Nitric Oxide Decreases Stability of mRNAs Encoding Soluble Guanylate Cyclase Subunits in Rat Pulmonary Artery Smooth Muscle Cells

Galina Filippov,* Donald B. Bloch,* and Kenneth D. Bloch†
*Cardiovascular Research Center and the Arthritis Unit of the General Medical Services, Massachusetts General Hospital; and †Department of Medicine, Harvard Medical School, Charlestown, Massachusetts 02129

Abstract

Nitric oxide stimulates soluble guanylate cyclase (sGC) to convert GTP to the intracellular second messenger cGMP. In rat pulmonary artery smooth muscle cells, sGC is an obligate heterodimer composed of α1 and β1 subunits. We investigated the effect of NO donor compounds on sGC subunit gene expression in rat pulmonary artery smooth muscle cells. Sodium nitroprusside and S-nitroso-glutathione decreased sGC subunit mRNA and protein levels, as well as sGC enzyme activity. 1H-[1,2,4]oxadiazolo[4,3-a]quinazolin-1-one, an sGC inhibitor, blocked the effect of sodium nitroprusside on sGC subunit gene expression, whereas 8-bromo cGMP decreased subunit mRNA levels, demonstrating that NO-mediated decrease in sGC subunit mRNA levels is cGMP-dependent. sGC subunit mRNA levels decreased more rapidly in rat pulmonary artery smooth muscle cells exposed to NO than in cells exposed to actinomycin D, suggesting that NO decreases sGC subunit mRNA stability. Actinomycin D and cycloheximide blocked the ability of NO to decrease sGC subunit mRNA levels. These results demonstrate that NO decreases sGC subunit mRNA stability via a transcription- and translation-dependent mechanism. (J. Clin. Invest. 1997. 100:942–948.) Key words: cGMP • NO-donor compound • actinomycin D • cycloheximide • receptor

Introduction

Nitric oxide, the active moiety of endothelium-derived relaxing factor, has a critical role in the regulation of vascular tone and patency (reviewed in reference 1). NO regulates vascular cell proliferation, migration, and apoptosis. In addition, NO inhibits platelet and leukocyte activation. NO also participates in nonvascular functions including neurotransmission and the response to infection. Excessive NO production contributes to cellular injury and the hemodynamic sequelae of sepsis (2). Extensive research has been directed toward identifying the mechanisms regulating the production of NO. However, mechanisms regulating responsiveness to NO are less well characterized.

NO acts in part by stimulating soluble guanylate cyclase (sGC), which converts GTP to cyclic GMP (cGMP). cGMP is an intracellular second messenger that activates cGMP-dependent protein kinase, as well as other cGMP receptor proteins (3), resulting in diverse effects including relaxation of vascular smooth muscle. sGC is a heterodimer composed of α and β subunits with two isoforms of each subunit encoded in the rat genome: α1, α2, β1, and β2 (4). α1 and β1 subunits are the predominant isoforms in the lung and vascular smooth muscle cells (reference 5 and our unpublished data).

Increasing evidence suggests that NO modulates gene expression. NO regulates gene transcription via both cGMP-dependent and -independent mechanisms. Peunova and Enikolopov (6) found, in rat pheochromocytoma PC12 cells, that the NO-mediated increase in c-fos gene expression was calcium- and cGMP-dependent and appeared to involve protein kinase A activation of the CREB transcription factor. Pilz et al. (7) observed, in rodent fibroblast and epithelial cell lines, that NO and a membrane-permeable cGMP analogue activated gene transcription via cis-acting nucleotide sequences containing a phorbol ester–response element. In contrast, Kourembanas et al. (8) found in endothelial cells that NO-mediated suppression of endothelin 1 and platelet-derived growth factor B chain gene transcription was cGMP independent. Similarly, DeCatarina et al. (9) and Shin et al. (10) reported that NO, but not cGMP analogues, inhibited transcription of genes encoding vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 in endothelial (9) and smooth muscle (10) cells, possibly by stabilizing the nuclear factor-κB inhibitor, IκBα (11). NO can also modulate gene expression by altering mRNA stability. Pantopoulos and Hentze observed that NO stabilized transferrin receptor mRNA by activating binding of the iron-regulatory protein to iron-response elements located in the 3′ untranslated region of the mRNA (12).

