p11 Expression in Human Bronchial Epithelial Cells Is Increased by Nitric Oxide in a cGMP-dependent Pathway Involving Protein Kinase G Activation*

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The effect of nitric oxide on p11 expression was studied in an immortalized human bronchial epithelial cell line (BEAS-2B cells). Three nitric oxide donors were used: spermine NONOate (SP), (±)-S-nitroso-N-acetylpenicillamine (SNAP), and S-nitrosoglutathione (SNOG). All three nitric oxide donors had similar effects resulting in dose-dependent and time-dependent accumulation of p11 protein and an increase of steady-state p11 mRNA. Studies using a reporter gene containing the region from −1499 to +89 of the p11 promoter demonstrated an increase in transcriptional activity after stimulation with NO donors for 4 h. These effects were abolished at the promoter and protein level using protein kinase G inhibitors (KT5823 and Rp-8-pCPT-cGMPS). Incubation of transfected cells with a cell permeable cGMP analogue (8-Br-cGMP) resulted in a dose-related increase of promoter activity. An electrophoretic mobility shift assay of nuclear proteins extracted from BEAS-2B cells identified an AP-1 site located at −82 to −77 of the p11 promoter region as an NO- and cGMP-dependent response element. These data were confirmed using a c-jun dominant negative mutant vector and a c-jun expression plasmid. Therefore, we conclude that nitric oxide-induced p11 expression in human bronchial epithelial cells is mediated at least in part through increased binding of activator protein one to the p11 promoter.

S-100 proteins are a family of proteins first described by Moore et al. (1) who initially characterized a group of low molecular weight (10,000–12,000) acidic proteins in neural tissue. The S-100 group consists of calcium-binding proteins that are expressed in a cell type-dependent fashion. There are at least 13 members, including S-100a, S-100b, and p11 (calpactin light chain). While p11 is a member of the S-100 family, it does not have the ability to bind Ca2+ ions due to crucial amino acid deletions and substitutions in the two EF-hand loops of the protein (2). p11 binds to and inhibits the phosphorylation of annexin II, and binds to the carboxyl region and inhibits cytosolic phospholipase A2 activity (3). p11-annexin II tetramer is also reported to be a binding protein for cathepsin B, facilitating tumor invasion and metastasis.

Nitric oxide is a lipophilic, short-lived, highly reactive free radical, pluripotent molecule involved in the regulation of blood pressure, neurotransmission, immune function, arachidonic acid metabolism, cell migration, learning and memory, hormone release, cell differentiation, and cell migration (4). Moreover, nitric oxide also plays a role as a messenger in a number of diseases including sepsis, stroke, diabetes mellitus, and rheumatoid arthritis (5). The main source of NO in humans is nitric oxide synthase (present in at least three isoforms), which catalyzes the oxidation of L-arginine to L-citrulline and NO (6). Nitric oxide influences the expression of many genes including cyclooxygenase-2 (7), tumor necrosis factor-α (8), interleukin-6 (9), and interleukin-10 (10).

Nitric oxide activates soluble, NO-dependent guanylate cyclase, causing an increase in intracellular cGMP concentration. This activates cGMP-dependent protein kinase I and II (G-kinase, PKG) (11) causing phosphorylation of target proteins or transcription factors. In addition, NO influences transcription via a cGMP-independent mechanism by decreasing the intracellular concentration of cAMP and affecting cAMP response elements (12) thereby influencing the transcription of various genes.

As the influence of nitric oxide on expression and activity of several enzymes located in downstream pathways involving arachidonic acid metabolism (such as cyclooxygenase-2) is well known, it was of interest to determine the effect of nitric oxide on p11 expression. Our aim was to study the influence of NO on p11 transcription, steady-state levels of p11 mRNA, and protein concentration in human epithelial cells.

EXPERIMENTAL PROCEDURES

Cell Culture—BEAS-2B cells, a human bronchial epithelial cell line transformed by an adenovirus 12-SV40 virus hybrid, were purchased from American Type Culture Collection (Rockville, MD). The cells were cultured in serum-free, hormonally defined LHC-8 culture medium obtained from BIOSOURCE (Rockville, MD) and grown in 6-well tissue culture plates coated with type I collagen (Becton Dickinson, Bedford, MA) for transfection experiments, in 75-cm² type I collagen-coated flasks (Becton Dickinson) for protein expression studies and in 150-cm² type I-collagen coated flasks (Becton Dickinson) for RNA and nuclear protein extraction. All experiments were done using 90% confluent cultures.

Nitric Oxide-related Reagents—All nitric oxide donors including spermine NONOate (SP), (±)-S-nitroso-N-acetylpenicillamine (SNAP),

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and S-nitrosoglutathione (SNOG) were purchased from Calbiochem (San Diego, CA).

