

# Nitric oxide reduces vascular smooth muscle cell elastase activity through cGMP-mediated suppression of ERK phosphorylation and AML1B nuclear partitioning

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**ABSTRACT** Nitric oxide (NO) reduces the severity of pulmonary vascular disease in rats as do elastase inhibitors. We therefore hypothesized that NO inhibits elastase by suppressing mitogen-activated protein kinases that *trans*-activate AML1B, a transcription factor for elastase. We used cultured pulmonary artery smooth muscle cells in which serum-treated elastin (STE) induces a > threefold increase in elastase activity as evaluated by solubilization of [<sup>3</sup>H]-elastin. NO donors (SNAP and DETA NONOate) inhibited elastase in a dose-dependent manner as did a cGMP mimetic (8-pCPT-cGMP). SNAP inhibition of elastase was reversed by coadministration of a cGMP-PKG inhibitor (Rp-8-pCPT-cGMP). The STE-induced increase in phospho-ERK was suppressed by NO donors and the cGMP mimetic, and reversed by cGMP-PKG inhibitor, as was expression of AML1B and DNA binding in nuclear extracts. A concomitant increase in p38 phosphorylation was also inhibited by SNAP, but whereas MEK inhibitor (PD98059) suppressed elastase and AML1B-DNA binding, a p38 inhibitor (SB202190) did not. Our study uniquely links NO with inhibition of elastase-dependent matrix remodeling in vascular disease by suggesting a cGMP-PKG-related mechanism suppressing ERK-mediated partitioning of AML1B in nuclear extracts.—Mitani, Y., Zaidi, S. H. E., Dufourcq, P., Thompson, K., Rabinovitch, M. Nitric oxide reduces vascular smooth muscle cell elastase activity through cGMP-mediated suppression of ERK phosphorylation and AML1B nuclear partitioning. *FASEB J.* 14, 805–814 (2000)

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IMPAIRED PRODUCTION OF nitric oxide (NO) is associated with a variety of vascular diseases characterized by extensive structural remodeling, which include pulmonary hypertension, atherosclerosis,

restenosis after angioplasty, and coronary arteriopathy after experimental heart transplantation (reviewed in ref 1). The changes observed in all these vascular pathologies are characterized by smooth muscle cell proliferation, migration, and accumulation of extracellular matrix glycoproteins such as collagen, fibronectin, and tenascin (2–5). The beneficial therapeutic effect of NO in patients with advanced pulmonary hypertension appears to be related to its vasodilator properties as well as to direct effects on the remodeling process, the nature of which are obscure (6, 7). The antiatherogenic effects of NO have also been described as independent of its vasodilator function (1) and may be related to its role as an intracellular signaling molecule. Administration of L-arginine, the substrate for NO, suppresses monocrotaline-induced pulmonary vascular disease in rats (7), and eNOS knockout mice have increased pulmonary hypertension and vascular disease when subjected to chronic hypoxia (8) and more severe neointimal formation after cuff injury of the femoral artery (9).

The features of vascular remodeling have been attributed to heightened activity of proteolytic enzymes, both elastases and matrix metalloproteinases (10–18). In experimental rabbits, increased activity of a serine elastase has been demonstrated in coronary arteries after cardiac transplant (14) and in vein grafts after arterial interposition (15). Increased activity of a 20 kDa endogenous vascular serine elastase (EVE) appears critical to the progression of experimental pulmonary vascular disease (18). The activity of EVE is increased in the early period after injection of the toxin monocrotaline (MCT) (10) in association with pulmonary endothelial injury (11). Furthermore, administration of serine elastase inhib-

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itors largely prevents development or retard progression of hypoxia (12) and monocrotaline-induced PVD in rats (13) and reduces the severity of the coronary arteriopathy after transplant (14) and the atherosclerotic degeneration of vein grafts in rabbits (15). Inhibition of elastase activity not only prevents the progression of vascular disease, but also appears to induce regression (16, 17).

The source of EVE appears to be smooth muscle cells (18), and the sequelae of EVE activity have been shown in cultured cells to be related to liberation of mitogenic growth factors from the extracellular matrix (19) and to induction of the proliferative glycoprotein tenascin (16, 17, 20) and of fibronectin-dependent smooth muscle cell migration in response to elastin peptides (21). EVE is produced in cultured smooth muscle cells (SMCs) after serum stimulation and appears to function in the microenvironment of the cell such that adhesion of elastin to the cell surface by serum factors is critical in demonstrating the activity of this enzyme (22, 23). The induction of EVE by serum-treated elastin (STE) is associated with tyrosine phosphorylation of focal adhesion kinase (FAK) and ERK1 (24) and by an increase in the transcription factor AML1 (A and B isoforms) (25). AML1 was initially described as a mutated gene in acute myeloid leukemia. While it exists as multiple isoforms, the products of alternative splicing of mRNA, the AML1B isoform is consistently found in the nucleus where it is transcriptionally active. Its expression is related to hematopoietic cell differentiation and proliferation and to the transcription of genes such as leukocyte elastase, myeloperoxidase, the receptor for macrophage colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, osteocalcin, and the T cell receptor (26–29).

We speculated there might be a link between NO and the MAP kinase signaling pathway that results in an increase in AML1B, the transcription factor for EVE, since NO inhibits ERK activation by a cyclic GMP-mediated activation of protein kinase G, which interferes with RAS/raf interaction (30). We now demonstrate that NO donors SNAP and DETA NONOate and a cGMP mimetic inhibit SMC elastase by repressing ERK phosphorylation, and that the mechanism is consistent with protein kinase G activity. We also report the novel finding that the reduction in ERK phosphorylation shifts distribution of the transcriptionally active isoform AML1B in the nucleus and the decrease in AML1B in nuclear extracts is associated with reduced DNA binding. These studies suggest a mechanism whereby NO might affect matrix remodeling by suppressing the intracellular signaling cascade necessary for the transcription of elastase.

