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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 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.

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 β1 subunit and an α1 or α2 subunit, and the sGCα1β1 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 (Ca^{2+}) homeostasis and Ca^{2+} sensitivity of contractile proteins, as well as the expression of genes that modulate VSMC phenotype [4]. cGMP activates

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-1β; 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-β3.

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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 Ca^{2+} and cyclic nucleotides. Phosphorylation of CREB at Ser^{133} 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.

Materials and methods

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

Cell culture

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

Microarray analysis

Two independent preparations of RPaSMCs were untreated or exposed to GSNO (100 μmol/L) for 1, 2, and 4 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 $\geqslant$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 4 h of GSNO stimulation using the CRE sequence database (http://natural.salk.edu/CREB/) [17].

Measurement of gene expression

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

Protein levels

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

NO exposure in vivo

Male WT mice (C57Bl/6) breathed air with or without 80 ppm NO for 2, 4, 8, and 24 h in specially-designed chambers, as described previously [18]. Additionally, WT mice and mice deficient for the α1 subunit of sGC [19] breathed air with or without NO (80 ppm) for 2 h. Lung tissue was harvested for measurement of ICER mRNA levels. Plasma nitrite and nitrate levels were measured as described in the Online supplement.

CRE-dependent gene transcription

RPaSMC were plated in 6-well dishes (2×10^5 cells/well) and transfected for 2 h 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 24 h, transfected cells were incubated in the presence and absence of GSNO with other agonists and antagonists as indicated in the Results section. After 4 h, 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.

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.-βgal) 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 Ser^{133} 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.βgal for 2 h in RPMI medium containing 10% NuSerum. Infected cells were then incubated in the same medium for 24 h.