**Protein Kinase G-related Reagents**—An adenosine triphosphate-binding site antagonist, KT5720, and guanosine 3',5'-cyclic monophospho- 
orothioate, 8-(-4-chlorophenylthio), R, isomer, triethylammonium salt form Calbiochem) were used as PKG inhibitors. A PKG cyclase inhibitor, 1H-[1,2,4]oxadiazolo[4,3-c]quinazolin-1-one (ODQ) and a PKG agonist 8-bromo-cGMP were also purchased from Calbiochem.

**Molecular Biology Reagents**—All primers were synthesized by Key- 
stone Labs (Camarillo, CA). The Fail-Safe PCR amplification system with buffer (Epicentre Technologies, Madison, WI) was used for all PCR reactions. All constructs were fully sequenced using Sanger’s method on an ABI Prism 377 Sequencer (Applied Biosystems, Foster 
City, CA) to confirm the sequence and orientation. Sequencer 3.1.1 
(Gene Codes Corporation, Ann Arbor, MI) software was used for se- 
quence analysis.

**Treatments**—Cells were incubated with the specified doses of sper- 
mie NONOate, SNAP, or SNOG for 24 h for dose-response experiments, or with 100 μM of each NO donor for periods of 4, 8, 16, and 24 h for time 
course experiments. Steady-state mRNA p11 levels were assessed after 4 
h incubation with specified doses of NO donors for dose-response 
experiments or for 4, 8, and 24 h at a dose of 100 μM for time course 
experiments. Cells incubated without NO donors were used as a control. 
For pretreatment studies, transfected cells were incubated with or without 
NO donors for 4 h prior to collection. All inhibitors and scavengers were 
added to cell cultures 30 min before incubation with NO donors or medi- 
a alone, and were maintained for the incubation period.

**Immunoblot of p11 Protein**—For the dose response experiments, the 
culture medium was replaced at the same time with medium containing 
the specified dose of NO donor and cells were harvested at 24 h. For the 
time course experiments, medium was changed in all cultures 24 h before 
harvesting cells. NO donors were added to the media at 4, 8, 16, 
or 24 h prior to harvesting the cells. Cells were harvested with trypsin 
(E-PET, Biofluidics, Rockville, MD) and gentle scraping, collected from 
flasks, and washed three times with cold PBS. Afterward, cells were 
transferred to 0.2 ml of homogenization buffer: 50 mM Hepes (pH 8.0), 
1 mM EDTA, 1 mM EGTA, 100 mM leupeptin, 1 mM dithiothreitol, 10 mM 
phosphomethylsulfonyl fluoride, 0.5 mM soybean trypsin inhibitor, 15 μM 
aprotilin, and 0.25% Triton X-100. Cells were then sonicated three 
times for 45 s and centrifuged at 10,000 × g for 15 min. Total protein 
concentration was determined using a BCA assay (Pierce, Rockford, IL). 
Samples containing 10 μg of crude cell lysate were separated on 16%
Tris glycine gels (Invitrogen) and 
and the sample was incubated at room temperature for 5 min, applied 
onto spin columns (Calbiochem), and centrifuged at 14,000 × g for 5 
min. After 2 washes with 100 μl of washing solution, the spin column 
was placed into a scintillation vial. Ten ml of BioSafe II scintillation 
fluid were added and sample was counted in a scintillation counter. 
Data are expressed as the percentage of control activity ± S.E. from three separate experiments. p < 0.05 by one way ANOVA.

**Immunoblot of Protein Kinase G Protein**—Cell harvesting and im-
munoblotting were done as described above except that 20 μg of crude 
cell lysate were separated on 10% Tris glycine SDS running buffer. The 
separated proteins were electrophoretically transferred onto a nitrocellulose membrane (Invitrogen), which was then 
blocked with 5% nonfat dry milk with 0.1% Tween 20 overnight at 4°C. 
The separated proteins were electroblotted onto a nitrocellulose membrane 
(Invitrogen), which was then 
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The separated proteins were electroblotted onto a nitrocellulose membrane. 
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**RNAse Protection Assay**—BEAS-2B cells were exposed to NO donors 
for the described times. The medium was removed and cells were 
washed three times with cold PBS, and then carefully scraped and collected 
by centrifugation for 5 min at 300 × g at 4°C. Total RNA was isolated 
using the RNAqueous kit from Ambion (Austin, TX). RNA was quantified 
using 260 nm optical density. To construct the probe for p11 mRNA, a 319-base pair precur- 
sor of p11 CDNA was amplified by PCR using the following sets of sense and antisense primers: 5′ primer: 5′- ACCACACCAATGCGCATCT-3′ (corresponding to bases 61–80 of the human p11 
cDNA sequence, GenBank™ accession number M81457); 3′ primer: 5′-CTGGTACATTGTGCTACT-3′ (which corresponds to bases 361– 
379 of the p11 cDNA sequence). The product was cloned into the 
pGEM-T Easy vector (Promega, Madison, WI). Orientation of the insert