## MATERIALS AND METHODS

### Materials

Medium 199 (M199), antibiotic-antimycotic solution, and trypan blue were purchased from Life Technologies, Inc. (Burlington, Ontario). Fetal bovine serum (FBS) was obtained from Intergen Co. (New York, N.Y.) and bovine serum albumin (BSA) from Boehringer Mannheim (Laval, Quebec, Canada). Purified elastin from bovine neck ligament was purchased from Elastin Products Co. Inc. (Owensville, Mo.). [ $^3\text{H}$ ]-NaBH $_4$  was purchased from New England Nuclear (Boston, Mass.) and [ $^{32}\text{P}$ ]- $\alpha$  ATP, HRP-conjugated anti-rabbit IgG antibody, and the enhanced chemiluminescence kit were purchased from Amersham (Oakville, Ontario). PD 98059 (2'-amino-3'-methoxyflavone) and SB202190 were obtained from Calbiochem (San Diego, Calif.). Genistein, uric acid (UA), 4,5-dihydroxy-1,3-benzene-disulfonic acid (Tiron), diamide, 2',3'-dideoxycytidine (DDC), and diphenyleneiodonium (DPI) were from Sigma, Mississauga, Ontario. Hydrogen peroxide was purchased from BDH Inc. (Toronto, Ontario). DETA NONOate, 8-pCPT-cGMP, Rp-8-pCPT-cGMP, peroxyntirite (ONOO $^-$ ), and [ $^1\text{H}$ ]-[1, 2, 4] oxadiazole [4,3-a] quinoxalin-1-one (ODQ) were from Alexis Corp. (San Diego, Calif.). S-nitro-N-acetylpenicillamine (SNAP) was obtained from BIOMOL Research Laboratories (Plymouth Meeting, Pa.). Antibodies against phospho-specific and -non-specific ERK, or phospho-specific and -nonspecific p38 were purchased from New England Biolabs (Beverly, Mass.); an antibody against a 17 amino acid amino-terminal peptide based on the human AML1 sequence was from Calbiochem (LaJolla, Calif.). The Bradford reagent came from Bio-Rad Laboratories (Hercules, Calif.). Tris-glycine gels were from Helix (Mississauga, Ontario) and polyvinylidene difluoride (PVDF) membranes from Millipore (Bedford, Mass.).

### Isolation and culture of vascular SMCs

Pulmonary artery SMCs were harvested from central vessels of juvenile pigs and cultured as in our previous studies (22–24). Explants of the vascular media were cultured in medium M199 supplemented with 10% FBS and 1% antibiotics-antimycotics (100 units/ml penicillin, 100  $\mu\text{g}/\text{ml}$  streptomycin, amphotericin B 250 ng/ml) in a humid 5% CO $_2$  environment at 37°C. The SMCs were used at passages 2–4.

### Preparation of radiolabeled elastin and STE

Purified elastin from bovine neck ligament was radiolabeled using [ $^3\text{H}$ ]-NaBH $_4$ , as described previously (31). The [ $^3\text{H}$ ]-elastin was reconstituted at 16 mg elastin/ml (specific activity 2,000 cpm/ $\mu\text{g}$  elastin) in Tris assay buffer (50 mmol/l Tris HCl, 150 mmol/l NaCl, 10 mmol/l CaCl $_2$  2H $_2$ O, 0.02% Brij, pH 8.0), boiled, and stored at  $-70^\circ\text{C}$  until use. Before each assay, [ $^3\text{H}$ ]-elastin was washed with Tris assay buffer until the background counts became less than 100 cpm/100  $\mu\text{l}$  supernatant. The [ $^3\text{H}$ ]-elastin suspension was then diluted to 10 $^5$  cpm/20  $\mu\text{l}$ . Pretreating the [ $^3\text{H}$ ]-elastin with FBS has been shown to stimulate elastin adhesion to SMC surfaces and induce endogenous vascular elastase activity (22–25). Radiolabeled insoluble elastin was incubated with FBS at a concentration of 10 mg/ml and rotated overnight at 37°C, then washed four times with Tris assay buffer to remove all unbound serum factors. The pretreated [ $^3\text{H}$ ]-elastin suspension was then diluted to 10 $^5$  cpm/20  $\mu\text{l}$  with Tris assay buffer and added to cultured SMCs for assay of elastase activity.

## Activity assay of elastase in cultured SMCs

The assay has been previously described in detail (22–25). Briefly, confluent SMCs were passaged to 24 wells of a 16 mm diameter multiwell tissue culture plate ( $\sim 5 \times 10^4$  cells per well). At confluence, SMCs were serum starved for 24 h in serum-free M199 with 0.1% BSA and 1% antibiotics. Cell monolayers were incubated for another 24 h with 20  $\mu$ l [ $^3$ H]-elastin either in serum-free medium (control) or with serum-treated elastin prepared as described above in a final volume of 1 ml in each well. To investigate the effects of NO donors SNAP (0.01–1 mM) and DETA NONOate (0.1–1 mM), and a cGMP mimetic 8-pCPT-cGMP (1 mM) on elastase activity, cells were pretreated with each compound for 30 min. To evaluate the effect of coadministration of a cGMP PKG inhibitor Rp-8-pCPT-cGMP (20  $\mu$ M) on SMCs treated with SNAP, cells were pretreated with Rp-8-pCPT-cGMP for 30 min. To check the effect of a specific p38 inhibitor on elastase activity, cells were pretreated with SB202190 (20  $\mu$ M) for 30 min; control cells and STE-stimulated cells were similarly pretreated with the vehicle 0.1% DMSO. SNAP, DETA NONOate, 8-pCPT-cGMP, and Rp-8-pCPT-cGMP were freshly prepared in phosphate-buffered saline (PBS), whereas SB202190 was dissolved in DMSO. The dose range of each compound was based on previous studies (32–38). Eight hundred  $\mu$ l of culture medium were harvested and microcentrifuged at 8160 *g* for 5 min. The amount of [ $^3$ H]-soluble elastin peptides in 600  $\mu$ l of supernatant was determined by scintillation counting (LKB Wallac 1219 Rackbeta counter, San Francisco, Calif.). To control for nonenzymatic degradation of the elastin substrate, we incubated 20  $\mu$ l of [ $^3$ H]-elastin with medium in cell-free wells in the presence of each test compound and subtracted the counts obtained as background. Compounds found to nonenzymatically degrade elastin (background counts above 200 cpm/well) were not used in these assays. To confirm the viability of cells in the presence of each test compound, the trypan blue exclusion method was used after incubation for 24 h.

