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Nitric Oxide Regulates Pulmonary Vascular Smooth Muscle Cell Expression of the Inducible cAMP Early Repressor Gene

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Abstract

Nitric oxide (NO) regulates vascular smooth muscle cell (VSMC) structure and function, in part by activating soluble guanylate cyclase (sGC) to synthesize cGMP. The objective of this study was to further characterize the signaling mechanisms by which NO regulates VSMC gene expression using transcription profiling. DNA microarrays were hybridized with RNA extracted from rat pulmonary artery smooth muscle cells (RPaSMC) exposed to the NO donor compound, S-nitroso-glutathione (GSNO). Many of the genes, whose expression was induced by GSNO, contain a cAMP-response element (CRE), of which one encoded the inducible cAMP early repressor (ICER). sGC and cAMP-dependent protein kinase, but not cGMP-dependent protein kinase, were required for NO-mediated phosphorylation of CRE-binding protein (CREB) and induction of ICER gene expression. Expression of a dominant-negative CREB in RPaSMC prevented the NO-mediated induction of CRE-dependent gene transcription and ICER gene expression. Pre-treatment of RPaSMC with the intracellular calcium (Ca^{2+}) chelator, BAPTA-AM, blocked the induction of ICER gene expression by GSNO. The store-operated Ca^{2+} channel inhibitors, 2-ABP and SKF-96365, reduced the GSNO-mediated increase in ICER mRNA levels, while 2-ABP did not inhibit GSNO-induced CREB phosphorylation. Our results suggest that

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induction of ICER gene expression by NO requires both CREB phosphorylation and Ca^{2+} signaling. Transcription profiling of RPaSMC exposed to GSNO revealed important roles for sGC, PKA, CREB, and Ca^{2+} in the regulation of gene expression by NO. The induction of ICER in GSNO-treated RPaSMC highlights a novel cross-talk mechanism between cGMP and cAMP signaling pathways.

Keywords

nitric oxide; vascular smooth muscle; cAMP-response element; cyclic GMP; protein kinase A

1. INTRODUCTION

Nitric oxide (NO) is a critical regulator of vascular remodeling via its effects on endothelial and vascular smooth muscle cell (VSMC) differentiation, proliferation, apoptosis and migration [1-3]. NO elicits its effects, in part, by stimulating soluble guanylate cyclase (sGC) to produce the intracellular second messenger cGMP. sGC is a heterodimer composed of $\beta 1$ subunit and an $\alpha 1$ or $\alpha 2$ subunit, and the sGC $\alpha 1\beta 1$ is the predominant isoform in the vasculature. cGMP, in turn, activates several intracellular target proteins including cGMP-dependent protein kinase (PKG), cGMP-gated channels, and cGMP-regulated phosphodiesterases (PDEs) [4], as well as under some circumstances, cAMP-dependent protein kinase (PKA) [5]. PKG phosphorylates serines and threonines on many cellular proteins, frequently resulting in changes in activity, function, or subcellular localization. In VSMC, the proteins that are phosphorylated by PKG regulate calcium homeostasis and calcium sensitivity of contractile proteins, as well as the expression of genes that modulate VSMC phenotype [4]. cGMP activates PDE2 and inhibits PDE3, thereby decreasing and increasing intracellular cAMP levels respectively [2], often in cell compartment-selective manner. The transcription factor, cAMP-response element (CRE)-binding protein (CREB) [6] is phosphorylated in response to a variety of stimuli including increases in the levels of intracellular calcium (Ca^{2+}) and cyclic nucleotides. Phosphorylation of CREB at Ser¹³³ by kinases, such as PKG and PKA [7], induces CREB to bind to CRE sequences in the promoters of multiple genes encoding c-fos, heme oxygenase 1 (HO1), hexokinase-2 (HK2), and inducible cAMP early repressor (ICER) [6, 8, 9]. The activity of CREB is limited, in part, by its induction of ICER, which binds to CRE sequences but lacks a transactivation domain [10].

In addition to activating gene transcription via cyclic nucleotide-stimulated phosphorylation of CREB, NO can modulate gene expression via Ca^{2+} -dependent mechanisms. Previous studies have identified several mechanisms by which NO can modulate intracellular Ca^{2+} levels including modulation of a variety of Ca^{2+} channels, for example voltage-dependent calcium channels (VDCC) and store-operated calcium channels (SOCC) [12]. NO regulates Ca^{2+} regulatory proteins and channels via cGMP/PKG-dependent mechanisms or, alternatively, via cGMP-independent, post-translational modifications [13, 14].

To further understand the mechanisms by which NO regulates gene expression in VSMC, we examined the transcriptional profile of rat pulmonary artery smooth muscle cells (RPaSMC) exposed to the NO-donor compound, S-nitroso-glutathione (GSNO). We observed that many of the genes induced by GSNO contain CRE sequences in their promoters. One of these CRE-containing genes, ICER, was markedly induced in RPaSMC exposed to GSNO and in lungs of mice breathing NO. We report here that NO increases ICER gene expression via mechanisms requiring sGC, PKA, and Ca^{2+} , but not PKG. Moreover, NO-mediated phosphorylation of CREB is required, but not sufficient, for induction of ICER gene expression by NO.

2. MATERIALS AND METHODS

All experimental studies using animals were approved by the MGH Subcommittee on Research Animal Care.

2.1) Cell culture

Cultures of primary RPaSMC were prepared as described previously [15].