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**Fig. 1. The effect of spermine NONOate on p11 protein levels**. **A.** Upper panel: the effect of time of incubation with SP on p11 protein levels in BEAS-2B cells. Cells were grown to near confluence and then were treated with SP (100 μM) for 4, 8, 16, and 24 h. Cell lysates from treated and untreated cells were processed as described under “Experimental Procedures,” and 10 μg of total protein were subjected to gel electrophoresis and immunoblotting. The lower panel represents the densitometric analysis of immunoblotting. Data shown are mean ± S.E. from three separate experiments. **B.** Upper panel: the effect of different spermine NONOate concentrations on p11 protein levels in BEAS-2B cells. Cells were grown to near confluence and then were treated with 0, 25, 50, 100, or 400 μM SP for 16 h. Cell lysates from treated and untreated cells were processed as described under “Experimental Procedures” and 10 μg of total protein were subjected to gel electrophoresis and immunoblotting. The lower panel represents the densitometric analysis of immunoblotting. Data shown are the mean ± S.E. from three separate experiments. p < 0.05 by one way ANOVA.

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**Experimental Procedures**—A universal Protein Kinase Assay Kit (Calbiochem) was used to assess PKG activity according to the manufacturer’s manual. Briefly, 5 μl of cell lysate were assayed to 20 μl of reaction mixture containing 4 mM Tris-HCl, 2 mM magnesium acetate, 5 μM cGMP, and 1 μCi/μl [γ-32P]ATP, 0.15 mM ATP, and 0.2 mM biotinylated substrate. After vortexing, the reaction mixture was incubated for 15 min at 30°C. The reaction was terminated by adding 10 μl of 8 M guanidine hydrochloride. Afterward, 8 μl of avidin solution were added and the sample was incubated at room temperature for 5 min, applied

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was determined by DNA sequencing. The p11 cRNA probe and a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe (Ambion) were radiolabeled using an <i>in vitro</i> transcription kit (Ambion) with T7 polymerase with [³²P]UTP (800 μCi (29.6 TBq)/mmol) (PerkinElmer Life Sciences, Boston, MA). An RPA assay kit (RPA III, Ambion) was used to quantitate total mRNA. Two micrograms of total RNA were mixed with 5,000 cpm (for GAPDH) or 20,000 cpm (for p11) of cRNA. The mixture was hybridized at 42°C overnight and digested by the addition of 1:100 dilution RNase A/T1 at 37°C for 30 min. Digestion was terminated by the addition of RNase inactivation and precipitation mixture. The protected fragments were separated on 6% polyacrylamide, 8 × urea gels (Invitrogen) and visualized by autoradiography using Kodak MR-X film at −70°C for 24–48 h.

**Transient Transfection of Reporter Gene Plasmids—**pCAT-basic vectors (Promega) containing the p11 promoter from −1499 to +89, −1049 to +89, and from −188 to +89 were created. Briefly, the following sense and antisense primers were used: 5′-GGTACCGAGATTTCTCTCCATGTTGA-3′ and 5′-GAGCTCACCTTGCCGAGGC-3′ (to obtain p11 promoter region from −1499 to +89); 5′-GGTACCCACCTGGCTGTGAACCTGAT-3′ and 5′-GAGCTCACCTGGCCAGGCC-3′ (to obtain p11 promoter region from −188 to +89); 5′-GGTACCCCTGCGAGGGCGG-3′ and 5′-GGGAGCCTGCGAGGGCGGCGG-3′ (to obtain p11 promoter region from −188 to +89). Each of the PCR reaction products was then run on 1% agarose gel, recovered from the gel using GelQuick gel purification kit (Qiagen, Valencia, CA), and cloned in pCR-TOPO vector. Each of the plasmids was cut using KpnI and SacI restriction endonucleases (Roche Molecular Biochemicals, Indianapolis, IN) and the insert was recovered from a 1% agarose gel using Millipore (Bedford, MA) Ultrafree-DA columns. Inserts were ligated overnight into dephosphorylated (shrimp alkaline phosphatase, Roche Molecular Biochemicals) pCAT-basic vector (Promega) using T4 DNA ligase (Invitrogen) and transformed into JM109 Escherichia coli competent cells. Plasmids were recovered from bacterial cultures using a MaxiPrep kit (Qiagen). The plasmids were fully, bidirectionally sequenced to ensure proper orientation and content. BEAS-2B cells were transfected with 1 μg of chloramphenicol acetyltransferase (CAT) basic vector and (to determine transfection efficiency) co-transfected with pCMV/β-galactosidase (0.2 μg) (CLONTECH, Palo Alto, CA) using LipofectAMINE Plus Reagent (Life Technologies, Inc., Rockville, MD) according to the manufacturer’s protocol. After 16 h, the medium was changed and cells were exposed to various concentrations of nitric oxide donors, inhibitors, or medium alone for 4 h. Cells were washed three times with cold PBS (BIOSOURCE) and lysed for 30 min at room temperature. Cell lysate was collected and stored at −70°C. CAT expression was determined in cell lysate using the colorimetric enzyme immunoassay kit (Roche Molecular Biochemicals). Results were normalized for transfection efficiency using β-galactosidase detection by the colorimetric enzyme immunoassay kit from the same manufacturer.