## Preparation of the total cell lysate

Total cell lysates were prepared to determine expression of phospho-specific or -nonspecific ERK or p38. After  $5 \times 10^5$  SMCs were plated and cultured for 36 h in 60 mm dishes, confluent monolayers of SMCs were serum starved for 36 h. The SMCs were exposed to STE or an equal amount of elastin (control) for 5, 10, or 30 min depending on the experiment after pretreatment with DETA NONOate (0.1–1 mM), SNAP (0.01–1 mM), a cGMP mimetic 8-pCPT-cGMP (1 mM), peroxynitrite ONOO<sup>-</sup> (0.1 mM), and the inactivated decomposed control for ONOO<sup>-</sup> for 30 min. To evaluate the effect of coadministration of a cGMP PKG inhibitor Rp-8-pCPT-cGMP (20  $\mu$ M) on SMCs pretreated with SNAP, cells were pretreated with Rp-8-pCPT-cGMP for 30 min, followed by treating cells with SNAP. After various treatments, cells were lysed by addition of boiling lysis buffer [1% sodium dodecyl sulfate (SDS), 1 mmol/l sodium vanadate, 10 mmol/l Tris pH 7.4], scraped into a microcentrifuge tube, and boiled for 5 min. Samples were centrifuged for 10 min to remove insoluble material.

## Preparation of subcellular fractions

Cytosolic fractions, nuclear extracts, and pellets were prepared to determine the distribution and relative expression of AML1 isoforms and whether there was a shift in molecular weight that would suggest an alteration in phosphorylation status. After  $2 \times 10^6$  cells were plated and cultured in 150 mm

dishes for 36 h, confluent monolayers of SMCs were serum starved for 36 h. Serum-starved cells pretreated with DETA NONOate (1 mM), SNAP (1 mM), or 8-pCPT-cGMP (1 mM) were stimulated with STE or control elastin for 30 min. To evaluate the effect of coadministration of a cGMP and PKG inhibitor, Rp-8-pCPT-cGMP (20  $\mu$ M), on SMCs pretreated with SNAP, cells were pretreated with Rp-8-pCPT-cGMP for 30 min, followed by treating the cells with SNAP. To check the effect of a specific p38 inhibitor, SB202190, on AML1 isoform expression, cells were pretreated with SB202190 (20  $\mu$ M) for 30 min; control cells and STE-stimulated cells were similarly pretreated with the vehicle, 0.1% DMSO.

Subcellular fractions were prepared according to a standard protocol (39). Briefly, after removing medium, cells were rinsed in cold PBS, scraped into microcentrifuge tubes, and the pellet was washed in PBS. Cells were lysed in cold hypotonic buffer [10 mmol/l N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES; pH 7.9), 1.5 mmol/l MgCl<sub>2</sub>, 19 mmol/l KCl, 0.2 mmol/l PMSF, 0.5 mmol/l DTT], followed by 15 passes with a type B Dounce homogenizer (VWR Scientific Ltd., London, Ontario). Nuclei were pelleted at 3300 *g* for 15 min at 4°C. The supernatant was mixed with 0.11 vol of 10 X cytoplasmic extract buffer (0.3 M HEPES pH 7.9, 1.4 M NaCl, 0.03 M MgCl<sub>2</sub>) and centrifuged at 16,000 *g* for 30 min. The supernatant is designated as S100. The nuclei pelleted were extracted in a high-salt buffer (20 mmol/l HEPES pH 7.9, 25% glycerol, 1.5 mmol/l MgCl<sub>2</sub>, 0.8 mol/l NaCl, 0.2 mmol/l EDTA, 0.2 mmol/l PMSF, 0.5 mmol/l DTT) with vortexing for 40 min, then microcentrifuged at 16,000 *g* for 30 min. The supernatants designated as nuclear extract were rapidly frozen and stored at -70°C. The residual nuclear pellet was washed with high-salt buffer once, boiled, and extracted by vortexing in SDS buffer (1% SDS, 10 mmol/l Tris pH 7.4) for 60 min. After microcentrifugation at 16,000 *g* for 30 min, the salt-resistant supernatants designated as nuclear pellet were frozen at -70°C until use.

## Western immunoblotting

Aliquots of 10  $\mu$ g of total protein from total cell lysate or from different subcellular fractions, as determined by Bradford protein assay were electrophoresed under reducing conditions by SDS-PAGE (SDS-polyacrylamide gel electrophoresis) on an 8–16% polyacrylamide Tris-glycine gel and transferred onto a PVDF membrane. Nonspecific binding was blocked by incubating the blot in blocking buffer (5% dry non-fat milk in 10 mmol/l Tris, pH 7.4, 50 mmol/l NaCl, and 0.5% Tween 20) for 1 h at room temperature.

The blot was then incubated with primary antibodies against phospho-specific or -nonspecific ERK, phospho-specific or -nonspecific p38 (1:1000), or AML1 (1:200). After the blots were washed with TBS-T (10 mmol/l Tris HCl, pH 7.4, 50 mmol/l NaCl and 0.5% Tween 20) for 30 min, they were incubated with HRP-conjugated anti-rabbit antibodies (1:5000) at room temperature for 1 h. After the blots were washed with TBS-T for 30 min, protein bands were visualized by enhanced chemiluminescence. The intensity of specific bands was quantified by scanning soft-laser densitometry (Bio-Rad Gel Doc 1000) and values from three different harvests in each group were averaged. Equal loading and transfer of proteins were confirmed by visualizing proteins after staining the gel with Coomassie blue.

