Transcriptional regulation of SP-B gene expression by nitric oxide in H441 lung epithelial cells

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Boggaram V, Chandru H, Gottipati KR, Thakur V, Das A, Berhane K. Transcriptional regulation of SP-B gene expression by nitric oxide in H441 lung epithelial cells. Am J Physiol Lung Cell Mol Physiol 299: L252–L262, 2010. First published April 23, 2010; doi:10.1152/ajplung.00062.2010.—Surfactant protein B (SP-B) is essential for the surface tension-lowering function of pulmonary surfactant. Surfactant dysfunction and reduced SP-B levels are associated with elevated nitric oxide (NO) in inflammatory lung diseases, such as acute respiratory distress syndrome. We previously found that NO donors decreased SP-B expression in H441 and MLE-12 lung epithelial cells by reducing SP-B promoter activity. In this study, we determined the roles of DNA elements and interacting transcription factors necessary for NO inhibition of SP-B promoter activity in H441 cells. We found that the NO donor diethylenetriamine-nitric oxide adduct (DETA-NO) decreased SP-B promoter thyroid transcription factor 1 (TTF-1); hepatocyte nuclear factor 3 (HNF-3), and Sp1 binding activities but increased activator protein 1 (AP-1) binding activity. DETA-NO decreased TTF-1, but not Sp1, levels, suggesting that reduced TTF-1 expression contributes to reduced TTF-1 binding activity. Lack of effect on Sp1 levels suggested that DETA-NO inhibited Sp1 binding activity per se. Overexpression of Sp1, but not TTF-1, blocked DETA-NO inhibition of SP-B promoter activity. DETA-NO inhibited SP-B promoter induction by exogenous TTF-1 without altering TTF-1 levels. DETA-NO decreased TTF-1 mRNA levels and gene transcription rate, indicating that DETA-NO inhibits TTF-1 expression at the transcriptional level. We conclude that NO inhibits SP-B promoter by decreasing TTF-1, Sp1, and HNF-3 binding activities and increasing AP-1 binding activity. NO inhibits TTF-1 levels and activity to decrease SP-B expression. NO inhibition of SP-B expression could be a mechanism by which surfactant dysfunction occurs in inflammatory lung diseases.

Nitric oxide (NO) is an important regulatory molecule that plays key roles in a variety of physiological and pathological processes. NO is produced from L-arginine by the actions of constitutively expressed and inducible NO synthases. The respiratory epithelium expresses constitutive and inducible NO synthases (43, 44) and is therefore capable of producing elevated levels of NO for extended periods of time during inflammatory challenges affecting the lung. Elevated NO production by activated alveolar macrophages, owing to its small size and lipophilic nature, can easily diffuse to affect the neighboring respiratory epithelium. Although NO is important for diverse physiological processes in the lung and is used clinically to treat a variety of lung disorders in adults and children, such as neonatal and adult respiratory distress syndrome, lung fibrosis, and pulmonary hypertension, elevated NO levels can be detrimental to lung function. Elevated NO levels are correlated with lung injury in ARDS (46), RSV infection (27), asthma (25), and other lung diseases. Reduced SP-B levels in ARDS (24), RSV infection (28), and other diseases point to a strong association between elevated NO and reduced SP-B levels, suggesting that NO could exert inhibitory effects on SP-B expression, causing surfactant dysfunction and development of lung injury. Indeed, our studies (39) and other studies (4, 7, 12, 29, 49) demonstrated that NO decreases SP-A and SP-B gene expression in lung cells in vitro and in animals. We found that the NO donor diethylenetriamine-nitric oxide adduct (DETA-NO) decreased SP-B mRNA levels and SP-B immunostaining in H441 cells, indicating that NO reduces SP-B protein levels (39). Our previous studies also demonstrated that NO inhibition of SP-B expression in H441 and MLE-12 lung epithelial cells is due to inhibition of SP-B promoter activity. Considering the important and diverse roles that surfactant plays in the maintenance of lung health, it is important to understand molecular regulation of surfactant protein expression by NO. In the present study, we determined the roles of DNA regulatory elements and interacting transcription factors necessary for NO inhibition of SP-B promoter activity in H441 cells. We used DETA-NO as the NO donor because of its prolonged half-life, which results in release of predictable amounts of NO (22). We found that DETA-NO decreases SP-B promoter activity by inhibiting thyroid transcription factor 1 (TTF-1), hepatocyte nuclear factor 1 (HNF-1), and Sp1 binding activities but increases AP-1 binding activity. DETA-NO inhibited SP-B promoter activity by reducing TTF-1, Sp1, and HNF-3 binding activities and increasing AP-1 binding activity.
factor 3 (HNF-3), and Sp1 DNA binding and increasing activator protein 1 (AP-1) binding. Previous studies showed that TTF-1, HNF-3, and Sp1 DNA elements are essential for the full expression of SP-B promoter activity (15, 33, 34) and that AP-1 elements are important for the basal and regulated expression of SP-B promoter (42). Our experiments also demonstrate that DETA-NO decreases TTF-1 expression at the transcriptional level.