Measurements of CAT and β-galactosidase were done in duplicate according to the manufacturer’s protocol. Data are expressed as fold increase of nitric oxide donors at their maximal concentration for 4 to 24 h to confirm that the promoterless vector does not respond to nitric oxide.

**Mutation Construct—**To confirm the role of the AP-1 site in NO-dependent p11 activation, this site was mutated in the −188+89 reporter gene construct using a PCR-based technique. The AP-1 site (GAGT) was mutated to Bbr PI restriction site (GACGTCATGGCGGCGGCGGC-3′) using primers: 5′-GACGTCATGGCGGCGGCGGC-3′ and 5′-GAGCTCACCTGGCCGAGGC-3′ in order to obtain a −188−60 PCR product and 5′-GAGCTCACCTGGCCGAGGC-3′ and 5′-GACGTCATGGCGGCGGCGGC-3′ to obtain a −85+89 PCR product. The −188+89 reporter gene plasmid was used as a template. Both products were separated on a 1% agarose gel as described above, mixed in equal molar concentrations, and used as a template for a third PCR reaction, using the same primers as for the −188+89 reporter gene cloning. The AP-1 MU vector was obtained from the third PCR product using TA cloning and subcloning to the pCAT basic vector as described above.

**Transient Transfection with c-jun Dominant Negative Mutant and c-jun Expression Plasmids—**TAM67 c-jun dominant negative plasmid, c-jun expression plasmid, and control vector were kind gifts from Dr. M. J. Birrer (13). TAM67 lacks amino acids 3–122 of c-jun. This
deletion removed most of the “transactivation domain” of c-jun but retains the DNA-binding and leucine zipper domains of c-jun. The c-jun expression plasmid contains the full wild type sequence of c-jun. The control vector lacks either of the inserts. All three plasmids are driven by a CMV promoter. BEAS-2B cells were transfected with the TAM67 and H11001p11 reporter gene, with the c-jun expression plasmid and H11002p11 reporter gene, or with control vector and H11002p11 reporter gene. All transfections were done with 5:1 ratio, respectively. All sets of cells were co-transfected with the pCMV/β-galactosidase vector to normalize for transfection efficiency. Transfection was done as described above and some cells were exposed to NO donor or media for 24 h. Collection of cells, CAT, and β-galactosidase assays were done as described above.

Nuclear Protein Isolation from BEAS-2B Cells—BEAS-2B cells were grown in T150 type I collagen-coated flasks to 90% of confluence and were incubated with NO donors or 8-Br-cGMP for periods of 2, 4, or 24 h. Culture medium was removed and cells were washed 3 times with ice-cold PBS, harvested by scraping into 4 ml of PBS, and centrifuged (500 × g, 5 min). The pellet was dispersed in 5 packed cell volumes of hypotonic buffer (10 mM Hepes-KOH, pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, apro-tein, pepstatin, and leupeptin, each 2 mg/ml). After 15 min on ice, Nonidet P-40 was added to a final concentration of 0.6% (v/v), and the nuclei were pelleted by centrifugation (5000 × g, 5 min). The pelleted nuclei were dispersed in a high salt buffer (20 mM Hepes-KOH, pH 7.9, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 25% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, aprotinin, pepstatin, and leupeptin, each 2 mg/ml) to solubilize DNA-binding proteins. The suspended nuclei were gently shaken horizontally for 30 min at 4 °C and centrifuged in a microcentrifuge (12,000 × g, 20 min). The supernatants containing nuclear proteins were stored at −70 °C until used for EMSA. Protein concentrations were determined using a BCA assay kit (Pierce) with bovine serum albumin as a standard.

Electrophoretic Mobility Shift Assays (EMSA)—Single-stranded oligonucleotides 4xAP-1WT (5′-GGAGTCGGAGTCGGAGTCGGAGTC-3′) representing the four repeats of the p11 promoter derived AP-1 site (GGAGTC) were obtained from Keystone Labs. Complementary strands in equal molar concentrations were mixed and annealed by slowly cooling to room temperature after heating to 95 °C for 5 min. Double-stranded oligonucleotides were stored at −20 °C in 50 mM NaCl at a concentration of 1.75 pmol/ml. For EMSA, oligonucleotide probes were labeled with [γ-³²P]ATP (Amersham Pharmacia Biotech) using T4

