inhibitors on DETA/NO induction of IL-8 mRNA levels. H441 cells were serum-starved for 24 h, treated with KT-5823 (5 μM), a protein kinase G inhibitor, or ODQ (5 μM), a guanylate cyclase inhibitor, for 2 h, and then incubated in control medium or medium containing DETA/NO (1 mM) for 12 h. IL-8 mRNA levels were analyzed by Northern blotting and normalized to 18S rRNA levels. IL-8 mRNA levels in control cells were arbitrarily set at 100, and the levels in treated cells are shown relative to control. Data are means ± SE of 3 independent experiments. *P < 0.05 and ***P < 0.001 for DETA/NO compared with control. The means of DETA/NO compared with DETA/NO + ODQ (P = 0.35) and DETA/NO vs. DETA/NO + ODQ (P = 0.24) were not significantly different according to unpaired t-test. B: effects of 8-bromo-cGMP, an activator of protein kinase G, on IL-8 mRNA levels. H441 cells were incubated in control medium (C) or medium containing 8-bromo-cGMP (1 and 2 mM) for 12 h, and IL-8 mRNA levels were analyzed. Data are means ± SE of 3 independent experiments.
different cells (40). We used pharmacological inhibitors of protein kinases to investigate their involvement in the NO induction of IL-8 mRNA levels. Cells were serum-starved for 24 h and then treated with the following inhibitors for 2 h: 5 μM U0126, 10 μM SB-203580, 5 μM bisindolylmaleimide, 100 nM wortmannin, 10 μM c-Jun NH2-terminal kinase (JNK) II inhibitor, or 10 μM 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolol[3,4-d]pyrimidine, and then exposed to 1 mM DETA/NO for 12 h. The effects of the protein kinase inhibitors on IL-8 mRNA levels were analyzed by Northern blotting. Results (Fig. 10) showed that extracellular signal regulated kinase (ERK) inhibitor U0126 significantly blocked DETA/NO induction of IL-8 mRNA levels, whereas p38, JNK, Src, and phosphatidylinositol 3 (PI3)-kinase inhibitors had no significant effect on DETA/NO induction. The NO induction of IL-8 mRNA was also significantly inhibited by bisindolylmaleimide, indicating the involvement of the protein kinase C pathway. These results indicate that ERK and protein kinase C signaling pathways may play important roles in mediating the inductive effects of NO on IL-8 gene expression.

Effects of inhibitors of NF-κB and AP-1 transcription factors on NO induction of IL-8 mRNA levels. Previous studies (41) have shown that AP-1 and NF-κB transcription factors play important roles in the cytokine induction of IL-8 gene expression. We used pharmacological inhibitors of NF-κB and AP-1 transcription factors to assess their role in the DETA/NO induction of IL-8 mRNA levels. Specifically, we investigated the effects of curcumin (diferuloylmethane) (17, 47) and resveratrol (26), dietary compounds found in turmeric and red wine, respectively, and PDTC (44), an antioxidant, and acetalsalicylic acid (23) on the DETA/NO induction of IL-8 mRNA levels. Cells were first treated for 2 h with resveratrol (50 μM), curcumin (30 μM), acetalsalicylic acid (15 μM), or PDTC (50 μM) and then incubated with DETA/NO (1 mM) for 12 h. Results (Fig. 11) showed that, whereas resveratrol and acetalsalicylic acid had no effect on NO induction of IL-8 mRNA levels, curcumin and phenylthiocarbamate significantly inhibited NO induction of IL-8 mRNA levels. Interestingly, resveratrol alone increased IL-8 mRNA levels. These data indicate possible involvement of AP-1 and NF-κB transcription factors in the NO induction of IL-8 mRNA.