EXPERIMENTAL PROCEDURES

Cell culture. NCI-H441 cells (HTB-174, American Type Culture Collection), a human pulmonary adenocarcinoma cell line with characteristics of bronchiolar (Clara) epithelial cells, and A549 cells (CCL-185, American Type Culture Collection), a human lung carcinoma cell line with certain characteristics of alveolar type II cells, were grown on plastic tissue culture dishes in RPMI 1640 and F-12K medium, respectively, containing 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 \(\mu\)g/ml), and amphotericin B (0.25 \(\mu\)g/ml). Cells were maintained in a humidified atmosphere of 95% air/5% CO\(_2\).

Cell viability. Cell viability was determined by measurement of lactate dehydrogenase activity in cell culture medium using the CytoTox 96 nonradioactive cytotoxicity assay kit (Promega). Materials. Diethylenetriamine-nitric oxide adduct (DETA-NO), spermine NONOate, and S-nitroso-N-acetylpenicillamine (SNAP) were obtained from Sigma, and Lipofectamine 2000 was obtained from Invitrogen. DETA-NO and spermine NONOate were directly dissolved in cell culture medium, and SNAP was dissolved in DMSO. The final concentration of DMSO in cells treated with SNAP was 0.2%, and DMSO at 0.2% had no effects on SP-B mRNA and promoter expression (data not shown). Dr. Shiko Kimura (National Cancer Institute, Bethesda, MD) and Dr. Robert Tjian (University of California, Berkeley, CA) kindly provided expression plasmids encoding TTF-1 and Sp1. Dr. Michael Birrer (National Cancer Institute) kindly provided expression plasmids encoding c-Jun, Jun B, Jun D, and Fra-1.

Transient transfection and reporter gene assay. The construction of luciferase reporter plasmids containing −911/+41, −517/+41, and −233/+41 bp of human SP-B 5′-flanking DNA is described elsewhere (39, 48). SP-B 5′-flanking DNA fragments were inserted into pBLCAT6 (16) to obtain chloramphenicol acetyltransferase (CAT) reporter plasmids. SP-B promoter plasmids were transiently transfected, along with pcDNA3.1 (Invitrogen), β-galactosidase expression plasmid, into cells by liposome-mediated DNA transfer with Lipofectamine 2000 according to the manufacturer’s instructions. After transfection, cells were incubated overnight and then subjected to treatments. Luciferase and β-galactosidase activities in cell extracts were measured by chemiluminescent assays (Tropix, Bedford, MA; Promega, Madison, WI), and CAT activity was determined by the liquid scintillation counting assay (41). Luciferase and CAT activities were normalized to cotransfected β-galactosidase activity or total cell protein to correct for variations in transfection efficiency.

Immunoblot analysis. SDS-PAGE separation and transfer of proteins to membrane were carried out with an XCell II Mini-Cell apparatus (Novex, San Diego, CA) according to the manufacturer’s instructions. Equal amounts (10 \(\mu\)g) of nuclear proteins were separated by SDS-PAGE on 10% Bis-Tris gels with 2-morpholinoethane-sulfonic acid running buffer and electropheretically transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were successively incubated with primary polyclonal thyroid-specific enhancer binding protein (T/EBP), Sp1, HNF-3α, c-Jun, or actin antibodies at 1:1,000 dilution overnight at 4°C and then with goat anti-rabbit alkaline phosphatase-conjugated secondary antibody (Cell Signaling) at 1:2,000 dilution for 1 h at room temperature. Protein bands were visualized by the enhanced chemiluminescence detection method (Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer’s instructions. Membranes were scanned for visualization of protein bands, and the intensities of bands were quantified using Quantity One image acquisition and analysis software (Bio-Rad). Polyclonal actin and Sp1 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Actin antibodies are against a peptide mapping at the COOH terminus of human actin and recognize α- and β-actin. Polyclonal rabbit antiseraum against the NH\(_2\)-terminal portion of rat T/EBP (TTF-1/Nkx2.1) was kindly provided by Dr. Shiko Kimura. Mouse monoclonal TTF-1 antibody was obtained from Thermo Scientific.