## Electrophoretic mobility shift assay (EMSA)

The protein AML1:DNA binding site interactions were analyzed by EMSA according to the protocol described by Meyers et al. (26). The wild-type AML1 binding site double-stranded

DNA oligonucleotides (similar for all isoforms) were prepared by annealing the complementary oligomers 5'-AATTC-GAGTAT *TGTGGTTAATACG*-3' and 5'-AATTCGTATTAAC-CACAATACTCG-3' (consensus binding site is italicized) and labeling with [ $\alpha^{32}$ P]dATP in a Klenow reaction. The oligomers 5'-AATTCGAGTATTGTTAGTAATACG-3' and 5'-AATTCGTATTAACAATACTCG-3' were annealed to form an AML1 mutant oligonucleotide. Briefly, the AML1 binding site reaction contained 10  $\mu$ g total protein from nuclear extracts and 1 ng of radiolabeled oligonucleotide in binding buffer (20 mmol/l HEPES, pH 7.8, 1 mmol/l MgCl<sub>2</sub>, 0.1 mmol/l EGTA, 0.4 mmol/l DTT, 40 mmol/l KCl, 10% glycerol and 60  $\mu$ g/ml salmon sperm DNA) and proceeded for 30 min at room temperature. In competition studies, 50 ng of unlabeled double-stranded wild type or mutant oligonucleotides or 0.5  $\mu$ g of AML1 antibody were preincubated for 30 min with the STE-treated nuclear extracts prior to the addition of radiolabeled oligonucleotides. The reactions were resolved on a 5% nondenaturing polyacrylamide gel in TBE buffer (50 mmol/l Tris borate and 1.0 mmol/l EDTA, pH 8.0) and exposed to Kodak XAR film.

### Statistical analysis

Statistical analyses were carried out on absolute values or ratios as indicated. Differences between the treatment groups were determined by a one-way ANOVA, followed by Student-Newman-Kuels test or a Fisher's protected LSD, if specified. A level of  $P < 0.05$  was accepted as statistically significant. Data are presented as mean  $\pm$  SE. The number of experiments is given in the figure legend.

## RESULTS

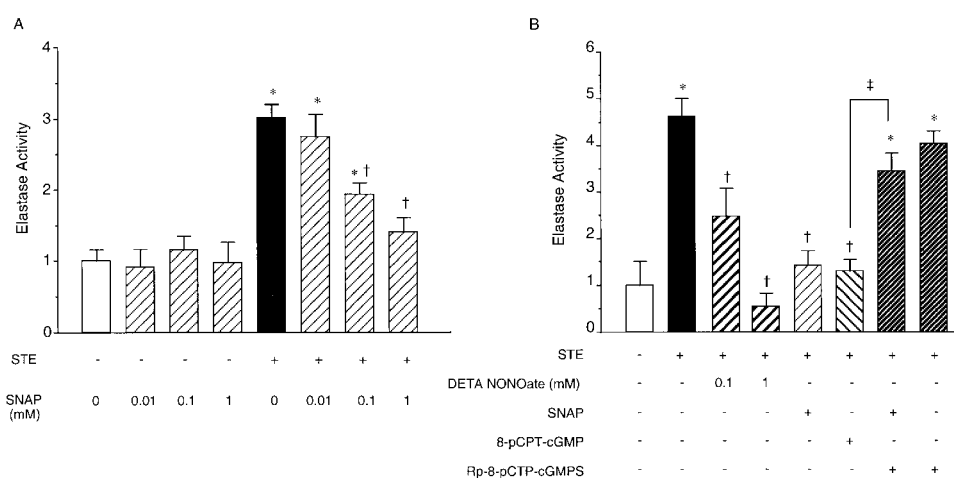
### Smooth muscle elastase activity is inhibited by NO via cGMP

To test the effect of NO in modulating the induction of elastase in cultured SMCs, we added the NO

donor SNAP. We demonstrated that the > threefold induction of SMC elastase activity mediated by STE was inhibited by SNAP in a dose-dependent manner. The decrease was significant at 0.1 mM ( $P < 0.05$ ) and there was complete suppression of stimulated activity at 1 mM (Fig. 1A). SNAP did not depress basal levels of elastase activity. Since SNAP can also produce small amounts of superoxide (35), we confirmed that the effect of SNAP was specifically related to NO by showing that DETA NONOate, a pure NO donor, similarly inhibited STE-induced elastase activity in a dose-dependent manner ( $P < 0.05$ ) (Fig. 1B). We next determined that the mechanism of NO-mediated suppression of elastase appeared to be related to cGMP-mediated PKG activity. Pretreatment with a cGMP mimetic, 8-pCPT-cGMP (1 mM), abolished elastase activity ( $P < 0.05$ ), and coadministration with the PKG inhibitor Rp-8-pCTP-cGMP (20  $\mu$ M) reversed the SNAP suppression of elastase activity ( $P < 0.05$ ) (Fig. 1B). There were no significant effects of any of these compounds on the basal levels of elastase activity, nor did they induce nonenzymatic degradation of labeled elastin in cell-free wells (data not shown). These compounds were not toxic to the cells, as evaluated by trypan blue exclusion tests, and the lack of the significant effect of STE on DNA synthesis had previously been confirmed.

To determine a direct link between NO suppression of elastase activity and soluble guanylate cyclase, we attempted to use the NO-sensitive soluble guanylate cyclase inhibitor ODQ (0.01 mM). This compound, however, caused nonenzymatic degradation of radiolabeled elastin. To negate the possibility that NO-mediated inhibition of elastase is related to the production of ONOO<sup>-</sup> that results from NO inter-

**Figure 1.** Influence of NO donors and a cGMP mimetic and inhibitor on smooth muscle cell (SMC) elastase activity. Elastase activity was evaluated by measured solubilized [ $^3$ H]-elastin in the culture medium after a 24 h incubation as described in Materials and Methods. A) Serum-starved SMCs were maintained serum free or incubated with serum-treated elastin (STE) after pretreatment with the NO donor SNAP (0.1–1 mM) for 30 min. B) Comparison of SMC elastase activity under control conditions or stimulated by serum-treated elastin (STE) after pretreatment with NO donors DETA NONOate (0.1–1 mM), SNAP (1 mM), and the cyclic GMP mimetic 8-pCPT-cGMP (1 mM) for 30 min. The effect of pretreatment with the cGMP-protein kinase G inhibitor Rp-8-pCPT-cGMP (20  $\mu$ M) on reversing SNAP suppression of elastase activity was also evaluated. Data were compared statistically based on cpm values but are illustrated as the ratio relative to the control value (which we have calculated as  $95.3 \pm 6.2$  ng degraded elastin/ $\mu$ g DNA) and reported as means  $\pm$  SE of quadruplicate wells, with similar results in three independent experiments. \* $P < 0.05$  vs. the control.  $P < 0.05$  vs. STE with no pretreatment. ‡ $P < 0.05$  between designated groups.



action with endogenous superoxide, several oxidants (ONOO<sup>-</sup>, hydrogen peroxide, diamide, and DDC) and antioxidants (UA, DPI, and Tiron) were tested. However, these compounds all induced nonenzymatic degradation of radiolabeled elastin.