Effects of antioxidants on the NO induction of IL-8 mRNA levels. NO can function both as a pro- and antioxidant molecule depending on the availability and concentration of potential reaction partners such as superoxide, hydrogen peroxide, or other reactive oxygen species. NO also reacts with thiol and thiol groups in proteins to alter cellular redox state and consequently protein function (49). To determine whether alterations in the cellular redox status plays a role, we investigated the effects of antioxidants such as N-acetylcysteine (NAC), DMSO, DMTU, and mannitol on the NO induction of IL-8 mRNA levels. NAC, a thiol, is a precursor of cysteine and glutathione synthesis as well as a scavenger of free radicals (2). It augments the synthesis of cellular glutathione to increase the ratio of reduced glutathione to oxidized glutathione (2). NO also reacts with thiols and other reactive oxygen species. NO can function both as a pro- and antioxidant molecule depending on the availability and concentration of potential reaction partners such as superoxide, hydrogen peroxide, or other reactive oxygen species. NO also reacts with thiol and thiol groups in proteins to alter cellular redox state and consequently protein function (49). To determine whether alterations in the cellular redox status plays a role, we investigated the effects of antioxidants such as N-acetylcysteine (NAC), DMSO, DMTU, and mannitol on the NO induction of IL-8 mRNA levels. NAC, a thiol, is a precursor of cysteine and glutathione synthesis as well as a scavenger of free radicals (2). It augments the synthesis of cellular glutathione to increase the ratio of reduced glutathione to oxidized glutathione to alter glutathione redox status. Glutathione redox status has been implicated to play a major role in the regulation of cellular function. DMSO, DMTU, and mannitol are known to scavenge hydroxyl radicals (54). Previous studies showed that IL-8 expression in stimulated whole blood and whole blood exposed to DETA/NO was decreased by DMSO, indicating the involvement of hydroxyl radicals in the induction process (55). Cells were first incubated with 2% DMSO, 15 mM DMTU, or 15 mM mannitol for 2 h and 20 mM NAC for 15 h and then exposed to DETA/NO for 12 h. Results (Fig. 12) showed that DETA/NO-induced IL-8 levels were unaltered by NAC but were significantly inhibited by DMSO and DMTU. Interestingly, mannitol did not alter DETA/NO induction of IL-8 mRNA levels. These data suggest that hydroxyl radicals and not changes in the intracellular glutathione redox status play important roles in mediating the DETA/NO induction of IL-8 mRNA levels.
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Fig. 12. Effects of antioxidants on DETA/NO induction of IL-8 mRNA levels. H441 cells were first incubated in control medium (C) or medium containing DMSO (2%), dimethyldithiourea (DMTU; 15 mM), or mannitol (15 mM) for 2 h, and then incubation continued in the presence or absence of DETA/NO (1 mM) for 12 h. In the case of N-acetylcysteine (NAC), cells were first incubated with NAC (20 mM) for 16 h and then exposed to DETA/NO (1 mM) for 12 h. IL-8 mRNA levels were analyzed by Northern blotting and normalized to 18S rRNA levels. IL-8 mRNA levels in control cells were arbitrarily set at 100, and the levels in treated cells are shown relative to control. Data are means ± SE of 3 independent experiments. **P < 0.001 for DETA/NO compared with control and for DETA/NO + DMSO and DETA/NO + DMTU compared with DETA/NO alone.

DISCUSSION

Respiratory epithelial cells have been implicated to play important roles in the initiation and regulation of inflammatory responses through the expression of inflammatory cytokines and growth factors (33, 50). Respiratory epithelium expresses all the forms of NOS (46) and is thus capable of producing elevated levels of NO, suggesting that it plays an important role in host defense in the lung. Many inflammatory diseases of the lung are associated with elevated levels of NO and IL-8. Because IL-8 levels are elevated in lung inflammatory diseases and IL-8 serves as a key chemoattractant for neutrophils, we proposed that NO increases IL-8 levels in the lung epithelium to contribute to the initiation of an inflammatory response.

Our data showed that NO donors increased IL-8 mRNA levels in lung epithelial cells in a dose- and time-dependent manner. The effects of NO on IL-8 mRNA levels appeared to be specific as NO elicited distinct effects on gene expression in H441 cells; whereas NO increased IL-8 mRNA levels, it decreased SP-B mRNA levels and had no effect on GAPDH mRNA levels. NO induction of IL-8 mRNA levels was associated with increases in IL-8 protein levels in the medium, although the increase was lower than the increase in RNA levels. The reasons for the discordant inductive effects of NO on IL-8 mRNA and protein levels are not clear. It is possible that IL-8 levels in the medium do not necessarily reflect total levels because significant amounts of IL-8 could be bound to cell surface and extracellular matrix. Previous studies in cell cultures have shown that DETA/NO (0.5 and 1 mM) and SNAP (2 mM) produced NO levels at concentrations of 0.5–1.5 μM and 3 μM, respectively (34, 38). The concentration of NO at sites of inflammation has been estimated to be in the range of 0.1–0.5 μM (6). Based on these studies, we predict that the NO donors at their highest concentration in our study produce NO levels similar to those found under pathophysiological conditions. NO and superoxide anion rapidly combine to form the potent oxidant peroxynitrite, which is a known inducer of IL-8 gene expression. It remains to be determined whether the inductive effects of NO observed in our study can be attributed to the formation of peroxynitrite. Our data also showed that the inductive effects of NO are not due to secondary changes in the levels of TNF-α and IL-1β, indicating that NO acts directly to induce IL-8 mRNA levels.