To investigate the effect of chronic exposure to NO on NO/cGMP signal transduction, sGC subunit mRNA and protein levels and sGC enzyme activity were measured in cultured rat pulmonary artery smooth muscle cells (RPaSMC) exposed to chemical compounds that generate NO. Evidence is presented that chronic exposure to NO decreases sGC enzyme activity by decreasing the stability of the mRNAs encoding its subunits and that destabilization of sGC subunit mRNAs is dependent on gene transcription and protein synthesis.
Methods

These investigations were approved by the Subcommittee for Research Animal Studies at the Massachusetts General Hospital.

Reagents. Sodium nitroprusside (SNP) and cycloheximide were purchased from Sigma Chemical Co. (St. Louis, MO). S-nitroso-glutathione (GSNO) and spermine NONOate were obtained from Alexis Corp. (San Diego, CA). Oxidized and reduced glutathione, S-nitroso-acetylpenicillamine (SNAP), 1H-[1,2,4]oxadiazolo[4,3-a] quinoxalin-1-one (ODQ), and actinomycin D were obtained from Calbiochem-Novabiochem Corp. (La Jolla, CA).

Cultured cells. Cultures of primary RPaSMC were prepared from explants of endothelium- and adventitia-stripped pulmonary arteries of adult Sprague-Dawley rats, as described by Yu et al. (13). RPaSMC were maintained in RPMI supplemented with 10% NuSerum (Collaborative Biomedical Products, Bedford, MA), penicillin, and streptomycin. Cells were used between passages 5 and 10.

RNA blot hybridization. RNA was extracted from RPaSMC using the guanidine isothiocyanate–cesium chloride method, as described previously (14). 10 μg of total cellular RNA were fractionated in 1.3% agarose-formaldehyde gels containing ethidium bromide. RNA was transferred to MAGNA CHARGE membranes (Micron Separations Inc., Westboro, MA) and cross-linked by exposure to ultraviolet light. Membranes were hybridized with rat sGC α1 and β1 subunit cDNA probes, as described previously (14) (cDNAs encoding sGC α1 and β1 subunits were generously provided by M. Nakane, Abbott Laboratories, Inc., Abbott Park, IL) (5). For some experiments, RNA blots were also hybridized with radiolabeled probes derived from a cDNA encoding the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH; kindly provided by M. Alexander-Bridges, Howard Hughes Medical Institute, Massachusetts General Hospital) (15) and a cDNA encoding rat c-jun (kindly provided by T. Curran, Roche Institute of Molecular Biology, Nutley, NJ) (16). Membranes were also hybridized with a 15-fold molar excess of a 32P-labeled oligonucleotide complementary to rat 18S RNA (17). Because ribosomal RNA represents the vast majority of cellular RNA and 18S RNA is a fixed proportion of ribosomal RNA, hybridization of this oligonucleotide was used as a measure for each sample of the quantity of total RNA loaded on gels.

Measuring sGC subunit protein levels in RPaSMC. RPaSMC were homogenized in buffer containing 50 mM Tris-HCl, pH 7.6, 1 mM EDTA, 1 mM DTT, and 2 mM PMSF. Extracts were centrifuged at 100,000 g for 1 h at 4°C. Supernatants (30 μg) of centrifuged cell extracts were fractionated using 8% SDS-PAGE and transferred to nitrocellulose filters (Micron Separations Inc.). Filters were blocked at room temperature for 1 h in phosphate-buffered saline, pH 7.4, containing 5% nonfat dry milk (PBS-milk), and then incubated at room temperature for 1 h with an immunooaffinity-purified polyclonal antiserum directed against the sGC β1 subunit (prepared as described below). Bound antibody was detected by incubation of the filters with horseradish peroxidase protein A (Boehringer Mannheim Biochemicals, Indianapolis, IN) and visualization using horseradish peroxidase protein A (Boehringer Mannheim Biochemicals, Indianapolis, IN) and visualized using chemiluminescence (ECL; Amersham Life Sciences, Arlington Heights, IL) and cross-linked by exposure to ultraviolet light. Membranes were also hybridized with a 15-fold molar excess of a 32P-labeled oligonucleotide complementary to rat 18S RNA (17). Because ribosomal RNA represents the vast majority of cellular RNA and 18S RNA is a fixed proportion of ribosomal RNA, hybridization of this oligonucleotide was used as a measure for each sample of the quantity of total RNA loaded on gels.