Preparation of nuclear extracts and EMSA. Methods for the preparation of nuclear extracts (40, 45) and double-stranded oligonucleotides (33) are described elsewhere. The sense strand sequences of human SP-B promoter and AP-1 consensus and mutant oligonucleotides, with binding sequences underlined, used in mobility shift analysis are as follows: 5′-GCCACCTGGAGGGCTTTAGAGC-CAA-3′ (−111/−87 bp, TTF-1/Nkx2.1), 5′-GCAAGACACAAACCTTCTGCAGGCTTA-3′ (−90/−73 bp, HNF-3), 5′-AGGCCCGAGCGCCTTCGACGAT-3′ (−53/−30 bp, Sp1), and 5′-CATGCTGTGATGGACACCTGCCTG-3′ (+15/+34 bp, AP-1) for SP-B, 5′-CGCTTGTAGTACCTGCACGGAA-3′ for consensus AP-1, and 5′-CGCTTGTAGTACCTGCACGGAA-3′ for mutant AP-1. Double-stranded oligonucleotides were 5′-end-labeled using [\(\gamma\)-\(^{32}\)P]ATP and T4 polynucleotide kinase. EMSAs were performed as described previously (33) by incubation of 0.5–1.0 ng (100,000 cpm) of labeled oligonucleotide with 5 μg of nuclear protein in 20 μl of binding buffer [13 mM HEPES, pH 7.9, containing 13% glycerol, 80 mM KCl, 5 mM MgCl\(_2\), 1 mM DTT, 1 mM EDTA, and 1 μg of poly(dI-dC) as nonspecific competitor DNA] at 30°C for 20 min. For antibody supershift assay, protein-DNA complex was formed and then incubated with antibody or antiseraum for 20 min at room temperature. After electrophoresis, the gel was exposed and X-ray film or storage phosphor screen.

Mutagenesis. Point mutations were introduced into SP-B promoter by PCR using pGL3 luciferase containing SP-B −233/+41 bp fragment as the template. The mutated promoter fragment was sequenced to verify mutations and inserted into pGL3 luciferase (basic) vector. The sequence of sense and antisense oligonucleotides for mutation of AP-1 site in SP-B promoter are as follows: 5′-CCCGAGCTCAGCACAAGTGTTCGACGAAAT-3′ (−233/−214 bp, sense) and 5′-CCCAAGGCTTCATCGAGAGGTGTTGACGTGACG-3′ (+16/+41 bp, antisense). The introduced SacI and HindIII sites are underlined, and the AP-1 site is shown in italics. The mutated nucleotides in the AP-1 site are underlined.

Chromatin immunoprecipitation assay. Typically, chromatin was extracted from 2–3 \(\times\) 10\(^6\) H441 cells. Cell fixation, enzymatic digestion of DNA, and immunoprecipitation reactions were performed using the Chip-IT express enzymatic kit (Active Motif) according to the manufacturer’s instructions. Normal IgG and TTF-1 (mouse monoclonal antibody, Thermo Scientific), Sp1 (Active Motif), and HNF-3c (FoxA1; Santa Cruz Biotechnology) antibodies were used at 2 μg/reaction in immunoprecipitation reactions. After isolation of DNA, SP-B promoter fragment was amplified by 36 cycles of PCR (GoTaq Hot Start Green Master Mix, Promega), and amplified DNAs were analyzed by agarose gel electrophoresis. The sequences of sense and antisense oligonucleotides for amplification of SP-B promoter are as follows: 5′-GGCAGGACGAAAGCCGTCATCGACG-3′ (−186/−167 bp, sense) and 5′-GACCTCTTGCACGCTTGGTAC-3′ (+6/+13 bp, antisense).

RNA isolation and real-time quantitative RT-PCR. Total RNA from cells was isolated using RNeasy mini kit (Qiagen) according to the manufacturer’s instructions, and then trace amounts of contaminating genomic DNA were removed by treatment with RNase-free DNase. Real-time quantitative RT-PCR was performed using a sequence detector (model 7700, Applied Biosystems, Foster City, CA). Specific quantitative assays for human TTF-1, SP-B, and actin mRNAs and 18S rRNA were developed using Primer Express software version 1.0.
Macintosh (Applied Biosystems) following the recommended guidelines based on sequences from GenBank. The names of the genes quantified, the sequences of the primers and probes, and other parameters of the assay are shown in Table 1. Synthesis of cDNA by RT and PCR amplification was performed according to standard protocols. Each sample was measured in triplicate, along with a control without reverse transcriptase. Because of the inherent inaccuracies in quantifying total RNA by absorbance, the amount of RNA added to an RT-PCR from each sample was more accurately determined by measurement of 18S rRNA level in each sample. The final data were normalized to 18S rRNA.