2.2) Microarray analysis

Two independent preparations of RPaSMCs were untreated or exposed to GSNO (100 $\mu\text{mol/L}$) for 1, 2, and 4 hours (h). RNA, cDNA, and labeled cRNA were generated as described in the online supplement. Fragmented cRNA was hybridized to Affymetrix rat U-34A GeneChips containing 8798 gene entries with the GeneChip Fluidics Station 450 and scanned with the GeneChip® Scanner 3000. Affymetrix GeneChip 5.0 software was used for data analysis according to Affymetrix protocols. The microarray data including information requested by the Microarray Gene Expression Data society can be accessed in the microarray database at NCBI Gene Expression Omnibus (GEO) using the following link: <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=thqflmmsosekire&acc=GSE19710>.

GSNO was determined to alter gene expression if, in both RPaSMC preparations, there was a 2-fold change in gene expression and if the gene was described as present (P) by Affymetrix detection call after GSNO stimulation for genes whose expression was increased or before GSNO stimulation for genes whose expression was decreased. In a second approach, we organized gene expression data into microarray pathway profiles (MAPPs) that represent NO-regulated biological pathways [16]. To facilitate the identification of NO-responsive MAPPs, a less stringent criterion for change in gene expression (1.5-fold) was used.

CRE sequences were identified in genes upregulated after 4h of GSNO stimulation using the CRE sequence database (<http://natural.salk.edu/CREB/>) [17].

2.3) Measurement of gene expression

mRNA levels were measured using RNA blot hybridization and quantitative RT-PCR, as described in the online supplement.

2.4) Protein levels

Levels of ICER, total CREB, and phospho-CREB proteins were measured using immunoblot techniques as described in the online supplement.

2.5) NO exposure in vivo

Male WT mice (C57Bl/6) breathed air with or without 80 ppm NO for 2, 4, 8 and 24h in specially-designed chambers, as described previously [18]. Additionally, WT mice and mice deficient for the $\alpha 1$ subunit of sGC [19] breathed air with or without NO (80 ppm) for 2h. Lung tissue was harvested for measurement of ICER mRNA levels. Plasma nitrite and nitrate levels were measured as described in the online supplement.

2.6) CRE-dependent gene transcription

RPaSMC were plated in 6-well dishes (2×10^5 cells/ well) and transfected for 2h with a control plasmid (pcDNA3) or a plasmid encoding ICER plasmid directing expression of firefly luciferase under the control of a CRE-containing promoter and a plasmid directing

expression of renilla luciferase (pRL-TK, Promega) to control for variations in transfection efficiency. After 24h, transfected cells were incubated in the presence and absence of GSNO with other agonists and antagonists as indicated in the Results section. After 4h, firefly and renilla luciferase activities were measured using the dual-luciferase reporter system (Promega). CRE-dependent gene transcription was estimated from the ratio of firefly luciferase activity to renilla luciferase activity.

2.7) Recombinant adenovirus generation

A replication-deficient adenovirus specifying rat ICER cDNA (Ad.ICER) was generated using the methods described by He and colleagues [20]. A full-length rat ICER cDNA was cloned into the HindIII and EcoRV restriction sites of the shuttle vector pAd.Track.CMV. A control adenovirus specifying β -galactosidase (Ad. β gal) generated using identical methods was provided by Dr. Anthony Rosenzweig (Beth Israel Deaconess Medical Center, Boston, Massachusetts). Both recombinant adenoviruses carry a second transcription cassette for green fluorescent protein (GFP). Wild-type adenovirus contamination was excluded by the absence of PCR-detectable E1 sequences.

The adenovirus carrying a dominant-negative CREB, in which Ser¹³³ was mutated to alanine (Ad.CREBml), was provided by Dr. William Walker (University of Pittsburgh).

For adenovirus-mediated gene transfer, cells were infected with Ad.CREBml, Ad.ICER, or Ad. β gal for 2h in RPMI medium containing 10% NuSerum. Infected cells were then incubated in the same medium for 24h.

2.8) Statistics

All data are expressed as mean $\pm$ standard deviation (SD). Data were analyzed using the student *t* test, 1-way ANOVA with Bonferroni post-hoc test for multiple comparisons, or 2-way ANOVA for multiple comparisons including Bonferroni post-hoc test. Statistical significance was considered for *p* values <0.05 with statement of the exact *p* values. Guidelines for reporting statistics in journals published by the American Physiological Society were followed [21]. For additional details, see the supplemental information containing detailed Materials and Methods, Supplemental Figures and Tables.

3. THEORY/ CALCULATION

Nitric oxide (NO) regulates vascular smooth muscle cell (VSMC) structure and function. The objective of this study was to further characterize the signaling mechanisms by which NO regulates VSMC gene expression using transcription profiling. We characterized the transcriptional profile of rat pulmonary artery smooth muscle cells treated with and without nitric oxide in order to further elucidate the signaling mechanisms by which NO regulates VSMC structure and function. We identified several genes whose expression was up- or down-regulated by NO. We focused on one gene that was among the most markedly induced by GSNO, the inducible cAMP early repressor (ICER), to elucidate the mechanisms by which NO modulates gene transcription in RPaSMC.