Fig. 3. The effect NO donors on protein kinase G protein levels and activity. A, cells were grown to near confluence and then were treated with 0, 2, 20, and 200 μM SP for 16 h. Cell lysates from treated and untreated cells were processed as described under “Experimental Procedures” and 20 μg of total protein were subjected to gel electrophoresis and immunoblotting. B, cells were grown to near confluence and then were treated with 100 μM SNOG for 30 min, 1 and 4 h. The protein kinase G activity assay was performed as described under “Experimental Procedures.” Data expressed are the percentage of PKG activity as compared with control ± S.E. (n = 3). The data presented are from one of three experiments, each with similar results.
polynucleotide kinase (Promega) and purified on G-50 columns (Amersham Pharmacia Biotech). Nuclear extracts (5 μg) were incubated with the hot AP-1 probe (0.5–1 × 10⁶ cpm) in binding buffer (10 mM Hepes, pH 7.8, 5% glycerol, 0.3 mM MgCl₂, 50 mM KCl, 0.1 mM ZnCl₂, 0.04 mM EDTA, 1 mM dithiothreitol, 40 μg/ml bovine serum albumin), for 20 min at room temperature. Samples were subjected to electrophoresis through 6% DNA-retardation gels (Invitrogen) in 0.5× Tris borate-EDTA buffer at room temperature at 200 V. Gels were dried at 75 °C and autoradiographed at −70 °C overnight or until adequate signal was developed. As a control for specificity, 100-fold molar excess of cold oligonucleotide (Promega), respectively, were preincubated with nuclear extracts for 20 min at room temperature prior to addition of hot AP-1 probe.

Statistical Analysis—Comparisons were made using a two-tailed unpaired Student’s t test. Dose-related effects were evaluated by one-way ANOVA. Differences were considered to be significant at p < 0.05. Microsoft Excel 98 software (Microsoft Corp, Redmond, WA) running on an iMac computer (Apple Computer Inc, Cupertino, CA) was used to perform statistical analysis.

FIG. 4. The effect of protein kinase G inhibitors on p11 expression induced by nitric oxide donors. Cells were preincubated in the presence of 1 μM of either the protein kinase G inhibitors KT5823 (A) or Rp-8-pCPT-cGMPS (B) or media for 30 min. Cells were then treated with 100 μM SNOG, SNAP, or SP for 16 h. Cells incubated with media containing Me₂SO for 30 min and then treated with similar media for 16 h served as controls. Cell lysates from treated and untreated cells were processed as described under “Experimental Procedures,” and 10 μg of total protein were subjected to gel electrophoresis and immunoblotting. The lower panel is densitometric analysis from three independent experiments. Data presented are the mean ± S.E. from three separate experiments. *, p < 0.01 as compared with cells treated with an NO donor and a PKG inhibitor. There was no difference between control and cells treated with an NO donor and either PKG inhibitor, p > 0.1.

FIG. 5. Effect of a nitric oxide donor on p11 promoter activity. BEAS-2B cells were grown on 6-well plates until 90% confluent. Cells were transfected with 1 μg of the −1499+89 p11 promoter in pCAT basic vector and CMV/β-gal as described under “Experimental Procedures.” After 16 h, cells were exposed to SNAP (0–500 μM) for 4 h. Cells were collected and pCAT and β-galactosidase activity were measured. Data are expressed as the mean ± S.E. of fold activation of promoterless pCAT basic vector from four independent experiments. The effect was dose related by ANOVA (p < 0.05).

FIG. 6. Hemoglobin and the guanylate cyclase inhibitor, ODQ, abolished the nitric oxide donor (SP) effect on p11 promoter activity. BEAS-2B cells were grown on 6-well plates to 90% confluence. Cells were transfected with 1 μg of the −1499+89 p11 promoter in pCAT basic vector and CMV/β-gal as described under “Experimental Procedures.” After 16 h, cells were preincubated with Hb (5 μM) or with ODQ (5 μM) for 30 min and then exposed to 100 μM SP or incubated with the NO donor only for 4 h. Afterward, cells were collected and pCAT and β-galactosidase activity were measured. Data are expressed as the mean ± S.E. of fold activation of promoterless pCAT basic vector (n = 4–6), Hb + SP and ODQ + SP, and ODQ alone were not different from control.

FIG. 7. cGMP analogue (8-Br-cGMP) mimics nitric oxide donors effect on p11 promoter activity. BEAS-2B cells were grown on 6-well plates until 90% confluent. Cells were transfected with 1 μg of −1499+89 p11 promoter in pCAT basic vector and CMV/β-gal as described under “Experimental Procedures.” After 16 h, cells were incubated with 8-Br-cGMP (dose range from 1 to 250 μM) for 4 h. Cells were collected and pCAT and β-galactosidase activity were measured. Data are expressed as mean ± S.E. of fold activation of promoterless pCAT basic vector (n = 6), p < 0.05 by ANOVA.
RESULTS

Nitric Oxide Increases the Level of the p11 Protein in BEAS-2B Cells—The effect of nitric oxide on the p11 protein expression was tested using three NO donors: spermine NONOate (SP), SNAP, and SNOG. Fig. 1 summarizes data obtained from immunoblots of p11 protein. Exposure of BEAS-2B cells to SP (100 μM) for 4–24 h resulted in an increase in cellular p11 protein (Fig. 1A). The effect of SP on p11 protein levels at 16 h was dose related for 25–400 μM (Fig. 1B). BEAS-2B cells exposed to SNAP exhibited a time and dose dependent increase in p11 protein expression (data not shown). Similarly, cells exposed to SNOG responded with an increase in p11 protein expression in a time dependent and dose dependent manner (data not shown).