Results

Exposure of rat pulmonary artery smooth muscle cells to NO donor compounds decreases sGC subunit mRNA and protein levels. To investigate the effect of exposure to NO on sGC subunit gene expression, RPaSMC were incubated with NO donor compounds, and the levels of mRNAs encoding sGC subunits were measured by RNA blot hybridization. Exposure of RPaSMC for 4 h to SNP decreased sGC α1 and β1 subunit mRNA levels in a dose-dependent manner (Fig. 1). Concentrations ≥ 10 μM SNP consistently decreased sGC subunit mRNA levels. The magnitude of the decrease in sGC subunit mRNA levels in RPaSMC exposed to SNP was dependent on the duration of exposure to SNP: minimum sGC subunit mRNA levels were evident after 4 h of exposure to SNP, and levels remained low for up to 24 h. Exposure of RPaSMC for 4 h to other NO donor compounds including GSNO and SNAP (Fig. 2), as well as spermine NONOate (data not shown), also decreased sGC subunit mRNA levels. Concentrations of GSNO and SNAP ≥ 1 and 10 μM, respectively, decreased sGC subunit mRNA levels (Fig. 2). By-products of NO generation from SNP and GSNO, cyanide and glutathione, respectively, did not alter sGC subunit mRNA levels (Fig. 2). These results suggest that NO generation by NO donor compounds was responsible for the observed effects on sGC subunit gene expression. Exposure of RPaSMC for 24 h to 1 mM SNP or 100 μM GSNO did not induce cell toxicity. The percentage of cells that failed to exclude trypan blue did not differ in

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Filippov et al. RPaSMC incubated in the presence or absence of an NO donor compound and was < 1% for both.

To determine whether NO-mediated changes in sGC subunit gene expression were correlated with changes in subunit protein levels, sGC β1 subunit levels were measured in RPaSMC exposed to 1 mM SNP for 0, 4, 8, and 24 h (top). sGC β1 subunit levels were also measured in RPaSMC incubated for 24 h in the absence (C) or presence of 10⁻³ to 10⁻⁴ M SNP (lower left) and 10⁻⁴ M GSNO (lower right). Proteins in the soluble fraction of cell extracts were fractionated using SDS-PAGE and transferred to nitrocellulose. Immunoreactive sGC β1 subunit (70 kD) was detected using an immunoaffinity-purified polyclonal antibody directed against the β1 subunit. Immunoreactive sGC α1 subunit (82 kD) was detected using a monoclonal antibody directed against the α1 subunit. These results demonstrate that chronic exposure of RPaSMC to NO donor compounds decreases sGC subunit protein levels.

Exposure of rat pulmonary artery smooth muscle cells to NO donor compounds decreases sGC enzyme activity. To determine the effect of chronic NO exposure on sGC function, RPaSMC were exposed to 1 mM SNP for 4, 8, and 24 h, and

Figure 1. NO decreases sGC subunit mRNA levels in rat pulmonary artery smooth muscle cells. RNA extracted from RPaSMC exposed to 10⁻⁶ to 10⁻³ M SNP for 4 h, from RPaSMC exposed to 1 mM SNP for 1, 2, 4, 8, 16, and 24 h, and from untreated RPaSMC (C) was fractionated on formaldehyde-agarose gels, transferred to nylon membranes, and hybridized with sGC α1 and β1 subunit cDNA probes and with an oligonucleotide complementary to 18S ribosomal RNA. (The band below the α1 subunit mRNA represents residual signal from the earlier hybridization with the β1 subunit cDNA probe.) These results demonstrate that the NO-mediated decrease in sGC subunit mRNA levels is exposure duration and dose dependent.