4. RESULTS

4.1) Exposure to GSNO alters the transcriptional profile of RPaSMC

To examine the impact of NO on VSMC gene expression, we used DNA microarrays (>8000 gene entries) and RNA extracted from two independent isolates of RPaSMC (which contain abundant sGC [22]) exposed to GSNO (100 μ mol/L) for 1, 2 and 4h. GSNO increased the expression of 65, 101, and 138 genes after 1, 2, and 4h, respectively. GSNO decreased expression of 18, 50, and 129 genes after 1, 2, and 4h, respectively (Supplemental

Fig. 1, Supplemental Table 1). Genes, whose expression was increased by GSNO as detected by microarray analysis and confirmed by RNA blot hybridization, include inducible cAMP early suppressor (Fig. 1A, HO1, HK2, tissue plasminogen activator (tPA), metallothionein 1 (MT1), γ -glutamylcysteine synthetase heavy chain (GCS:hc) and light chain (GCS:lc), and p21^(Waf1/Cip1) (Supplemental Fig. 2, Supplemental Table 2). Genes, whose expression was decreased by GSNO, include those encoding sGC α 1 and β 1 subunits, endothelin 1 (ET1), angiotensin II receptor (AT2R), and transforming growth factor- β 3 (TGF β 3).

GenMAPP was used to organize gene expression data into MAPPs, that represent specific biological pathways and functionally grouped genes modulated by GSNO based on the gene ontology (GO) system [16]. NO-modulated MAPPs included pathways involved in oxidative stress, apoptosis, and glutathione biosynthesis (Supplemental Fig. 3A-C). Examination of the 5' flanking sequences of genes, whose expression was induced by GSNO at 4h, revealed that 61% contain a cAMP-response element (10 occurrences of full site CREs, TGACGTCA, and 68 half site CREs, TGACG/CGTCA; data not shown) [17]. Subsequent studies focused on ICER gene expression as an example of a CRE-containing gene, whose expression is robustly induced by NO.

4.2) Nitric oxide increases ICER gene expression in RPaSMC

To validate the microarray data, ICER mRNA levels were measured in RPaSMC incubated with or without GSNO (100 μ mol/L) for 1, 2, 4, 16, and 24h (Fig. 1A). ICER mRNA levels increased starting at 1h after GSNO stimulation, reaching maximum levels between 2 and 4h. ICER gene expression remained elevated for at least 16h and declined to basal levels at 24h. Incubation of RPaSMC with GSNO for as little as 2 hrs increased ICER protein levels (data not shown). The GSNO-mediated increase in ICER gene expression was dose-dependent: increased ICER mRNA levels were detected with exposure to as little as 1 μ mol/L GSNO with maximal effects observed with 10 μ mol/L (Fig. 1B). Similar dose-dependent effects of GSNO were observed when ICER protein levels were measured (Fig. 1C).

4.3) Cytokines increase ICER gene expression in RPaSMC

Exposure of RPaSMC to the cytokines interleukin 1 β (IL-1 β) and tumor necrosis factor α (TNF α) induces expression of NO synthase 2 (NOS2) resulting in increased NO levels [22]. To determine whether endogenous NO levels produced in response to cytokines are sufficient to induce ICER gene expression, NOS2 and ICER mRNA levels were measured in RPaSMC exposed to a combination of IL-1 β and TNF α . Incubation of RPaSMC with IL-1 β and TNF α for 4h induced both NOS2 and ICER gene expression. Pretreatment of RPaSMC with L-NIL (0.5 mol/L), a selective NOS2 inhibitor, blocked the ability of cytokines to induce ICER, but not NOS2 gene expression (Fig. 2A). These results suggest that endogenously-produced levels of NO are sufficient to induce ICER gene expression in RPaSMC.

4.4) Inhaled NO induces pulmonary ICER gene expression

To determine whether NO induces ICER gene expression *in vivo*, we measured pulmonary ICER mRNA levels in C57Bl/6 (WT) mice breathing air supplemented with NO (80 ppm) gas for 0, 2, 4, 8, and 24h. Increased pulmonary ICER mRNA levels were evident in WT mice within 2h of initiating NO inhalation, and elevated ICER mRNA levels persisted at least 8h (Fig. 2B). Interestingly despite continued NO inhalation, pulmonary ICER mRNA levels returned to baseline by 24h. Consistent with mRNA levels, pulmonary ICER protein levels in WT mice breathing NO for 2 hrs were greater than those in mice breathing air (Supplemental Fig. 4).

4.5) Mechanisms by which NO induces ICER gene expression

NO is known to regulate cellular functions via both cGMP-dependent and -independent mechanisms. To investigate the role of cGMP signaling in induction of ICER gene expression by GSNO, we studied RPaSMC incubated in the presence and absence of the sGC inhibitor ODQ. Pretreatment of RPaSMCs with ODQ markedly inhibited the ability of GSNO (Fig. 2C) or cytokines (Fig. 2A) to increase ICER mRNA levels. To confirm the importance of sGC in the upregulation of ICER gene expression by NO, we compared pulmonary ICER mRNA levels in WT and sGC α 1^{-/-} mice [19] breathing NO (Fig. 2D). Inhalation of NO (80 ppm) for 2 hours increased pulmonary ICER mRNA levels to a much greater extent in WT than in sGC α 1^{-/-} mice, whereas plasma nitrite and nitrate levels increased similarly in the two genotypes (Supplemental Fig. 5). These results suggest that sGC α 1 β 1 is critical to the ability of NO to induce ICER gene expression.

To elucidate the mechanisms by which cGMP modulates ICER gene expression, we studied RPaSMC pretreated with the PKG inhibitors, Rp-8-Br-cGMPS, Rp-8-pCPT-cGMPS and KT5823. PKG inhibition did not alter the ability of GSNO or cytokines to increase ICER gene expression (Fig. 2A, C). In complementary studies, treatment of RPaSMC with membrane-permeable cGMP analogues that specifically activate PKG, Sp-8-pCPT-cGMP and Sp-8-Br-cGMP, did not augment ICER gene expression (Fig. 2C). The inability of PKG activators and inhibitors to modulate ICER gene expression was not attributable to the absence of PKG in RPaSMC, as the I α isoform of PKG was readily detected in RPaSMC using RNA blot and immunoblot techniques (data not shown).