Isolation of nuclei and nuclear run-on assay. H441 cell nuclei were isolated according to the method described previously (23). Equal numbers of nuclei were labeled (23) by incubation with an equal volume of 2× reaction buffer (10 mM Tris-Cl, pH 8.0, containing 5 mM MgCl₂, 150 mM KCl, 5 mM DTT, and ATP, CTP, and GTP at 1 mM each) and 100 μCi of [³²P]UTP (3,000 Ci/mmol) for 30 min at 30°C. Total RNA was isolated using TriReagent as described previously, and equal amounts of radioactive RNAs were hybridized to nitrocellulose filters immobilized with plasmids containing cDNA probes. Protocol for hybridization and washing has been described previously (14). Radioactivity bound to filters was quantified with a PhosphorImager.

Cloning of human TTF-1 5’-flanking DNA. TTF-1 5’-flanking DNA encompassing −501/+99 bp was amplified by PCR using H441 cell genomic DNA as template and the following primers: 5’-TCGCCAAGTTGGTTGCAAAAAG-3’ (forward) and 5’-TTGGACCTACGCGACAG-GATTC-3’ (reverse). The reaction conditions for 32-cycle amplification were denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 90 s, followed by a final extension step at 72°C for 10 min. After cloning and sequence verification, the two translation initiation codons, ATGs, of the amplified fragment were mutated to ACG to prevent interference with luciferase reporter ATG. The initiation codons were mutated by PCR using the TTF-1 −501/+99-bp fragment as the template and the same forward primer and the following reverse primer: 5’-TTGGACCTACGCGACAG-GATTC-3’. The amplified DNA fragment was inserted into pGL3 luciferase (basic) reporter vector (Promega), and the insert sequence was verified.

Statistical analyses. Values are means ± SD or SE. In experiments where SP-B mRNA/protein levels or promoter activity were arbitrarily set at 100%, statistical significance was analyzed by one-sample t-test. For other samples, unpaired t-test was used to analyze the statistical significance. One-tailed P < 0.05 was considered significant.

RESULTS

SP-B minimal promoter contains DNA elements necessary for NO inhibition. Our previous studies demonstrated that DETA-NO inhibition of SP-B mRNA levels was suppressed by the transcriptional inhibitor 5,6-dichloro-1-β-d-ribofuranosyl-benzimidazole and that DETA-NO inhibited SP-B promoter activity, indicating the involvement of transcriptional mechanisms (39). We mapped SP-B promoter region(s) responsible for NO inhibition by determining the effects of DETA-NO on the transcriptional activities of SP-B genomic regions containing deletions at the 5’ end. Deletion of −911-bp fragment to −517 and −233 bp reduced promoter activity by 20% and 50%, respectively, but the deleted fragments were still sensitive to DETA-NO inhibition similar to the −911-bp fragment (Fig. 1B). These data indicate that the SP-B minimal promoter (∼233/+41 bp) contains DNA elements necessary for NO inhibition. NO donors were recently demonstrated to inhibit luciferase reporter expression in a promoter-independent manner by shortening the half-life of luciferase mRNA (21). We found that the NO donors DETA-NO and SNAP at 1 mM inhibited CAT reporter activity from the SP-B fragment −911/ +41 bp (control = 100, DETA-NO = 57 ± 7.8, and SNAP = 56.2 ± 5.7, n = 3) and luciferase reporter activity similarly, indicating that the inhibitory effects of NO donors on luciferase activity are indeed due to inhibition of SP-B promoter activity, and not destabilization of luciferase mRNA.

Determination of lactate dehydrogenase activity in cell medium showed an ∼3% increase in cell death in cells treated with 1 mM DETA-NO for 24 h. Similar lack of toxic effects of other NO donors has been reported for H441 (4) and primary alveolar type II cells (29).