Nitric Oxide Effect on Steady-state p11 mRNA Level in BEAS-2B Cells—To investigate whether this NO effect is associated with changes in the steady-state level of p11 mRNA, a ribonuclease protection assay was performed after extracting total RNA from BEAS-2B cells. Exposure of BEAS-2B cells to SP (Fig. 2A), SNAP (data not shown), or to SNOG (data not shown) resulted in time-dependent increases in the steady-state levels of p11 mRNA starting within 4 h. This effect on steady state levels of p11 mRNA was dose dependent for each of the NO donors (Fig. 2B and data not shown).

Protein Kinase G Expression and Activity in BEAS-2B Cells—To confirm the presence of PKG protein in BEAS-2B cells, immunoblotting was performed. BEAS-2B cells were incubated with SP at 2, 20, and 200 μM concentrations for 16 h. PKG I was expressed constitutively and SP did not influence PKG I protein expression (Fig. 3A). A PKG assay was performed to study PKG activity in BEAS-2B cells. Incubation with SNOG (100 μM) significantly increased protein kinase G activity at 30 min with a maximum increase at 1 h (79% above control). This change was still present at 4 h (Fig. 3B).

Effect of Nitric Oxide on p11 Promoter Activity—To further explore the effect of NO donors on p11 production, a reporter gene containing the p11 promoter region from −1499 to + 89

**FIG. 8. The effect of nitric oxide donors on p11 mutated promoter activity (A).** The effect of cotransfection with c-jun dominant negative vector and c-jun expression vector on −188+89 p11 promoter activity (B). A, the effect of SNAP (100 μM) and SP (100 μM) on the wild type −188+89 p11 promoter and a mutated −188+89 promoter. AP-1-binding site in the −188+89 promoter was mutated to Bbr PI restriction site (MU). Cells were transfected with 1 μg of the −188+89 p11 (WT) or MU promoters in pCAT basic vector and CMV/β-galactosidase as described under “Experimental Procedures.” After 16 h, cells were incubated with SNAP or SP (100 μM) for 4 h. Cells were collected and pCAT and β-galactosidase activity were measured. Data are expressed as mean ± S.E. of fold activation of promoterless pCAT basic vector from four independent experiments. *, p < 0.05 as compared with control. B, the effect of co-transfection with TAM67 or a c-jun expression vector on −188+89 promoter activity. BEAS-2B cells were transfected with TAM-67 and −188+89 p11 reporter gene, with a c-jun expression vector and −188+89 p11 reporter gene, or with control vector and −188+89 p11 reporter gene (5:1 ratio, respectively). All sets of cells were co-transfected with pCMV/β-galactosidase to normalize for transfection efficiency. Transfection was done as described above and cells were exposed to SP (100 μM) or media for 24 h. Collection of cells, CAT and β-galactosidase assay were done as described above. *, p < 0.05 as compared with pCMV, +TAM67, +TAM67+SP (n = 6).
was cloned into the basic pCAT vector and transfected into BEAS-2B cells. Incubation of cells with the nitric oxide donor SNAP caused statistically significant increases of pCAT activity as shown in Fig. 5. To confirm that this activity is due to the action of NO, transfected cells were subsequently preincubated with the NO scavenger, hemoglobin, for 30 min and then incubated with SP and Hb for 4 h. As shown on Fig. 6, preincubation and co-incubation of SP with Hb resulted in similar promoter activity to control cells. Incubation with Hb alone did not alter reporter gene activity. To confirm the mechanism of NO action on p11 promoter activity, transfected cells were preincubated with the soluble guanylyl cyclase inhibitor, ODQ, and then co-incubated with ODQ and the NO donor, SP. SP increased reporter gene activity. ODQ alone had no effect on reporter gene activity. However, the increase in reporter gene activity noted in response to SP alone was substantially reduced by ODQ (Fig. 6). In order to confirm this finding, transfected cells were incubated with increasing concentrations of 8-Br-cGMP (range from 1 to 250 μM) for 4 h as shown in Fig. 7. Incubation of cells transfected with p11 promoter with 8-Br-cGMP resulted in a dose-related increase p11 promoter activity.

To further investigate the nitric oxide transcriptional effect, deletion constructs were created in which the promoter region was truncated to form two shorter p11 promoters from −1089 to −89 and −188 to +89. Two shorter p11 promoters were ligated into pCAT basic vector and BEAS-2B cells were transfected. Cells were incubated with SP (100 μM). SP activated both of the shorter p11 promoters suggesting that an NO/cGMP response element is located in −188+89 p11 promoter fragment (data not shown). Analysis of this part of the p11 promoter revealed an AP-1 site located at −82 to −77 of this promoter. To confirm the role of the AP-1 site in NO-dependent p11 promoter activation, BEAS-2B cells were transfected with the −188+89 p11 reporter gene and with the −188+89 MU AP-1 reporter (obtained as described above). The mutated promoter had diminished activity and did not respond to SP as shown in Fig. 8A. To confirm this finding, co-transfection with the TAM67 c-jun dominant negative vector or with a c-jun expression vector were used (Fig. 8B). TAM67 decreased the NO effect on p11 promoter activity, whereas the c-jun expression mimicked the NO effect.