### NO suppresses ERK phosphorylation via cGMP

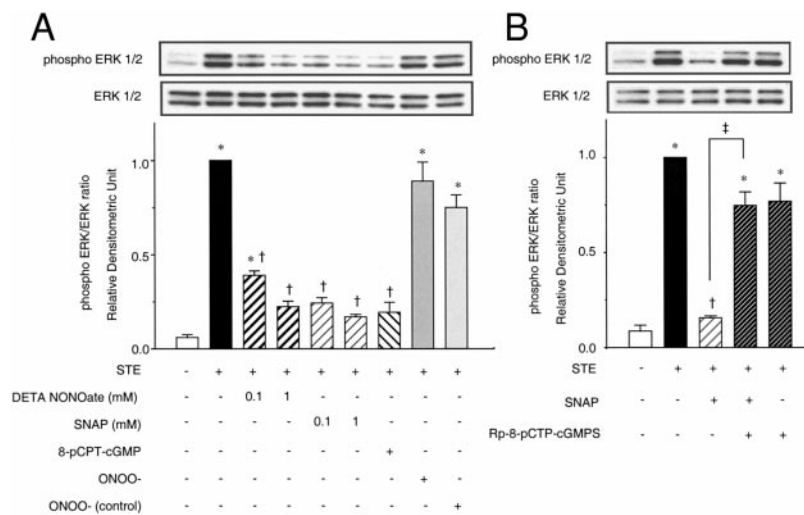
Our previous studies showed that STE-induced elastase activity was mediated by ERK phosphorylation (24). We therefore determined whether NO might suppress ERK phosphorylation. Protein expression of ERK is not modulated by STE or any of the compounds used (Fig. 2A, B). STE pretreatment of SMCs induced a >10-fold increase in phosphorylation of ERK ( $P < 0.05$ ). This induction was reduced by > 60% with DETA NONOate and completely reversed by 1 mM of DETA NONOate as well as 0.1–1 mM of SNAP ( $P < 0.05$  for all comparisons) (Fig. 2A). Although for the technical reasons described above we could not determine whether ONOO<sup>-</sup> produced from NO and endogenous superoxide suppressed elastase activity, we were able to show that when cells were pretreated with ONOO<sup>-</sup> (0.1 mM) or its decomposed inactive control, ERK phosphorylation was not inhibited (Fig. 2A). Consistent with this, coadministration of a ONOO<sup>-</sup> scavenger uric acid (0.1 mM) did not reverse SNAP suppression of ERK phosphorylation in two independent experiments (data not shown).

The effect of NO donors was consistent with PKG suppression of ERK phosphorylation, since pretreatment of cells with the cGMP mimetic 8-pCPT-cGMP (1 mM) abolished STE-induced ERK phosphorylation and administration of the cGMP PKG inhibitor Rp-8-pCTP-cGMP (20 μmol/l) reversed SNAP suppression of STE-induced ERK phosphorylation ( $P < 0.05$ ) (Fig. 2B).

### NO-cGMP mechanism inhibits nuclear expression and DNA binding of AML1

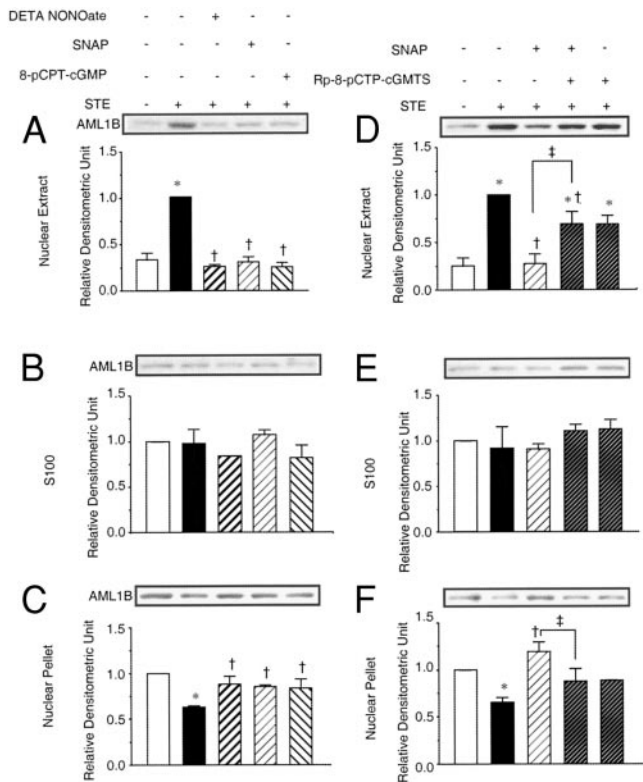
Our previous studies showed that STE induction of ERK phosphorylation is necessary for nuclear expression and DNA binding of the transcription factor for elastase, AML1 (B isoform) (24). We therefore determined whether we could correlate the inhibitory effect of NO donors on ERK phosphorylation with a reduction in nuclear expression of AML1B. STE induced a > threefold increase in nuclear expression of AML1B (the 50 kDa transcriptionally active isoform) (Fig. 3A). Pretreatment of SMCs with DETA NONOate (1 mM), SNAP (1 mM), or 8-pCPT-cGMP (1 mM) abolished STE-induced nuclear expression of AML1B (Fig. 3A). Consistent with a cyclic GMP-mediated PKG effect, coadministration of Rp-8-pCTP-cGMP (20 μmol/l) reversed SNAP inhibition of STE-induced nuclear expression of AML1B (Fig. 3D).