It is known that ICER gene expression is induced by cAMP signaling and PKA [7]. To test whether PKA activity is required for the induction of ICER gene expression by GSNO, we studied RPaSMC incubated in the presence and absence of H89, an inhibitor of PKA [23, 24]. Pretreatment with H89 prevented the increase in ICER mRNA levels induced by cytokines (Fig. 2A) and GSNO (Fig. 2C, Supplemental Fig. 6). Taken together, these results suggest that activation of sGC and PKA, but not PKG, is required for the induction of ICER gene expression by NO.

4.6) GSNO stimulates CRE-dependent gene transcription

Since ICER gene expression is known to be regulated by CRE-dependent transcription factors [25], and because the 5' flanking sequences of many of the NO-inducible genes contain a cAMP-response element, we examined the ability of GSNO to activate CRE-dependent gene transcription in RPaSMC. RPaSMC were transiently transfected with a luciferase reporter plasmid containing an upstream CRE site and subsequently exposed to GSNO. Incubation with GSNO for 4h markedly increased CRE-dependent luciferase activity (Fig. 3A). Pretreatment with ODQ prevented the induction of CRE-dependent gene transcription by GSNO, whereas PKG inhibitors had no effect. Moreover, PKG agonists did not stimulate CRE-dependent gene transcription (Supplemental Fig. 7), whereas the cAMP analogue, 8-Br-cAMP, increased luciferase activity. On the other hand, inhibition of PKA with H89 blocked the ability of GSNO to induce CRE-dependent gene transcription (Fig. 3B). Taken together, these findings suggest that NO activates CRE-dependent gene transcription via a cGMP synthesis- and PKA-dependent mechanism.

To investigate the mechanisms by which NO can activate CRE-dependent gene transcription via PKA, we studied the effects of milrinone, a PDE3 inhibitor, and rolipram, an inhibitor of the cAMP-specific PDE4. Incubation of CRE-luciferase-transfected RPaSMC with milrinone or rolipram augmented CRE-dependent gene transcription (Fig. 3B). However, milrinone did not augment the ability of GSNO to induce luciferase activity, whereas rolipram markedly augmented the induction of CRE-dependent gene transcription by

GSNO. These findings suggest that NO/cGMP signaling and milrinone activate CRE-dependent gene transcription via the same mechanism, inhibition of PDE3. Moreover, the importance of cAMP signaling in the stimulation of CRE-dependent gene transcription by NO is highlighted by the ability of rolipram to augment the effects of GSNO.

4.7) Activation of CREB is required for GSNO-mediated ICER induction

When CREB undergoes phosphorylation at Ser¹³³, it binds to CRE sequences and induces gene transcription [25]. Since we observed that GSNO induces CRE-dependent gene transcription, we examined whether GSNO can induce CREB phosphorylation. Incubation of RPaSMC with either GSNO or forskolin (an activator of adenylate cyclase) rapidly induced CREB phosphorylation (Fig. 4A). ODQ inhibited the ability of GSNO, but not forskolin, to induce CREB phosphorylation. In contrast, H89 inhibited phosphorylation of CREB by both GSNO and forskolin. These results suggest that PKA is required for GSNO-mediated CREB phosphorylation.

To further examine the role of CREB in the induction of CRE-dependent gene expression by GSNO, we transfected RPaSMC with a CRE-luciferase reporter plasmid together with a plasmid encoding CREBml, a dominant-negative CREB mutant in which Ser¹³³ is replaced with an alanine [26]. Co-transfection with CREBml prevented the GSNO-mediated increase in CRE-luciferase expression (Fig. 4B). To test whether CREB activation was required for the induction of ICER gene expression by NO, RPaSMC were infected with an adenovirus specifying CREBml (Ad.CREBml) or a control adenovirus specifying β -galactosidase (Ad. β gal). Infection with Ad.CREBml, but not Ad. β gal, markedly attenuated the ability of GSNO to increase ICER mRNA levels (Fig. 4C). Taken together, these results suggest that NO-mediated phosphorylation of CREB is sGC- and PKA-dependent and that NO-mediated induction of CRE-dependent gene transcription, as well as ICER gene expression, requires phosphorylation and activation of CREB.

4.8) Induction of ICER gene expression by NO requires intracellular calcium

Previous studies found that the ability of NO to induce gene expression required the synergistic effects of PKA activation and an increase in Ca²⁺ signaling [2, 4, 11, 12]. Moreover, CREB is phosphorylated in response to increases in intracellular Ca²⁺ levels [27, 28]. To test whether intracellular Ca²⁺ was required for the induction of ICER mRNA levels by NO, we studied RPaSMC pretreated with the Ca²⁺ chelator, BAPTA-AM. Chelation of intracellular Ca²⁺ with BAPTA-AM markedly attenuated the ability of GSNO to induce ICER gene expression in RPaSMC (Fig. 5A). Similarly, 2-ABP and SKF-96365, inhibitors of SOCCs, blocked the induction of ICER mRNA levels by GSNO (Fig. 5B) and forskolin (Supplemental Fig. 8). In contrast, inhibition of the voltage-dependent Ca²⁺-channels (VDCCs) with nifedipine did not prevent the ability of GSNO to induce ICER gene expression (Fig. 5C). Our results suggest that Ca²⁺-mediated ICER induction requires SOCCs.