**EMSA—**To confirm that nitric oxide increased p11 expression through this particular AP-1 site electrophoretic mobility gel shift assays with nuclear protein extract from BEAS-2B cells were employed. The AP-1 (−82–77) binding site is partially overlapping with a TCE site. This fact made it difficult to define NO influence on AP-1 complex binding from other potential binding proteins. Therefore, we synthesized an oligonucleotide consisting of four AP-1 wild type sites: 5′-GGAGTCCGGAGTCCGAGTCCGTCG-3′. This oligonucleotide (4xAP-1WT) was used in EMSA studies. To confirm that this oligonucleotide binds to the AP-1 complex, EMSA with an excess of cold AP-1 consensus oligonucleotide (Promega) was performed (Fig. 9). The AP-1 consensus oligonucleotide completely inhibited 4xAP-1WT binding. To confirm binding specificity, ×100 excess of cold 4xAP-1WT oligonucleotide also inhibited AP-1 binding as shown in Fig. 9.

Nuclear protein derived from BEAS-2B cells incubated for 2, 4, and 24 h with SP (100 μM), SNAP (100 μM), or SNOG (10 μM) significantly increased AP-1 binding (Fig. 10A and data not shown). Similarly, EMSA using nuclear proteins obtained from BEAS-2B cells after incubation with 10 μM 8-Br-cGMP increased AP-1 binding (Fig. 10B).

To confirm the role of cGMP in nitric oxide modulation of p11 activity, BEAS-2B cells were incubated with or without a PKG inhibitor (KT5823, 1 μM for 4 h). The EMSA shown in Fig. 11 demonstrated increased binding for nuclear protein extract obtained from cells incubated with NO donors and decreased binding of nuclear protein from extracts of cells which were co-incubated with the PKG inhibitor and a nitric oxide donor. Therefore, three different NO donors increased the p11 cellular protein levels and steady state levels of p11 mRNA.

**DISCUSSION**

p11, or calpactin light chain, is a member of the S-100 family of small, calcium-binding proteins; however, it has several unique features. S-100 proteins contain two EF-hands that function as calcium-binding domains (2). p11 does not have the ability to bind Ca²⁺ ions due to amino acid deletions and substitutions in the two EF-hand motifs. Instead, p11 is present in a variety of cells separately or as a heterotetramer with annexin II (p36). The heterotetramer is composed of two copies of the 36-kDa heavy chain, annexin II, subunits and two copies of 11-kDa light chain (p11) subunits as (p36)₂(p11)₂ (2, 14). Annexin I has been reported to inhibit phospholipase A₂ activity in vitro. These observations led to the hypothesis that the inhibition of phospholipase A₂ by annexins is the mechanism of the anti-inflammatory action of glucocorticoids. Yao and colleagues (15) have shown that glucocorticosteroids increased p11 levels in BEAS-2B and HeLa cells and inhibited phospholipase A₂ activity and arachidonic acid release. These studies taken together suggest a potential role for p11 in regulation of arachidonic acid metabolism in epithelial cells. The (p36)₂(p11)₂ complex may play an important role in exocytosis allowing cross-linking between vesicle and cellular membranes (16). Since annexin II is a substrate for src kinase and PKC, its role in regulation of cell growth and differentiation has been postulated (17). Phosphorylation of annexin II heavy chain reduces the affinity for p11 binding and on the other hand, p11 binding to p36 interferes with the phosphorylation of p36. Therefore, it may influence several signal transduction path-
ways (including insulin receptor sorting and internalization) (18). Studies in Nb2 (T lymphoma) cells suggested that an increase in p11 level may decrease cell proliferation (19). (p36)2(p11)2 complex may also increase c-raf-1 kinase activity (20). Annexin II-p11 tetramer may also act as a surface protein receptor for plasminogen and tissue plasminogen activator. Extracellular p11 binds to plasminogen and increases plasmin generation (21).

Nitric oxide is a potent transcriptional regulator influencing a variety of genes including genes involved in arachidonic acid metabolism. Modulation of promoter activity by NO has been reported to occur by either cGMP-dependent or -independent mechanisms and can result in either up-regulation or down-regulation of gene transcription (7–10). Many promoter studies were done using relatively long promoter regions that appear to be involved in NO-mediated responses. Some studies also concentrated on identification of transcription factors involved in NO-dependent promoter regulation. These studies have identified the following nitric oxide response elements: NF-κB (22), AP-1 (23), heat shock factor 1 (24), and SP-1 (12), all of which undergo increased or decreased DNA binding upon exposure of cells or cell extract to exogenous NO. Redox-sensitive transcription factors (such as SP-1) bind to DNA via three zinc fingers of the Cys2His2 (24) and zinc is easily displaced by a redox reaction or thiol compounds (25). Some data also suggest that nitric oxide decreased transcription factor binding (NF-κB and c-jun) by S-nitrosylation (26). These studies taken together also suggest that nitric oxide may act through a variety of nuclear proteins and that the final effect is promoter-specific.