To exclude the possibility that STE-mediated induction of nuclear expression of AML1B or its suppression by the NO-PKG pathway was associated with a translocation from or to the cytoplasm, we also monitored AML1B in cytoplasmic extracts but found no appreciable changes with STE, NO donors, or the PKG stimulator or inhibitor at the 30 min time point (Fig. 3B, E). AML1B has also been found in the nuclear pellet, so it was of interest that STE decreases AML1B in the nuclear pellet ( $P < 0.05$ ); this decrease was reversed by DETA NONOate (1 mM), SNAP (mM), or 8-pCPT-cGMP (1 mM) ( $P < 0.05$  by Fisher's protected LSD) (Fig. 3C). The SNAP-induced reversal of AML1 expression in the nuclear pellet was inhibited by coadministration of Rp-8-pCTP-cGMP (20 μmol/l;  $P < 0.05$  by Fisher's protected LSD) (Fig. 3F). This suggests that NO-cGMP-PKG-mediated



**Figure 2.** Influence of NO donors, cGMP mimetic and inhibitor, and peroxynitrite (ONOO<sup>-</sup>) on ERK phosphorylation. A) Serum-starved cells pretreated with DETA NONOate (0.1–1 mM), SNAP (0.1–1 mM), 8-pCPT-cGMP (1 mM), ONOO<sup>-</sup> (0.1 mM), and an inactivated negative control for ONOO<sup>-</sup> (0.1 mM) or B) pretreated with SNAP (1 mM) in the presence or absence of coadministration with Rp-8-pCPT-cGMP (20 μM) were stimulated by STE or control elastin for 5 min. Cell lysates (10 μg) were analyzed by SDS-PAGE and immunoblotted with phospho-specific or -nonspecific ERK antibodies. Densitometric units of phospho-ERK and ERK are evaluated and represented graphically as a ratio relative to the STE ratio assigned as 1. Values were calculated as means ± SE of three independent experiments. Statistical comparisons were based on

actual densitometric units in each experiment \* $P < 0.05$  vs. the control elastin.  $P < 0.05$  vs. STE with no pretreatment. ‡ $P < 0.05$  between designated groups.



**Figure 3.** Influence of NO donors and cGMP mimetic activator and inhibitor on STE-stimulated AML1B expression in three subcellular components of SMCs. Serum-starved cells pretreated with or without DETA NONOate (1 mM), SNAP (1 mM), and 8-pCPT-cGMP (1 mM) for 30 min were stimulated by STE for 30 min. Samples (10  $\mu$ g) from *A*) nuclear extract, *B*) S100, and *C*) nuclear pellet were analyzed by SDS-PAGE, and immunoblotted with an antibody that recognized AML1B. Samples from *D*) nuclear extract, *E*) S100, and *F*) nuclear pellet in cells pretreated with SNAP (1 mM) in the presence or absence of Rp-8-pCPT-cGMP (20  $\mu$ M) were similarly analyzed. Values are evaluated as densitometric units and depicted graphically as a ratio relative to the value in STE-treated cells assigned as 1 and as mean  $\pm$  SE of three independent experiments. Statistical comparisons were based on relative values in each experiment. \* $P < 0.05$  vs. the control.  $P < 0.05$  vs. STE with no pretreatment. † $P < 0.05$  between designated groups.

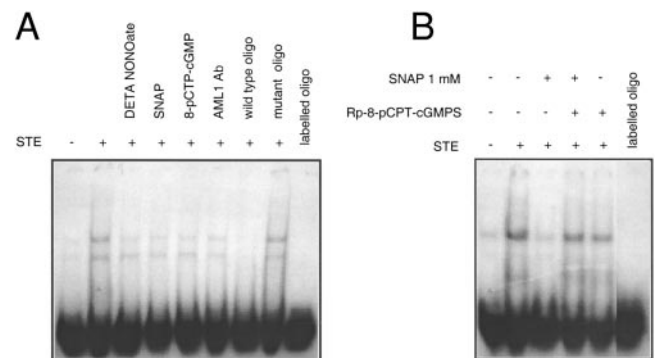
ERK phosphorylation might redistribute AML1B in the nucleus.

To investigate whether this change might be associated with alteration in the phosphorylation status of AML1B, the nuclear extracts, cytoplasmic extracts and nuclear pellets from all the above conditions were analyzed by 8–16% SDS-PAGE run for a maximal time (>4 h) and immunoblotted with an antibody recognizing all AML1 isoforms. We could not identify a band shift among different subcellular fractions or among different treatment groups, which would suggest an altered phosphorylation state, nor could we show differences upon phosphatase treatment of the various fractions (data not shown).

To confirm that the form of AML1B found in the

nuclear extract could bind DNA, EMSA was carried out as described in Materials and Methods using a radiolabeled DNA oligonucleotide encoding the AML1B binding sequence (similar in all isoforms). The protein:DNA complexes formed after stimulation with STE were inhibited by DETA NONOate (1 mM), SNAP (1 mM), or 8-pCPT-cGMP (1 mM) (Fig. 4A). Consistent with changes in AML1 protein expression, SNAP suppression of STE-induced protein:DNA binding was reversed by coadministration of Rp-8-pCPT-cGMP (20  $\mu$ mol/l) (Fig. 4B). Competition experiments were also carried out to confirm the specificity of the protein:DNA binding. A 50-fold increase in unlabeled wild-type AML1 oligonucleotide, but not the mutant oligonucleotide, inhibited the formation of the protein:DNA complexes. The formation of these complexes was also inhibited by the AML1 antibody, even though a ternary AML1 antibody/protein/DNA complex (supershift) was not found. The failure to produce a supershift might be due to the different source of AML1 polyclonal antibody from that used in our previous experiments where supershifts were demonstrated (25).

To negate the possibility that NO suppression of nuclear expression of AML1B is due to ONOO<sup>-</sup>, SMCs were pretreated with a ONOO<sup>-</sup> scavenger UA or a cell-permeant scavenger of superoxide, but SNAP suppression of STE-induced nuclear expression and DNA binding of AML1B was not reversed in two independent experiments (data not shown).



**Figure 4.** Influence of NO donors, cGMP mimetic activator, and inhibitor on AML1-DNA binding as evaluated by electrophoretic mobility shift assay. Serum-starved SMCs, preincubated with or without *A*) DETA NONOate (1 mM), SNAP (1 mM), and 8-pCPT-cGMP (1 mM) for 30 min or pretreated with *B*) SNAP (1 mM) for 30 min in the presence or absence of Rp-8-pCPT-cGMP (20  $\mu$ M) for 60 min were treated with or without STE for an additional 30 min. Radiolabeled wild-type AML1 consensus DNA oligonucleotide was incubated with the nuclear extracts (10  $\mu$ g) and analyzed on a 5% nondenaturing polyacrylamide gel. In competition experiments to confirm the specificity of the protein:DNA interaction, nuclear extracts were incubated with 0.5  $\mu$ g of AML1 antibody or a 50-fold excess of unlabeled wild-type or mutant oligonucleotide as described in Materials and Methods. This gel is representative of three independent experiments with similar results.