DETA-NO alters DNA binding activities of TTF-1, HNF-3, Sp1, and AP-1 elements in the SP-B promoter. Previous studies from our laboratory and others showed that the SP-B minimal promoter (∼233/+41 bp) is selectively active in H441 and MLE lung epithelial cells, indicating the presence of lung cell-specific DNA elements. Previous studies also demonstrated that the SP-B minimal promoter contains functionally important TTF-1/Nkx2.1, HNF-3, Sp1/Sp3, and activating transcription factor/cAMP response element (ATF/CRE) elements that act in a combinatorial manner to activate SP-B promoter (11, 15, 33, 34). The importance of the AP-1 element located at +20/+27 bp in the human SP-B promoter function is not clear. However, mutation of the AP-1 element significantly reduced transcription in vitro (35), indicating its importance in SP-B promoter function. A DNA element, TGAAG-GTCA, in rabbit SP-B promoter that binds ATF/CRE binding protein and AP-1 transcription factors was found to be important for promoter activity (10). Although the sequence and the location of this element are similar in mouse and human SP-B promoters, the human DNA element TGAGGTCG differs by a
DNA binding activities were found to be dose-dependent, with the effects of DETA-NO on TTF-1, HNF-3, Sp1, and AP-1 activity increased in a dose- and time-dependent manner. TTF-1, HNF-3, and Sp1 DNA binding activities, AP-1 binding decreased. In contrast to the inhibitory effects of DETA-NO on TTF-1 and HNF-3, the effect on Sp1 binding activity was not. At 24 h of treatment, the luciferase activity for each construct in cells treated with DETA-NO (D/NO) for 24 h was determined and normalized to cotransfected plasmid.

Effects of DETA-NO on TTF-1, HNF-3, and Sp1 levels. We next studied the effects of DETA-NO on the levels of TTF-1, HNF-3, and Sp1 transcription factors (Fig. 4) to determine whether the reduced DNA binding activities of these factors are due to reduced protein level or activity per se or a combination of both. Treatment with 1 mM DETA-NO decreased TTF-1, but not Sp1, levels in nuclear extracts of cells in a dose- and time-dependent manner. Treatment with 1 mM DETA-NO for 24 h decreased TTF-1 levels by >50%, whereas Sp1 levels were not significantly affected. We were unable to determine the effects of DETA-NO on HNF-3 levels, as the antibodies (Santa Cruz Biotechnology) failed to detect a protein band of the expected size. We also found that DETA-NO increased c-Jun levels (Fig. 5), consistent with the increase in AP-1 DNA binding activity. Previous studies reported cytosolic trapping of TTF-1 in H441 cells exposed to transforming growth factor-β (28a) and PMA (28b). To determine whether NO alters cellular distribution of TTF-1, we used Western immunoblot analysis to evaluate the effect of DETA-NO on the cytosolic and nuclear TTF-1 levels (Fig. 6). Results demonstrated that DETA-NO reduced nuclear TTF-1 levels without affecting those in cytosolic TTF-1 levels. In fact, TTF-1 could not be detected in the cytosolic fraction of control or DETA-NO-treated cells.

Effects of TTF-1 and Sp1 overexpression on DETA-NO inhibition of SP-B promoter activity. DETA-NO decreased TTF-1 and Sp1 DNA binding activities to inhibit SP-B promoter activity. To gain further insights into mechanisms mediating DETA-NO inhibition, we determined the effects of overexpression of TTF-1 and Sp1 on DETA-NO inhibition of SP-B promoter activity in H441 cells. We found that overexpression of Sp1, but not TTF-1, blocked DETA-NO inhibition of SP-B promoter activity (Fig. 7A). The inability of TTF-1 to block DETA-NO inhibition of SP-B promoter activity suggests that NO could be inactivating TTF-1 binding, for example, by posttranslational modification. To gain evidence for NO inactivation of TTF-1, we studied the effects of DETA-NO on SP-B promoter activation by TTF-1 in A549 lung cells. A549 cells express very low or undetectable levels of TTF-1, making them suitable to study the effects of exogenous TTF-1 on SP-B.
promoter activity. We found that exogenous TTF-1 and Sp1 increased SP-B promoter activity by severalfold and that TTF-1, but not Sp1, activation was significantly inhibited by DETA-NO (Fig. 7B). The lack of effect of DETA-NO on inhibition of SP-B promoter activity in cells overexpressing Sp1 was certainly due to high levels of Sp1, as SP-B promoter activity was inhibited in cells transfected with a lower concentration (0.05 μg) of Sp1 expression plasmid (data not shown). In separate experiments, we found that DETA-NO did not alter the levels of cotransfected TTF-1 in A549 cells (Fig. 7C), suggesting that DETA-NO inhibition of TTF-1 activation of SP-B promoter activity could be due to inactivation of TTF-1 activity.