Figure 2. NO generation by NO donor compounds decreases sGC subunit mRNA levels. RPaSMC were incubated for 4 h in the absence (C) and presence of 10⁻³ M SNP, 10⁻³ M sodium cyanide (CN), 10⁻³ M SNAP, 10⁻⁴ to 10⁻³ M S-nitroso-glutathione (GSNO), and 10⁻³ M oxidized glutathione (GS). RNA blots were prepared and hybridized with sGC α1 and β1 subunit cDNA probes and with an oligonucleotide complementary to 18S ribosomal RNA. These results suggest that sGC subunit mRNA levels decrease in response to the generation of NO from NO donor compounds.

Figure 3. NO decreases sGC subunit protein levels in rat pulmonary artery smooth muscle cells. sGC α1 and β1 subunit levels were measured in RPaSMC exposed to 1 mM SNP for 0, 4, 8, and 24 h (top). sGC β1 subunit levels were also measured in RPaSMC incubated for 24 h in the absence (C) or presence of 10⁻³ to 10⁻⁴ M SNP (lower left) and 10⁻⁴ M GSNO (lower right). Proteins in the soluble fraction of cell extracts were fractionated using SDS-PAGE and transferred to nitrocellulose. Immunoreactive sGC β1 subunit (70 kD) was detected using an immunoaffinity-purified polyclonal antibody directed against the β1 subunit. Immunoreactive sGC α1 subunit (82 kD) was detected using a monoclonal antibody directed against the α1 subunit. These results demonstrate that sGC subunit protein levels decrease in RPaSMC exposed to NO.
sGC enzyme activity was measured in the soluble fraction of cell extracts (Fig. 4). In extracts prepared from untreated RPaSMC, addition of SNP to the reaction mixture increased sGC enzyme activity > 10-fold (from 0.38±0.07 to 5.4±0.58 pmol cGMP synthesized per min/mg protein). In extracts prepared from cells exposed to SNP for 4 h, basal and NO-stimulated sGC enzyme activity decreased by 47 and 87%, respectively (0.20±0.05 and 0.68±0.26; P < 0.01 for both). Similar decreases in basal and NO-stimulated sGC enzyme activity were observed in RPaSMC exposed to 4 h to 100 μM GSNO (data not shown). NO-activated sGC enzyme activity in RPaSMC exposed to SNP for 24 h was < 5% of that in control cells. These results demonstrate that chronic exposure to NO decreases the capacity of RPaSMC to synthesize cGMP.

**Figure 4.** sGC enzyme activity is decreased in rat pulmonary artery smooth muscle cells chronically exposed to NO. The soluble fraction of extracts derived from untreated RPaSMC (control) and RPaSMC exposed to 1 mM SNP for 4, 8, and 24 h (three separate experiments for each time point) was assayed for sGC enzyme activity in the absence (closed boxes) and presence (open boxes) of 1 mM SNP. sGC enzyme activity is expressed as picomoles of cGMP produced per minute per milligram of cell extract supernatant (±SD). *Significant differences from control values (P < 0.01). These results show that basal and NO-activated sGC enzyme activity is reduced in RPaSMC chronically exposed to NO.