To test whether Ca²⁺ is required for the activation of CREB in RPaSMC, we measured CREB phosphorylation in RPaSMC exposed to 2-ABP. In the presence of 2-ABP, GSNO was able to induce CREB phosphorylation (Fig. 5D). Together, these findings suggest that intracellular Ca²⁺ is required for the induction of ICER gene expression by GSNO. However, phosphorylation of CREB in response to GSNO does not require SOCC activity. Furthermore, CREB phosphorylation, alone, is not sufficient to induce ICER gene expression in RPaSMC.

4.9) ICER inhibits cAMP signaling in RPaSMC

To explore the impact of ICER gene expression on CRE-dependent gene transcription, we studied RPaSMC transiently transfected with the CRE-luciferase reporter plasmid and a plasmid encoding ICER (pcDNA3-ICER). Transfection of pcDNA3-ICER attenuated the ability of GSNO or forskolin to activate CRE-dependent luciferase expression (Fig. 6A). Moreover infection of RPaSMC with an adenovirus specifying ICER (Ad.ICER), but not with a control adenovirus Ad. β gal, inhibited the ability of forskolin to increase expression of genes encoding HK2 and GCS:hc (Fig. 6B). These results suggest that induction of ICER in RPaSMC inhibits cAMP-stimulated CRE-dependent gene transcription.

5. Discussion

Using transcription profiling, we found that exposure to NO for up to 4h dramatically changed expression of a large number of genes in RPaSMC (Supplemental Fig. 1, 2). The majority of the genes up-regulated by GSNO contained cAMP-response elements. One of the CRE-containing genes encodes ICER, which was induced more than 100-fold by GSNO. To further elucidate the pathways by which NO regulated the expression of CRE-containing genes in RPaSMC, we focused our studies on the ICER gene (Fig. 1). We found that induction of ICER gene expression by NO was mediated by a sGC- and cGMP-dependent mechanism (Fig. 2, 3), PKA signaling, and activation of the CRE-binding transcription factor, CREB (Fig. 4). The proposed mechanisms regulating ICER gene expression are summarized in Figure 7.

To further characterize the mechanisms by which NO regulates VSMC structure and function, we compared the transcription profile (~8000 gene elements) of RPaSMC incubated in the absence or presence of GSNO. Two approaches were used to analyze the microarray data. First, we looked for common transcription factor-binding sites in genes whose expression was changed by 2-fold or more. We found that ~100 genes were up-regulated by GSNO, and a similar number of genes were down-regulated (Supplemental Fig. 1). Of the genes whose expression was induced by NO, 61% contained CRE sequences in their promoter regions (Supplemental Table 2). In a second approach, we organized gene expression data into microarray pathway profiles (MAPPs) that represent NO-regulated biological pathways (Supplemental Fig. 3A-C).

The findings that multiple genes, whose expression is up-regulated by GSNO, share CRE regulatory sequences suggests that CREB plays an important role in the NO-mediated regulation of VSMC phenotype. To further characterize the role of CRE/CREB signaling in the regulation of RPaSMC gene expression by NO, we focused on the gene encoding ICER, which is highly responsive to NO and is known to be regulated by CREB [7, 10]. We used a high concentration of GSNO (100 μ M) as a tool to identify genes that are modulated by NO. It is conceivable that the use of 100 μ M GSNO might lead to the generation of non-physiological concentrations of NO during the initial few minutes of GSNO exposure. Once ICER was identified as a gene whose expression was markedly increased by high concentrations of GSNO, we studied the effects of lower concentrations of GSNO, as well as inhaled NO, at doses that induce vasorelaxation. We found that ICER mRNA levels were markedly increased in RPaSMC exposed to GSNO at 1 and 10 μ M, in RPaSMC expressing NOS2 (Fig. 2A), and in lungs of WT mice breathing 80 ppm NO gas (Fig. 2B, D).

NO signals, in part, by activating sGC to synthesize cGMP. We observed that inhibition of sGC prevented the induction of ICER in RPaSMC exposed to GSNO, as well as in RPaSMC induced to express NOS2 (Fig. 2A). Moreover, the ability of inhaled NO to induce pulmonary ICER gene expression was markedly attenuated in mice lacking one sGC isoform, α 1 β 1 (Fig. 2D) [19]. The modest residual induction of ICER gene expression in

sGC α 1^{-/-} mice may attributable to the remaining sGC isoform, α 2 β 1. These results in *vitro* and in *vivo* implicate sGC and cGMP signaling in the induction of ICER gene expression by NO.

cGMP is well known to modulate gene expression in VSMC via PKG [29, 30]. Pilz and colleagues reported that PKG activation induces gene expression in VSMC in a CRE- and CREB-dependent manner [6]. In contrast, Aizawa et al. [31] found that NO and cGMP can induce NF κ B-dependent gene expression via a mechanism that does not require PKG. Similarly, we previously reported that NO/cGMP signaling induced PDE4B gene expression in RPaSMC via mechanisms that do not require PKG [32]. In the current study, we observed that PKG inhibitors did not block the ability of GSNO or endogenous NO to induce ICER gene expression in RPaSMC (Fig. 2A, C). In addition, PKG inhibition did not prevent the induction of CRE-dependent gene transcription by GSNO. Moreover, PKG-specific agonists were unable to induce ICER gene expression in these cells (Fig. 2C). Taken together these experiments suggest that sGC/cGMP signaling can regulate RPaSMC gene expression via a PKG-independent mechanism. We considered the possibility that PKG expression levels in RPaSMC may have been lost as the cells were passaged [33]: losing PKG activity would explain why compounds that modulate PKG activity had no effect in our experiments. However, PKG-I α mRNA was readily detectable in the RPaSMC that we studied.