Role of AP-1 in NO regulation of SP-B promoter activity. We found that DETA-NO inhibition of SP-B promoter activity in H441 cells was associated with increases in AP-1 DNA binding activity and c-Jun levels, suggesting that NO elevation of AP-1 DNA binding inhibits SP-B promoter activity. We
examined the involvement of AP-1 factors, as there is limited information on their role in the regulation of human SP-B promoter activity. H441 cells express predominantly c-Jun, Jun B, Jun D, and Fra-1 members of the AP-1 family (52), and we determined the effects of overexpression of these proteins on SP-B promoter activity (Fig. 8A). Overexpression of c-Jun, Jun B, and Jun D, but not Fra-1, significantly inhibited SP-B promoter activity (Fig. 8A). To determine whether AP-1 members inhibit SP-B promoter activity by binding to the AP-1 site (+20/+27 bp), we evaluated the effects of overexpression of c-Jun on wild-type and AP-1 mutant SP-B minimal promoters (−233/+41 bp). The SP-B minimal promoter contains a single AP-1 site at +20/+27 bp. Overexpression of c-Jun inhibited SP-B promoter activity by >60%, whereas it inhibited the AP-1 mutant promoter to a significantly lesser degree, indicating that the AP-1 site mediates inhibition of SP-B promoter activity (Fig. 8B). Mutation of the AP-1 site reduced SP-B promoter activity by 60%, indicating its importance for basal promoter activity.

**Effect of DETA-NO on TTF-1 gene transcription and TTF-1 mRNA levels.** Western immunoblotting and immunofluorescence microscopy data (not shown) show that DETA-NO treatment decreased TTF-1 levels. We investigated the effect of DETA-NO on TTF-1 mRNA levels by quantitative RT-PCR to determine the inhibitory effects of DETA-NO on TTF-1 expression. Treatment with DETA-NO decreased TTF-1 mRNA expression by ~50%, similar to inhibition of SP-B mRNA (Fig. 9A). We next investigated the effect of DETA-NO on TTF-1 promoter activity and TTF-1 gene transcription rate...
to determine whether the effects are mediated at the transcriptional level. We found that treatment with DETA-NO decreased TTF-1 promoter activity (~50%; Fig. 9B) and TTF-1 gene transcription rate by ~50% (Fig. 9C), indicating that DETA-NO decreases TTF-1 expression at the transcriptional level.

**DISCUSSION**

Abnormal surfactant function is believed to play important roles in the pathogenesis of acute lung injury (24). Decreased expression of surfactant proteins and lipids is one mechanism by which surfactant function can be altered. There appear to be strong associations between surfactant dysfunction (24), elevated NO levels, and lung injury, suggesting that elevated NO levels, as encountered in ARDS and bronchopulmonary disease, could inhibit surfactant protein expression. Indeed, our study and other studies show that treatment of lung epithelial cell lines (4, 7, 39) and primary cells (29) with NO donors results in decreased expression of surfactant proteins. Specifically, expression of NO synthase type 2 in the lung epithelium was found to be responsible for endotoxin-induced decrease of SP-B expression (7), further supporting the inhibitory effects of NO on SP-B levels. The effects of NO on surfactant protein levels in experimental animals vary depending on the dose and duration of treatment. At 40 ppm, NO decreased SP-A and SP-B protein, but not mRNA, levels in lambs after 24 h of exposure (49). Similar inhibitory effects of 40 ppm NO on SP-B and SP-C levels were reported in experimental lung transplantation in pigs (51). In a primate model of chronic lung disease, 5 ppm NO increased tissue levels of SP-B and SP-C after 14 days of exposure (5).

NO donors decreased SP-B promoter activity in H441 and MLE-12 (39) and MLE-15 (7) lung epithelial cells, indicating the involvement of transcriptional mechanisms in the inhibition of SP-B expression. In primary cultures of alveolar type II epithelial...
In this study, we investigated molecular mechanisms mediating NO inhibition of SP-B gene expression. The highest concentration of DETA-NO, i.e., 1 mM, used in our study is expected to produce NO at a steady-state level of 0.5 μM over a 24-h period (37), which is similar to levels found in vivo (30) under pathological conditions, making our findings physiologically relevant. Elevated NO levels have been found to cause cytostasis and cytotoxicity in tumor cells (37). The doubling time for H441 cells is reported to be 58 and 98–133 h in serum-containing and serum-free medium, respectively (American Type Culture Collection), indicating that the cells grow slowly. We consistently found that DETA-NO (1 mM) inhibited SP-B transcription and mRNA levels at 24 h of treatment, suggesting that the inhibition may not be due to inhibition of cell growth. Exposure of H441 cells to DETA-NO also suggests that the cells grow partially reversed the NO-mediated decrease in the levels of SP-B and TTF-1 mRNAs, indicating that endothelin levels may partly mediate the inhibitory effects (29).