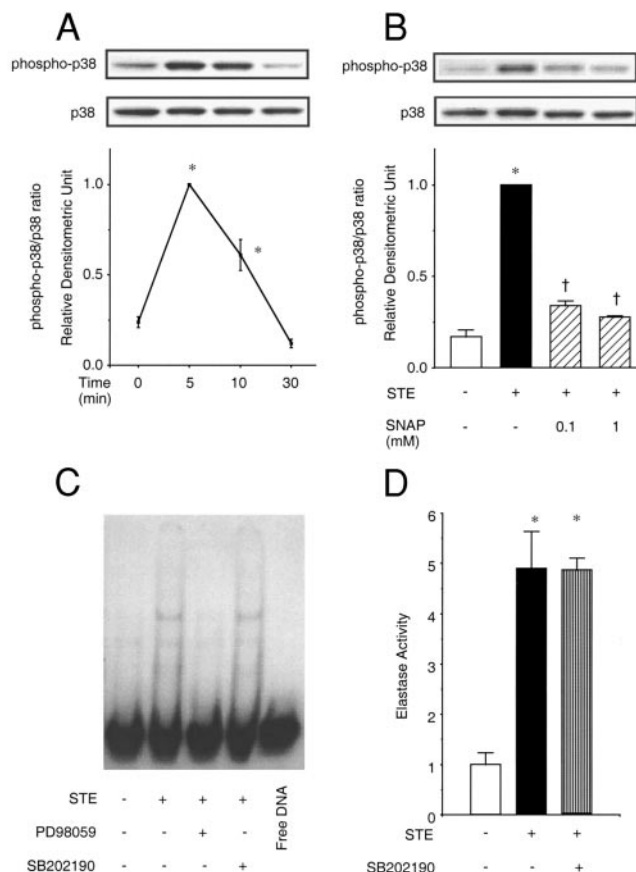
## STE activation of ERK, not p38, causes NO suppression of AML1 and elastase

To confirm the specificity of ERK among MAP kinases in inducing AML1 expression and elastase activity, we investigated whether another MAP kinase, p38, is phosphorylated by STE and whether SNAP inhibits phosphorylation of p38. STE induced a > fivefold increase in phosphorylation of p38 at 5 min ( $P < 0.05$ ) and values returned to baseline by 30 min; protein expression of p38 was unaffected (Fig. 5A). Pretreatment of SMCs with SNAP (0.1 mM and 1 mM) abolished the induction of p38 phosphorylation ( $P < 0.05$ ) (Fig. 5B). However, pretreatment of SMCs with a specific p38 inhibitor, SB202190, did not prevent the increase in AML1B in nuclear extracts (not shown) or its DNA binding (Fig. 5C) induced by STE, and inhibited by the MEK inhibitor PD 98059. Consistent with this finding, SB202190 did not inhibit STE-induced elastase activity. The lack of effect of SB202190 on nonenzymatic degradation of elastin or unstimulated control cells was confirmed (data not shown).

## DISCUSSION

Proteolytic degradation of the extracellular matrix is a feature of a broad spectrum of vascular diseases including pulmonary hypertension, post-transplant coronary arteriopathy, atherosclerosis, and restenosis (10, 14, 15, 49, 41). Its pathophysiologic significance has been documented in cultured smooth muscle cells, where alterations in cell:extracellular matrix interactions induced by heightened proteolytic activity are major determinants of increased cell proliferation and migration (16, 17, 19, 20). Impaired endothelial NO production is also a feature of vascular disease, and restoration of endothelial function or administration of NO donors ameliorates these disorders (reviewed in ref 1).

A variety of studies have suggested that the beneficial effect of NO may be related to its role as an intracellular signaling molecule as well as to its vasodilatory properties. NO can inhibit the mitogenic response of SMCs to growth factors and the production of extracellular matrix proteins such as proteoglycans, osteopontin, and thrombospondin, the latter via protein kinase G (1, 30, 37, 38). We now report that NO can inhibit vascular remodeling by suppressing the signaling mechanism required for the transcription of elastase. We established that different NO donors inhibit elastase activity induced in cultured vascular smooth muscle cells by STE and that the effect is mediated by cGMP. Since elastase activity is dependent on phosphorylation of ERK and on increased AML1B in nuclear extracts, we docu-



**Figure 5.** STE activation of ERK, but not p38, is involved in NO suppression of AML1B expression and elastase activity. A) Serum-starved SMCs were treated with STE for 0, 5, 10, and 30 min, and B) SMCs pretreated with or without SNAP (0.1–1 mM) for 30 min were treated with STE for 5 min. Cell lysates (10  $\mu$ g) were analyzed by SDS-PAGE and immunoblotted with phospho-specific or -nonspecific p38 antibodies. Densitometry of phospho-specific p38 and -nonspecific p38 were evaluated and the ratios relative to STE-stimulated cells are reported. Values are mean  $\pm$  SE of three independent experiments. C) Serum-starved SMCs were preincubated with a MEK inhibitor PD98059 (100  $\mu$ mol/l) or a p38 inhibitor SB202190 (20  $\mu$ mol/l), and analyzed by EMSA as described in Fig. 4. This is a representative gel of three independent experiments with similar results. D) Serum-starved SMCs were pretreated with or without a p38 inhibitor SB202190 and elastase activity was determined as in Fig. 1. Data were analyzed statistically based on absolute values and are represented as the ratio relative to the control value, which corresponds to  $95.3 \pm 6.2$  ng degraded elastin/ $\mu$ g DNA. Means  $\pm$  SE of quadruplicate wells are shown with similar results in three independent experiments. \* $P < 0.05$  vs. the control.  $P < 0.05$  vs. STE with no pretreatment.

mented that NO-mediated cGMP suppresses this pathway in association with an alteration in the nuclear distribution of AML1B. The mechanism is likely related to cGMP-mediated protein kinase G activity, which interferes with upstream events, namely, the interaction between ras and raf that is necessary for the activation of the MAP kinase kinase (MEK) that phosphorylates ERK (30). The specificity of the link between ERK and AML1B/elastase was

further established when a MEK inhibitor, but not a p38 inhibitor, reduced AML1B:DNA interaction in nuclear extracts and elastase activity. These findings suggest not only a novel link between NO and elastase-mediated matrix remodeling in vascular disease, but also propose a new paradigm whereby AML1B-mediated gene expression may be one of the downstream targets of NO/cGMP-generating vasodilators in myeloid as well as nonmyeloid cells.