We present several lines of evidence suggesting that PDE3 and PKA are responsible for the induction of ICER gene expression by NO and cGMP. First, the PKA inhibitor, H89, inhibited NO-induced CRE-dependent gene transcription and ICER gene expression (Fig. 2A, C). Second, treatment of RPaSMC with rolipram, an inhibitor of the cAMP-specific PDE4, markedly potentiated the effect of GSNO on CRE-dependent gene transcription, whereas the PDE3 inhibitor, milrinone, did not (Fig. 3B). Our findings suggest that milrinone and GSNO acted on CRE-dependent gene transcription using the same mechanism, inhibition of PDE3. Moreover, the observation that rolipram augmented the effects of NO supports the concept that NO activates CRE-dependent gene transcription via cAMP. Recently, Kumar et al. reported that, in COS-7 cells, NO increased cAMP levels and induced GTP cyclohydrolase I gene expression in a PKA dependent manner [34]. However, we were unable to detect an increase in cAMP levels in RPaSMC exposed to GSNO. Yan and colleagues proposed that PDE3 inhibition by NO leads to localized increases of cAMP levels that were not detectable in whole cell extracts [35]. As further confirmation that NO increased cAMP levels in RPaSMC, we observed that GSNO induced phosphorylation of VASP at Ser¹⁵⁷, the residue targeted by PKA, but not Ser²³⁹, the residue targeted by PKG [36] (data not shown). Taken together, these findings suggest that NO and cGMP signaling can regulate VSMC gene expression via cAMP-mediated PKA activation. cAMP activates gene transcription by phosphorylating the CRE-binding protein CREB at Ser¹³³ [7, 37]. Others have observed that NO/sGC/cGMP signaling can induce CREB phosphorylation in a variety of cell types,, including glial cells of avian retina [38], neurons in the nucleus tractus solitarii [39], in sGC- and PKG-expressing BHK cells [6], and in COS-7 cells [34]. We observed that incubation of RPaSMC with GSNO led to CREB phosphorylation via sGC- and PKA-dependent mechanisms. Moreover, as we showed previously for PDE4B [32], expression of a dominant-negative CREB prevented GSNO induced CRE-dependent gene transcription and ICER gene expression.

In rat pheochromocytoma cells (PC12 cells), previous reports suggested that the ability of NO to induce gene expression required the synergistic effects of PKA activation and an increase in Ca²⁺ signaling [11]. We similarly found that chelation of intracellular Ca²⁺ with BAPTA-AM abrogated the ability of NO to induce ICER gene expression (Fig. 5A). Our results suggest that Ca²⁺ is required for NO to modulate ICER signaling in VSMC.

To identify the source of the Ca^{2+} participating in the NO response, we used selective Ca^{2+} -channel inhibitors, 2-ABP and SKF, to block SOCCs and nifedipine to inhibit VDCCs. Importantly, our study suggests that the ability of NO to modulate ICER mRNA levels is dependent upon Ca^{2+} derived from SOCCs and not VDCCs (Fig. 5B-C). Prior studies have shown that Ca^{2+} signaling can induce CREB phosphorylation [40]. However, the ability of NO to phosphorylate CREB in RPaSMC was insensitive to SOCC inhibition (Fig. 5D). These results suggest that induction of ICER gene expression by NO requires both CREB phosphorylation and Ca^{2+} signaling. Prior studies have shown that Ca^{2+} has a critical role in regulating ICER gene transcription and potential downstream transcription factors have been identified, including thyrotropic embryonic factor (TEF) [41]. The identities of the transcription factors, in addition to CREB, that are responsible for activation of ICER gene transcription by NO in RPaSMC remain to be determined.

ICER is known to be a potent inhibitor of cAMP/PKA/CRE-dependent gene transcription [7]. Similarly, we found that transient expression of ICER in RPaSMC attenuated GSNO-dependent gene transcription. Moreover, that overexpression of ICER markedly inhibited forskolin-stimulated gene expression (Fig. 6). These results suggest that induction of ICER gene expression by NO signaling can modulate gene expression stimulated by subsequent exposure to cAMP.

NO and ICER share the ability to inhibit proliferation and increase apoptosis of VSMC, as well as to inhibit neointima formation in rat carotid arteries subjected to balloon injury [30, 42, 43]. It is conceivable that the induction of ICER expression by NO may be responsible, at least in part, for the ability of the latter to modulate VSMC functions. Additional in vitro and in vivo studies will be required to elucidate the role of ICER in the ability of NO to modulate VSMC phenotype.

In summary, we used transcription profiling to characterize the mechanisms by which NO regulates gene expression in RPaSMC (summarized in Fig. 7). NO induces expression of a wide variety of genes, many of which contain CRE regulatory sequences. By focusing on one of these NO-induced genes, ICER, critical roles were found for sGC, cGMP, and PDE3 in the regulation of CRE-dependent gene transcription. Unexpectedly, PKA, but not PKG, is required for the induction of CRE-dependent gene transcription, CREB phosphorylation, and ICER gene expression by NO. Moreover, a critical CREB-independent role for Ca^{2+} signaling in NO-induced ICER gene expression was identified. ICER induction represents a negative feedback loop for NO-stimulated gene expression and a novel “cross talk” mechanism between cGMP and cAMP signaling pathways.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