In deletion mapping experiments, we found that the SP-B minimal promoter region −233/+41 bp was sensitive to DETA-NO inhibition, indicating the presence of DNA elements necessary for promoter inhibition. The SP-B minimal promoter contains functional TTF-1, HNF-3, Sp1, ATF/CRE, and AP-1 elements (10, 15, 34) and is active in in vitro cell cultures and transgenic mice (1) to support cell/tissue-specific expression of reporter gene. Treatment with DETA-NO differentially altered TTF-1, HNF-3, Sp1, and AP-1 DNA binding activities: whereas TTF-1, HNF-3, and Sp1 binding were decreased, AP-1 binding was increased. Immunoblotting experiments showed that the SP-B promoter activity was decreased, AP-1 binding was increased, and Sp1, GAPDH, and actin levels are unaltered. The differential effects of DETA-NO on gene expression in H441 cells also suggest that its inhibitory effects on SP-B levels may not be due to cytostasis. DETA-NO had no significant adverse effects on H441 cell viability, indicating that inhibition of SP-B levels is not due to cytotoxicity.

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**Fig. 8.** A: effects of overexpression of AP-1 members on SP-B promoter activity. H441 cells were cotransfected with pGL3 luciferase (basic) plasmid containing SP-B fragment −911/+41 bp and empty expression vector (pCMV) or expression plasmids encoding c-Jun, Jun B, Jun D, or Fra-1. After incubation for 40 h, luciferase activity was determined and normalized to total cell protein. Values are means ± SE of 3 independent experiments. *P < 0.05 vs. cells transfected with pCMV vector. B: effects of c-Jun on wild-type and AP-1 mutant SP-B promoter activity. pGL3 luciferase (basic) plasmid containing wild-type and AP-1 mutant SP-B promoter (−233/+41 bp) was transiently transfected into H441 cells along with c-Jun expression plasmid. After 40 h of incubation, luciferase activity in cell lysates was determined and normalized to total cell protein. Values are means ± SE of 3 independent experiments. *P < 0.05 vs. cells transfected with pCMV vector. P = 0.0919, pCMV vs. c-Jun on AP-1 mutant promoter.

**Fig. 9.** DETA-NO decreases TTF-1 expression at the transcriptional level. A: effect on SP-B and TTF-1 mRNA levels. H441 cells were treated with (1 mM) or without (C) DETA-NO for 24 h, and the effect on SP-B and TTF-1 mRNA levels was analyzed by quantitative RT-PCR. SP-B and TTF-1 levels in control cells were arbitrarily set at 100. Values are means ± SD of 3 independent experiments. *P < 0.05 vs. control. B: effect on TTF-1 promoter activity. Human TTF-1 promoter plasmid containing −501/+99 bp of TTF-1 5′-flanking DNA inserted upstream of luciferase reporter gene was transiently transfected into H441 cells, which were then treated with (1 mM) or without (C) DETA-NO for 24 h. Luciferase activities of cell extracts were determined and normalized to cotransfected β-galactosidase activity. Luciferase activity in control cells was arbitrarily set at 100. Values are means ± SD of 3 independent experiments. ***P < 0.001 for 1.0 mM DETA-NO-treated cells vs. control. C: effect on TTF-1 gene transcription rate. H441 cells were treated with (1 mM) or without (C) DETA-NO for 24 h, and nuclei were isolated and labeled with [α-32P]UTP. Equal amounts of radioactivity were hybridized to nitrocellulose membranes immobilized with plasmids containing cDNA probes. DETA-NO decreased TTF-1 transcription rate by >50%. Similar results were obtained for a second independent experiment. Lane 1, TTF-1 cDNA; lane 2, plasmid only; lane 3, actin cDNA.
experiments showed that TTF-1, but not Sp1, levels were decreased in cells exposed to DETA-NO, indicating that reduced TTF-1 expression may be responsible for the reduced TTF-1 binding activity. The levels of Sp1 were not affected by DETA-NO, suggesting that the observed decrease in Sp1 binding activity could be due to an effect on the binding activity per se. Posttranslational modifications, such as phosphorylation, acetylation, and oxidation, are known to influence the activities of transcription factors. Specifically, changes in phosphorylation, glycosylation, and acetylation influence Sp1 DNA binding and transcriptional activities to regulate gene expression (17, 32). It remains to be determined whether treatment with DETA-NO alters phosphorylation, acetylation, or glycosylation of Sp1 to decrease its DNA binding activity.