**Discussion**

Exposure of rat pulmonary artery smooth muscle cells to NO donor compounds decreased expression of genes encoding both the α1 and β1 subunits of the heterodimeric sGC enzyme. Diminished sGC subunit gene expression was accompanied by

**Figure 5.** NO decreases sGC subunit mRNA levels via a cGMP-dependent mechanism. RPaSMC were incubated with 3 μM ODQ, an sGC inhibitor, for 30 min, and then were exposed to 1 mM SNP for 4 h. Other RPaSMC were untreated (C) or were incubated with either SNP, ODQ, or 1 mM 8-bromo cGMP (cGMP) alone. RNA extracted from RPaSMC was hybridized with α1 and β1 subunit cDNA probes and the oligonucleotide complementary to 18S RNA. The decrease in sGC subunit mRNA levels detected in cells exposed to 8-bromo cGMP but not in cells exposed to the combination of SNP and ODQ suggests that NO decreases sGC subunit gene expression via a cGMP-dependent mechanism.
Figure 6. Inhibition of gene transcription or protein synthesis blocks the ability of NO to decrease sGC subunit mRNA levels. (A) RPaSMC were pretreated with 10 μM actinomycin D for 30 min, and then were exposed to 1 mM SNP for 2, 4, and 6 h. Other cells were treated with actinomycin D alone, SNP alone, or were untreated (C). RNA extracted from RPaSMC was hybridized with α1 and β1 subunit, c-jun, and GAPDH cDNA probes, and with an oligonucleotide complementary to 18S RNA. These results show that the NO-induced decrease in sGC subunit mRNA levels is selective and actinomycin D sensitive. (B) RPaSMC were pretreated with 100 μM cycloheximide (CHX) for 30 min, and then were exposed to 1 mM SNP for 4 h. Other cells were untreated (C) or were treated with cycloheximide or SNP alone. RNA extracted from RPaSMC was hybridized with α1 and β1 subunit cDNA probes and an oligonucleotide complementary to 18S RNA. These results demonstrate that the NO-induced decrease in sGC subunit mRNA levels is cycloheximide sensitive.

decreased subunit protein levels and decreased basal and NO-activated sGC enzyme activity. The concentrations of NO donor compounds used in this study are comparable with those required to modulate expression of genes encoding other proteins, including endothelin 1 (8), macrophage-colony stimulating factor (22), and the endothelin A receptor (23). Experiments using NO synthase inhibitors demonstrated that levels of NO sufficient to modulate expression of these genes are attainable in endothelial cells (8, 22) and in cytokine-treated smooth muscle cells (23). Moreover, using an NO-selective electrode, Ichimori et al. observed that concentrations of NO generated by 10 μM SNAP in biological fluids are similar to those produced by endothelial cells stimulated with 1 μM acetylcholine (24). Taken together, these observations suggest that concentrations of NO sufficient to alter sGC subunit mRNA levels are attainable in vivo.

Exposure of RPaSMC to a membrane-permeable cGMP analogue, 8-bromo cGMP, decreased sGC subunit gene expression, whereas an inhibitor of sGC enzyme activity, ODO, attenuated the ability of NO to alter sGC subunit mRNA levels. These results suggest that the effect of NO on sGC subunit gene expression is cGMP dependent. Other investigators have also suggested that cGMP regulates sGC subunit gene expression. Ujie et al. (25) observed that incubation of cultured rat medullary interstitial cells with agents that increased intracellular cGMP concentrations decreased sGC enzyme activity and that exposure of these cells to 0.3 mM SNP modestly decreased sGC α1 and β1 subunit mRNA levels. Similarly, Papapetropoulos et al. (26) observed that incubation of aortic smooth muscle cells with Zaprinast, an inhibitor of cGMP degradation by type 5 phosphodiesterase, decreased sGC subunit gene expression. Increased cGMP levels may alter sGC subunit gene expression via activation of cGMP-dependent protein kinase. Alternatively, cGMP may inhibit phosphodiesterase activity (27), leading to increased cAMP levels that in turn may decrease sGC subunit mRNA levels, as described previously in other cell types (28–30).