NO	nitric oxide
ICER	inducible cAMP early repressor
RPaSMC	rat pulmonary artery smooth muscle cells
VSMC	vascular smooth muscle cells
PKG	cGMP-dependent protein kinase
sGC	soluble guanylate cyclase
sGCα1^{-/-}	mice deficient for the soluble guanylate cyclase α subunit
IL-1β	interleukin-1 β
CRE	cAMP-response element
CREB	CRE-binding protein
P-CREB	phosphorylated CREB
CREM	cAMP-response element modulator
PDE	phosphodiesterase
SOCC	store-operated, non-voltage gated calcium channels
VDCC	voltage-dependent calcium channels
NOS2	nitric oxide synthase 2
Ad	adenovirus
βgal	beta-galactosidase
M.O.I	multiplicity of infection
KID	kinase-inducible domain
VASP	vasodilator-stimulated phosphoprotein
HO1	heme-oxygenase 1

tPA	tissue plasminogen activator
HK2	rat hexokinase 2
MT1	metallothionein 1
GCS:hc	γ -glutamylcysteine synthetase heavy chain
GCS:lc	γ -glutamylcysteine synthetase light chain
ET1	endothelin 1
AT2R	angiotensin II receptor
TGF-β3	transforming growth factor- β 3

Fig. 1.

Panel A: Incubation with S-nitroso-L-glutathione (GSNO) induces ICER gene expression in rat pulmonary artery smooth muscle cells (RPaSMC) in a time- dependent manner. RNA was extracted from untreated RPaSMC and RPaSMC treated with GSNO for 1, 2, 4, 16, and 24h. RNA blot hybridization was performed using radiolabeled DNA probes specifying ICER. Ethidium bromide staining of 28S rRNA in the gel was scanned and confirmed the equal loading of RNA samples. A representative blot of at least 3 experiments is shown.

Panel B: Incubation with S-nitroso-L-glutathione (GSNO) induces ICER gene expression in RPaSMC in a dose-dependent manner. RNA was extracted from untreated RPaSMC and RPaSMC exposed to varying concentrations of GSNO for 2h. RNA blot were hybridized with DNA probes specifying ICER. Ethidium bromide staining of 28S rRNA confirmed the equal loading of RNA samples. A representative blot of at least 3 experiments is shown.

Panel C: Proteins were extracted from RPaSMC treated without or with 0.1, 1, 10, 100 $\mu\text{mol/L}$ of GSNO for 4h. Immunoreactive ICER was detected using CREM-1 antibodies, and GAPDH was used as a loading control.

Fig. 2.

Panel A: Cytokines increase ICER gene expression in RPaSMC via NO- and cGMP-dependent mechanisms. RNA was extracted from untreated RPaSMC or RPaSMC incubated with IL-1 β (20 ng/ml) and TNF α (100 ng/ml) for 4h. Similarly-treated RPaSMC were pretreated without or with the NOS2 inhibitor, L-NIL (0.5 mmol/L); the sGC inhibitor, ODQ (10 μ mol/L); PKG antagonists, Rp-8-pCPT-cGMP (100 μ mol/L) and Rp-8-Br-cGMP (100 μ mol/L); or the PKA inhibitor, H89 (10 μ mol/L). Membranes were hybridized with DNA probes specifying rat ICER and NOS2; the experiment shown is representative of 3 separate experiments. **Panel B:** Inhaled nitric oxide (NO) increases pulmonary ICER gene expression in vivo. RNA was extracted from lungs of wild-type mice that breathed air supplemented with NO gas (80 ppm) for 2, 4, 8, or 24h. Membranes were hybridized with DNA probes specifying ICER; the experiment shown is representative of 3 different experiments. **Panel C:** GSNO induces ICER in RPaSMC via PKA. RNA blots were prepared from RPaSMC pretreated with or without ODQ (10 μ mol/L), PKG inhibitors, Rp-8-pCPT-cGMP (100 μ mol/L) and KT5823 (1 μ mol/L); H89 (10 μ mol/L); or cGMP analogues, Sp-pCPT-cGMP (100 μ mol/L) and Sp-8-Br-cGMP (100 μ mol/L) for 30 min followed by incubation with and without GSNO (100 μ mol/L) for 2h. Membranes were hybridized with DNA probes specifying ICER; the experiment shown is representative of 3 different experiments. **Panel D:** WT and sGC α 1^{-/-} mice breathed air supplemented with or without NO gas (80 ppm) for 2h. Pulmonary ICER mRNA levels were induced in WT mice breathing air supplemented with NO (* p <0.0001 lungs of WT mice breathing air vs breathing air supplemented with NO). sGC α 1^{-/-} mice showed a minor trend to induce ICER mRNA levels when breathing NO, that was significantly less than the induction of WT mice that breathed air supplemented with NO († p <0.0001 sGC α 1^{-/-} mice breathing NO vs WT mice breathing air with iNO. WT mice increased $n=5$ mice in each group; mean (SD).

Fig. 3.

Panel A: GSNO mediated cAMP response element (CRE)-dependent gene transcription is sGC-dependent. RPaSMC transfected with CRE-luciferase reporter plasmid were preincubated with ODQ (10 $\mu\text{mol/L}$), KT5823 (1 $\mu\text{mol/L}$), Rp-8-pCPT-cGMP (100 $\mu\text{mol/L}$), or Rp-8-Br-cGMP (100 $\mu\text{mol/L}$) for 30min and then stimulated with or without GSNO (100 $\mu\text{mol/L}$) for 4h. The results are expressed as firefly luciferase activity normalized to control renilla luciferase activity. * $p < 0.0005$ GSNO treated cells vs control cells, either unstimulated, or pretreated with KT5823, Rp-8-pCPT-cGMP or RP-8-Br-cGMP; and † $p < 0.0001$ ODQ- vs vehicle-pretreated treated RPaSMC stimulated with GSNO. **Panel B:** RPaSMCs incubated with Rolipram (Rol) plus GSNO induce CRE-dependent gene transcription more effectively than RPaSMC treated with GSNO alone RPaSMC treated with GSNO plus milrinone (mil). RPaSMC transfected with CRE-luciferase reporter plasmid were pretreated with H89 (10 $\mu\text{mol/L}$), Mil (30 $\mu\text{mol/L}$) or Rol (10 $\mu\text{mol/L}$) for 30 min and stimulated with or without GSNO (100 $\mu\text{mol/L}$) for 4h. Firefly and renilla luciferase activities were measured. * $p < 0.001$ versus control, # $p < 0.001$ vs GSNO only. $n = 3$ for each condition; mean (SD).