Overexpression of Sp1, but not TTF-1, blocked DETA-NO inhibition of SP-B promoter activity. The reasons for the inability of TTF-1 to block inhibition of SP-B promoter activity are not clear, but they suggest that NO may inactivate TTF-1 through posttranslational modifications. Our finding that DETA-NO inhibited SP-B promoter induction by exogenous TTF-1 without altering TTF-1 levels further supports a role for TTF-1 inactivation in NO inhibition of SP-B promoter activity. Our data also suggest that TTF-1 is highly susceptible to NO inactivation compared with Sp1. NO could inactivate TTF-1 by oxidation, S-nitrosylation, tyrosine nitration, or phosphorylation. Studies have shown that treatment of TTF-1 in vitro with oxidizing agents, such as oxidized glutathione or diamide, inactivated TTF-1 DNA binding activity, which was reversed by DTT (33). The decrease in TTF-1 binding activity was found to be due to the oxidation of two specific cysteine residues located outside the TTF-1 homeodomain, which resulted in the formation of higher-order oligomers (3). Redox effector factor-1 (Ref-1) was found to directly interact with TTF-1 and mediate redox effects on the TTF-1 homeodomain (50). It remains to be determined whether NO or its metabolites, such as peroxynitrite or nitrogen dioxide, directly modify TTF-1 by oxidation, S-nitrosylation, or tyrosine nitration or act via Ref-1 to oxidize TTF-1. Increased TTF-1 phosphorylation by PKA activated SP-B (54) and SP-A (31) gene transcription in H441 and primary alveolar cells, respectively, suggesting that alterations in TTF-1 phosphorylation modulate target gene expression. Whether DETA-NO alters TTF-1 phosphorylation to decrease SP-B promoter activity is not known.

DETA-NO treatment increased AP-1 DNA binding and c-Jun levels, suggesting that elevated AP-1 binding may mediate inhibition of SP-B promoter activity. Consistent with a role for elevated AP-1 binding in the inhibition of SP-B promoter activity, overexpression of c-Jun, Jun B, and Jun D inhibited SP-B promoter activity. Overexpression of AP-1 family members is known to inhibit SP-B promoter activity in MLE-12 (42) and H441 (11) lung epithelial cells. Molecular mechanisms mediating inhibition of SP-B promoter activity by AP-1 transcription factors are not well understood but may involve interference with the binding of stimulatory factors such as TTF-1 (42). A DNA element in the rabbit SP-B promoter that binds ATF/CRE and AP-1 factors is essential for basal promoter activity and shows enhanced binding in cells treated with PMA, TNF-α, or cAMP, indicating that it may play roles in the regulation of promoter activity (10). Although the human SP-B promoter contains a similar DNA element, we found that the human DNA element did not bind H441 nuclear proteins. The human DNA element differs from the rabbit element, with a single nucleotide change that could explain its inability to bind proteins.

Molecular mechanisms underlying NO regulation of gene expression are incompletely understood. Acute responses to NO are thought to involve posttranslational modifications of preexisting cellular proteins, including transcription factors, and long-term effects may be mediated by alterations in their levels. Redox-sensitive transcription factors, such as NF-κB, AP-1, hypoxia-inducible factor-1α, and others, such as Sp1, Egr-1, and glucocorticoid receptor are targets of NO actions (13, 38). Oxidation of critical cysteine residues via NO-mediated S-nitrosylation modulates the activities of Sp1, AP-1, heat shock protein, and NF-κB. In murine lymphocytes, NO inhibition of IL-2 gene expression is associated with decreased Sp1 binding (9), and in human U937 cells, NO stimulation of TNF-α promoter activity involves decreased Sp1 binding (53).

Treatment with DETA-NO decreased TTF-1 mRNA content and TTF-1 promoter activity and gene transcription rate, indicating that DETA-NO decreases TTF-1 gene transcription to reduce TTF-1 levels. As TTF-1 is a common activator for SP-A, SP-B, SP-C, and Clara cell secretory protein gene transcription in lung cells, it might be expected that DETA-NO inhibition of TTF-1 expression would lead to decreased expression of surfactant proteins and Clara cell secretory protein. Indeed, treatment of H441 lung epithelial cells with the NO donor SNAP decreases SP-A expression without reducing mRNA stability (4). A better understanding of molecular mechanisms mediating NO inhibition of SP-B expression may provide information for the design of novel treatments for inflammatory lung diseases, such as ARDS.

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DISCLOSURES

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