The induction of IL-8 mRNA levels was sensitive to transcriptional inhibitors, indicating that continued transcription and transcriptional mechanisms are necessary for DETA/NO induction. DETA/NO increased IL-8 promoter activity, further indicating the importance of transcriptional mechanisms in the induction process. DETA/NO induction of IL-8 mRNA levels was not correlated with a similar increase of IL-8 gene transcription as determined by nuclear run-on and transient transfection promoter assays, indicating that posttranscriptional mechanisms play a significant role in the induction process. Indeed, we found that DETA/NO increased the half-life of IL-8 mRNA significantly. Thus both transcription and mRNA stability contribute to the DETA/NO induction of IL-8 mRNA.

IL-8 gene expression is subject to regulation by a wide variety of agents, including cytokines, bacterial cell wall products, viral agents, and others (41). In the lung epithelium, IL-8 expression is induced by a wide variety of agents, including IL-1 (1), RSV infection (13), oxidants (24), bacterial cell wall products (28), and TNF-α (50). Transcriptional activation of the IL-8 gene and stabilization of IL-8 mRNA contribute to the induction of IL-8 gene expression. Nucleotides spanning −133/+44 bp in the 5’ flanking region of IL-8 gene are necessary and sufficient for the basal and TNF-α activation of IL-8 gene transcription (33). The transcriptional response region contains functionally important DNA binding sites for NF-IL-6, NF-κB, and AP-1 transcription factors (4, 24, 51, 59). These factors appear to act both independently and synergistically to activate IL-8 promoter in response to stimulatory agents in a cell-specific manner. Stabilization of IL-8 mRNA was also found to contribute to the induction of IL-8 mRNA by cytokines and stress factors (7, 20, 21, 52). The p38 MAPK signaling pathway was found to control the stability of IL-8 mRNA through adenine and uracil-rich elements located in the 3’ untranslated region (15). Molecular mechanisms that mediate NO induction of IL-8 gene expression remain to be determined. Our data suggest that mechanisms underlying the inductive effects of NO on IL-8 gene expression are different from those for TNF-α and IL-1β. NO increases the intracellular levels of cGMP via binding and activation of sGC (43). Indeed, the sGC has been termed the intracellular receptor for NO. Blocking discrete components of the cGMP signaling pathway, such as sGC and protein kinase G with pharmacological inhibitors, had no effect on the DETA/NO induction of IL-8 mRNA levels, indicating a lack of involvement of the cGMP pathway in the induction process. Consistent with this observation, cell-permeable analogs of cGMP did not alter DETA/NO induction of IL-8 mRNA levels. NO activates all three MAPK signaling pathways, ERK, p38, JNK, and PI3-kinase signaling pathways in different cells to alter gene expression (40). Using pharmacological inhibitors that specifically inhibit protein kinases, we found that the ERK inhibitor U0126 and the protein kinase C inhibitor bisindolylmaleimide significantly blocked the inductive effects of DETA/NO. On the other hand, inhibitors of p38, JNK, PI3-kinase, and Src kinases did not significantly alter DETA/NO induction of IL-8 mRNA levels. Interestingly, inhibition of the PI3-kinase path—
way by wortmannin enhanced DETA/NO induction of IL-8 mRNA. These data indicate that NO induction of IL-8 gene expression may be dependent on the activation of ERK and protein kinase C signaling pathways. Cytokine induction of IL-8 gene expression is dependent on the activation of different MAPK signaling pathways. Whereas activation of JNK and ERK MAPK pathways are necessary for transcriptional induction, activation of p38 MAPK pathway is required for transcription and mRNA stability. These data indicate that NO induction of IL-8 gene expression in lung epithelial cells cannot be ruled out. Hydroxyl radicals were implicated in the NO induction of IL-8 mRNA levels in U-937 cells (53) and whole blood (55). Pathways underlying NO-mediated generation of hydroxyl radicals and other oxidants that mediate induction of IL-8 gene expression in lung epithelial cells remain to be elucidated.

In conclusion, our studies have shown that NO is a powerful inducer of IL-8 gene expression in lung epithelial cells. NO induced IL-8 mRNA levels by increasing gene transcription and mRNA stability. The inductive effects of NO were independent of cGMP signaling pathway but appeared to be dependent on ERK and protein kinase C signaling pathways. Cytokine-induced stabilization of IL-8 mRNA (16, 58).

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REFERENCES


28. Margana RK and Boggarra V. Transcription and mRNA stability regulate developmental and hormonal expression of rabbit surfactant