Fig. 4.

Panel A: GSNO mediated CREB phosphorylation is sGC- and PKA-dependent. RPaSMC preincubated without or with ODQ (10 μ mol/L) or H-89 (10 μ mol/L) were stimulated with GSNO or forskolin. Phosphorylated CREB (p-CREB) and total CREB were detected with immunoblotting. **Panel B:** Activation of CREB is required for induction of CRE luciferase activity in GSNO-stimulated RPaSMC. RPaSMC were transfected with a CRE-responsive luciferase plasmid without or with a plasmid specifying a dominant-negative CREB (CREBml) and stimulated with and without GSNO. Four hours later, firefly and renilla luciferase activities were measured. $n=3$; means (SD); * $p<0.001$ vs control, † $p<0.001$ vs GSNO control). **Panel C:** Activation of CREB is required for GSNO-mediated ICER induction. Uninfected cells and cells infected with Ad.CREB-ml (100 and 200 M.O.I.) or Ad.βgal (200 M.O.I.) were incubated with GSNO. Membranes were hybridized with DNA probes specifying ICER. Ethidium bromide staining of 28S rRNA confirmed the equal loading of RNA samples.

Fig. 5.

Intracellular calcium is required for GSNO-mediated ICER induction. RPaSMC were pretreated with the intracellular Ca^{2+} -chelator, BAPTA-AM (50 $\mu\text{mol/L}$; **Panel A**) store-operated Ca^{2+} -inhibitors, 2-ABP and SKF-96365 (**Panel B**); or the voltage dependent Ca^{2+} -channel inhibitor, nifedipine (100 $\mu\text{mol/L}$) (**Panel C**), followed by incubation with or without GSNO (100 $\mu\text{mol/L}$). ICER mRNA levels were detected using qRT-PCR. $n=3$; mean (SD). (**Panel A**) $*p=0.041$ control vs GSNO-treated cells, $\dagger p=0.042$ vs GSNO-treated cells without BAPTA-AM. (**Panel B**) Left panel: $*p=0.037$ control vs GSNO-treated cells, $\dagger p=0.017$ RPaSMC pretreated with 2-ABP followed by GSNO vs. GSNO-treated cells. Right panel: $*p=0.007$ control vs GSNO-treated cells, $\dagger p=0.008$ RPaSMC pretreated with SKF followed by GSNO vs. GSNO-treated cells. (**Panel C**) $*p=0.02$ control vs GSNO treated cells. Incubation with nifedipine did not alter ICER mRNA levels. (**Panel D**) GSNO stimulates CREB phosphorylation in presence of 2-ABP. Cells were preincubated with or without 2-ABP for 30 min and exposed to GSNO (100 $\mu\text{mol/L}$) for 4hrs. p-CREB and CREB phosphorylation were detected using immunoblot. Incubation of RPaSMC with GSNO induced CREB phosphorylation. CREB phosphorylation was not prevented by preincubation with the store-operated Ca^{2+} -inhibitor 2-ABP. A representative result of 3 different experiments is shown.

Fig. 6.

Panel A: ICER inhibits cAMP-dependent gene transcription. RPaSMC were transiently transfected with the CRE-luciferase reporter plasmid and a control plasmid (pcDNA3) or a plasmid encoding ICER (pcDNA3-ICER) for 24h. Transfected cells were treated with GSNO (100 μ mol/L) or forskolin (FK; 10 μ mol/L) for 4h. Firefly and renilla luciferase activities were measured. $n=3$, mean (SD); * $p<0.001$ RPaSMC transfected with pcDNA3 and stimulated with GSNO vs cells transfected with pcDNA3-ICER stimulated with GSNO, † $p<0.001$ RPaSMC transfected with pcDNA3 and stimulated with forskolin vs cells transfected with pcDNA3-ICER and stimulated with forskolin. **Panel B:** ICER inhibits cAMP-stimulated CRE-dependent gene expression. Uninfected RPaSMC and RPaSMC infected with Ad.ICER or Ad.βgal (100 M.O.I. each) were incubated with FK (10 μ mol/L) for 2h. RNA blot hybridization was performed using radiolabeled DNA probes specifying hexokinase2 (HK2) and γ -glutamylcysteine synthetase heavy chain (GCS:hc) probes.

Fig. 7.

Proposed pathway of NO-mediated ICER induction. NO induces ICER expression in RPaSMC via cGMP-mediated inhibition of phosphodiesterase3 (PDE3), thereby elevating cAMP concentrations (likely in a specific intracellular compartment), leading to activation of PKA and CREB phosphorylation. Phosphorylated CREB binds to the cAMP-response element (CRE) leading to activation of gene transcription. Ca^{2+} is also required for ICER gene induction by GSNO. Once the ICER gene is induced, it inhibits CRE-dependent transcription and its own expression via a negative feedback loop.