In this study, we show that p11 expression in human bronchial epithelial cells is stimulated by nitric oxide and that this process is mediated by cGMP activation and the binding of AP-1 complex to an AP-1-binding site located at +78 to +77 of the p11 promoter. This effect was confirmed using three different nitric oxide donors (SP, SNAP, and SNOG) in a relatively broad range of concentrations from 10 to 500 μM. SNAP and SNOG are able to enhance nuclear protein binding by S-nitrosylation, whereas SP is unable to mediate this process. A similar effect of all three nitric oxide donors suggests that p11 accumulation is not mediated through S-nitrosylation. These effects appeared to be time- and dose-dependent.

To confirm that this effect is mediated at the transcriptional level, we assayed steady state mRNA levels showing that p11 mRNA levels are increased in response to NO donors in a time- and dose-dependent manner. A p11 reporter gene containing 188 base pairs 5' of the transcription start site responded to NO donors. Incubation of epithelial cells with nitric oxide donors also significantly increased nuclear protein binding to the AP-1-binding site in the p11 promoter. This effect was blocked when cells were preincubated with a PKG inhibitor and was mimicked by a cell permeable cGMP analogue. To reconfirm this finding we mutated the AP-1 site in a −188+89 reporter

![Fig. 10. The influence nitric oxide donors and a cGMP cell permeable analogue (8-Br-cGMP) on AP-1 binding in the p11 promoter measured by EMSA. BEAS-2B cells were incubated with SP (100 μM) (panel A) or 8-Br-cGMP (10 μM) (panel B) for 2, 4, or 24 h. Cells were lysed. Nuclear protein was extracted as described under “Experimental Procedures.” From each sample, 5 μg of nuclear protein were incubated with 32P-labeled AP-1 WT oligonucleotide probe. The autoradiographs are representative of three experiments with similar results. The arrow indicates protein-DNA complexes.](http://www.jbc.org/)

![Fig. 11. The effect of a protein kinase G inhibitor on an NO donor stimulated AP-1 binding. BEAS-2B cells were preincubated (for 30 min) and co-incubated for 4 h with or without the PKG inhibitor (KT 5823, 1 μM) and 100 μM SNAP, SP, or SNOG for 4 h. Cells were lysed, and nuclear protein was extracted as described under “Experimental Procedures.” From each sample, 5 μg of nuclear protein were incubated with 32P-labeled AP-1 WT oligonucleotide probes. The autoradiographs are representative of three experiments with similar results. The arrow indicates protein-DNA complexes.](http://www.jbc.org/)
construct and this reporter gene did not respond to an NO donor. Similarly, the p11 reporter gene did not respond to SP in cells co-transfected with an AP-1 dominant negative plasmid.

Nitric oxide may modulate activator protein-1 binding activity by S-nitrosylation. This has been reported to decrease the AP-1 complex binding to DNA (23). On the other hand, protein kinase G is able to mediate phosphorylation of transcription factors including activator protein-1 complex proteins. This effect significantly increased binding to DNA (27). Due to the fact that KT 5823 and Rp-8-pCPT-cGMPS, PKG inhibitors, were found to decrease AP-1 complex binding activity and abolished the NO effect on the expression of p11 protein and p11 promoter activity we conclude that the p11 promoter is activated through AP-1 complex phosphorylation leading to increased binding. We confirmed the fact that protein kinase G is playing a crucial role in nitric oxide-induced p11 activation using a soluble guanylate cyclase inhibitor (ODQ) and a cell permeable cGMP analogue. ODQ preincubation completely inhibited the NO effect on p11 promoter activity in transfected BEAS-2B cells suggesting guanylate cyclase involvement. The fact that a cGMP analogue is able to mimic the NO effect on p11 promoter activity and protein expression further confirms this hypothesis.

Recently the fos gene was reported to be regulated via soluble guanylate cyclase and type I cGMP-dependent protein kinase although a putative NO/cGMP responsive promoter element was not described for this gene (28). An NO/cGMP/PKG/AP-1 pathway was crucial for the tumor necrosis factor-α promoter (29) in pulmonary endothelial cells. Similarly, nitric oxide stimulates transforming growth factor β-1 release from human alveolar epithelial cells through protein kinase G activation (30), although the transcription factor involved in this process was not postulated. These data suggest the importance of NO-dependent phosphorylation of activator protein one complex in promoter activation in human lung cells.

While p11 has been reported to play a variety of roles in cellular function (17, 19–21), it seems clear that p11 levels in many cell types are finely controlled (20). We believe that nitric oxide activation of AP-1 binding in the p11 promoter is one of the mechanisms by which this effect may occur.

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