NO can modulate intracellular signaling in SMCs through cGMP-dependent or -independent mechanisms (reviewed in ref 1). Our findings demonstrate that NO donor suppression of STE-induced elastase activity was associated with the inhibition of ERK phosphorylation mediated by cGMP. cGMP involvement in NO suppression of elastase negates the possibility that NO directly inhibits elastase enzymatic activity shown for cysteine proteinases (42). The NO donor SNAP can produce ONOO<sup>-</sup>, which could be a weak releaser of cGMP (36). However, DETA NONOate, a pure NO donor that does not release superoxide or generate ONOO<sup>-</sup>, had a similar inhibitory effect on ERK phosphorylation. Intracellular generation of ONOO<sup>-</sup> was also ruled out when we showed that administration of exogenous ONOO<sup>-</sup> did not inhibit ERK phosphorylation, and coadministration of a ONOO<sup>-</sup> scavenger UA did not reverse SNAP suppression of ERK phosphorylation.

We speculate that the mechanism whereby NO/cGMP interferes with STE-induced ERK phosphorylation is related to induction of PKG suppressing Ras:raf interaction and MEK activity, as was shown in EGF-stimulated rat aortic smooth muscle cells (30). Other possibilities also need to be considered, such as the effect of NO-cGMP on integrin-SMC interactions required for the induction of elastase activity (24). We showed that STE stimulation of vascular cells results in phosphorylation of focal adhesion kinase (FAK) as well as ERK and that pretreatment with RGD peptides to disrupt integrin:matrix interactions inhibits elastase activation (24). Recent studies in chondrocytes demonstrated that NO/cGMP can inhibit  $\alpha 5\beta 1$  integrin-mediated interaction with fibronectin and subsequent actin polymerization, FAK translocation, MAP kinase activation, and proteoglycan synthesis (43).

The mechanism whereby NO reduces expression of AML1B in nuclear extracts in a cGMP-dependent manner is related to inhibition of ERK phosphorylation. Although another member of the MAP kinase family, p38 could be involved in inducing expression of downstream genes (33, 44), such as AML1B through cross talk with ERK, inhibition of p38 does not alter nuclear expression of AML1B even though STE induces p38 phosphorylation, which is inhibited by SNAP.

An interesting aspect of this work addresses how ERK phosphorylation induces AML1B functional activity. The subnuclear localization of transcriptionally active AML1B has been investigated in a number of studies. Tanaka et al. (45) reported that in COS cells transfected with human AML1B, localization of the transcription factor in nuclear extracts is associated with DNA binding and reporter activity of a downstream gene, the T cell receptor. Studying Jurkat cells transfected with human AML1B, Zeng et al. (46, 47) demonstrated that AML1B is found exclusively in nuclear pellets, where it is transcriptionally active when colocalized with RNA polymerase II. Our studies in vascular smooth muscle cells are more comparable to those of Tanaka's in COS cells in that when STE stimulation shifts the distribution of AML1B from the nuclear pellet to the extract, expression and DNA binding property support its transcriptional activity in terms of elastase activity. Recent studies suggest that functional activity of certain transcription factors is related to their ability to partition between the nuclear matrix and the extract, i.e., mutated transcription factors remain bound to the matrix (48, 49). Thus, whereas 'functional' transcription factors are bound to the matrix, 'functioning' transcription factors may not be (48). Binding of transcription factors to the nuclear matrix has also been associated with transcriptional inhibitory activity (50). We have not, however, specifically evaluated the AML1B in the pellet associated with RNA polymerase II; this subfraction might be affected differently by STE and NO donors when compared to the portion in the pellet as a whole.

The mechanism whereby ERK phosphorylation induces AML1B redistribution in the nucleus and its functional activity remains to be determined. Tanaka et al. (45) demonstrated a band shift in AML1B on immunoblots from COS cells transfected with human AML1B after stimulation in 10% fetal calf serum. This phosphorylation was associated with up-regulation not of DNA binding, but of transcriptional activity, probably by modulating interaction with other transcription factors.

In our studies, no AML1B band shift was evident under different treatments or in the different subnuclear fractions associated with ERK phosphorylation, making it unlikely that ERK directly phosphorylates AML1B. Moreover, the site of ERK-mediated AML1B phosphorylation is different from the nuclear matrix targeting signal, perhaps making it less likely that ERK-mediated phosphorylation is responsible for the shift in nuclear distribution (45, 46). It is therefore possible that phosphorylation of other transcription factors by ERK may modulate AML1B nuclear localization. For example, Ets requires phosphorylation and activation by ERK in order to interact with AML1B through autoinhibitory domains,

and this increases AML1B:DNA binding and transcriptional activity (51). It would be interesting to investigate whether binding to phosphorylated Ets also alters the nuclear distribution of AML1B in smooth muscle cells.

Although the functional significance of AML1B as a transcription factor in vascular smooth muscle cells has only recently been investigated by our group, (25), AML1B regulates the transcription of various genes that are important in hematopoiesis such as leukocyte elastase, myeloperoxidase, granulocyte-macrophage-colony stimulating factor, and the receptor for macrophage-stimulating factor (27). In addition to hematopoiesis, AML1 isoforms are involved in osteocalcin production, fibroblast transformation, and skeletal muscle development (52–54). Our findings therefore suggest that NO/cGMP-generating vasodilators could influence AML1B-mediated gene expression not only in vascular remodeling, but also in remodeling of bone and other tissues as well as in hematopoiesis and neoplastic transformation. Thus, in myeloid or nonmyeloid cells, AML1B could be a therapeutic target of a variety of cGMP-generating vasodilators and vasculoprotective compounds such as natriuretic peptides, carbon monoxide, and phosphodiesterase 5 inhibitors. **FJ**

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