Nitric oxide appears to decrease sGC subunit mRNA stability via a transcription-dependent mechanism. This conclusion is based on the observations that sGC subunit mRNA levels decrease more rapidly in RPaSMC exposed to NO than in cells exposed to actinomycin D and that pretreatment of RPaSMC with actinomycin D blocked the ability of NO to alter sGC subunit mRNA levels. The observations that levels of c-jun and GAPDH mRNAs did not change markedly in RPaSMC exposed to SNP are consistent with NO mediating a selective effect on sGC subunit mRNAs. Incubation of RPaSMC with the protein synthesis inhibitor cycloheximide also blocked the ability of NO to decrease sGC subunit mRNA levels, supporting the hypothesis that NO induces synthesis of an mRNA destabilizing protein. However, it should be noted that, for some mRNAs, low levels of mRNA translation are required for the degradation of the molecule (31). We recently reported that incubation of PC12 cells with nerve growth factor decreases sGC subunit mRNA levels via a similar transcription- and translation-dependent mechanism (30). These observations suggest that shared regulatory mechanisms may control sGC subunit gene expression in these two cell types.

NO-induced modulation of mRNA stability has been reported previously. Pantopoulos and Hentze (12) observed that NO stabilized the mRNA encoding the transferrin receptor by activating binding of an iron-regulatory protein (IRP) to sequences (iron-response elements, IREs) in the 3' untranslated region of the mRNA. It is unlikely that the IRP/IRE system (reviewed in reference 32) is responsible for the effect of NO on sGC subunit mRNA stability because IRP/IRE modulation of mRNA stability does not require gene transcription and consensus IRE sequences are absent from the reported sequences of the mRNAs encoding α1 and β1 subunits (5).
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NO-mediated transcription/translation-dependent destabilization of sGC subunit mRNAs appears to represent a novel mechanism by which NO modulates gene expression.

Chronic exposure of RPaSMC to SNP decreased sGC enzyme activity. However, whereas NO-activated sGC enzyme activity decreased 87% within 4 h, more than 4 h were required to decrease sGC subunit protein levels by 50%. These results suggest that chronic exposure to NO donor compounds decreases the specific activity of the sGC enzyme, as well as the levels of its subunits. Mechanisms regulating the specific activity of the sGC enzyme have been extensively investigated (reviewed in reference 27). Other investigators have observed that chronic exposure of cultured vascular smooth muscle cells decreased sGC enzyme activity (26, 33). Papapetroupolos et al. (26) observed that pretreatment with reducing agents such as ascorbic acid restored NO-stimulated cGMP synthesis in RPaSMC chronically exposed to SNP and suggested that chronic exposure to NO may impair sGC enzyme activity by altering the redox state of the enzyme.

The decrease in sGC enzyme activity associated with chronic exposure to NO may have important clinical implications. Downregulation of sGC function may have a protective effect under conditions associated with elevated NO levels. For example, cells respond to viral, bacterial, or parasitic infection by producing high concentrations of NO (1). It is possible that NO-mediated downregulation of sGC function represents a homeostatic mechanism preventing excessive activation of cGMP-dependent protein kinase and other cGMP-dependent mechanisms and, thereby, attenuating NO-mediated host cell toxicity or hemodynamic compromise. Chronic administration of NO donor compounds, such as nitroglycerin, impairs the ability of vascular smooth muscle to respond to additional NO. Clinically, this phenomenon, referred to as nitrate tolerance, is important for patients with coronary artery disease to whom nitroglycerin is chronically administered. The etiology of nitrate tolerance is multifactorial (34) with decreased sGC enzyme activity having an important role (35, 36). Although the concentrations of NO donor compounds required to elicit nitrate tolerance are typically greater in cultured cells than in vivo, the observations presented here suggest that decreased sGC subunit mRNA levels may contribute to impaired NO responsiveness in nitrate tolerance.

In summary, chronic exposure of rat pulmonary artery smooth muscle cells to NO donor compounds decreased sGC subunit mRNA and protein levels and decreased sGC enzyme activity. The NO-induced decrease in the levels of mRNAs encoding both sGC subunits was cGMP, actinomycin D, and cycloheximide dependent. These results suggest that NO induces synthesis of a factor (probably a protein) that selectively destabilizes sGC subunit mRNAs. NO-induced modulation of sGC subunit mRNA levels illustrates a novel mechanism by which NO regulates gene expression.

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References


