Online data Supplement for "Nitric Oxide Regulates Pulmonary Vascular Smooth Muscle Cell Expression of the Inducible cAMP Early Repressor Gene"

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Expanded Materials and Methods

Materials

S-nitroso glutathione (GSNO) was obtained from Alexis Corp. (San Diego, CA). Forskolin and 8-bromo-cAMP were purchased from Sigma-Aldrich, Inc. (St. Louis, Missouri). L-N^6-(1-iminoethyl) lysine (L-NIL), ODQ, H89, KT5823, BAPTA-AM, 2-ABP and SKF-96365 were obtained from Calbiochem, Novabiochem, and Novagen Inc. (brands of EMD chemicals, Darmstadt, Germany). Sp-8-pCPT-cGMPS, Sp-8-Br-cGMPS, Rp-8-pCPT-cGMPS, and Rp-8-Br-PET-cGMPS were purchased from Biomol Research Laboratories Inc. (Plymouth Meeting, PA). IL-1β and TNFα were obtained from R&D Systems Inc. (Minneapolis, MN). Anti-CREM/ICER antibody (X-12: sc-440) was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Antibodies directed against CREB and phosphorylated CREB (Ser^{133}) were obtained from Cell Signaling Technology, Inc. (Beverly, MA).

Transcription profiling

RNA was extracted from either untreated RPaSMC or RPaSMC exposed to GSNO (100 μmol/L) for 1, 2, and 4 hours using the Trizol reagent (Invitrogen Life Technologies Inc., Carlsbad, CA). RNA was purified using the Qiagen RNeasy total RNA isolation method (Quiagen Inc. Valencia, CA). Purified RNA (10μg) was used to generate first strand cDNA by reverse transcription in the presence of dT_{24} primer followed by incubation with DNA polymerase to synthesize double-stranded cDNA using Superscript Choice system (Invitrogen). Biotin-labeled cRNA was generated from the double-stranded cDNA by in *vitro* transcription using the ENZO Bioarray High Yield transcript labeling method (Affymetrix Inc., Santa Clara, CA). Labeled cRNA (40 μg)

was fragmented in fragmentation buffer (40 mmol/L Tris, pH 8.1, 100 mmol/L potassium acetate, 30 mmol/L magnesium acetate) for 35 min at 94°C. Following confirmation of cRNA quality by hybridization to a test chip (Affymetrix), fragmented cRNA was hybridized to Affymetrix rat U-34A GeneChips containing 8798 gene elements in the GeneChip Fluidics Station 450 and was washed and scanned with the GeneChip® Scanner 3000. Affymetrix GeneChip 5.0 software was used for scanning and data analysis according to Affymetrix protocols. Transcription profiling was performed using two independent preparations of RPaSMCs. The results were confirmed for the genes named in Supplemental Figure 1 by RNA blot analysis and quantitative RT-PCR.

Evaluating gene expression data

For each transcription profiling experiment, gene expression data from untreated RPaSMC were compared to that from cells exposed to GSNO for 1, 2, and 4 hours. GSNO was identified as altering gene expression if, in both transcription profiling experiments, 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. The differentially expressed genes were extracted to generate two groups: Up-regulated and Down-regulated (Supplemental Table 1 and Supplemental Table 2).

Biological pathways of interest, such as oxidative stress responses, apoptosis, cell cycle regulation, and cellular metabolism, were evaluated for up-regulation or down-regulation by GSNO in RPaSMCs using the Gene Microarray Pathway Profiler (http://www.GenMAPP.org).[1, 2]

For pathway analysis, criteria for differential gene regulation included a ≥ 1.5-fold change in gene expression after 4h of GSNO exposure in both RPASMC preparations with an Affymetrix call of present at 4h for those genes whose expression was increased or at baseline for genes whose expression was decreased.

RNA blot hybridization

Total RNA from RPaSMC was extracted using Trizol® reagent (Invitrogen) according to the manufacturer's protocol. RNA (15 µg) was fractionated in 1.5 % agarose-formaldehyde gels, transferred to MAGNA CHARGE membranes (Micron Separation, Inc., Westborough, MA), and cross-linked by exposure to UV light. Membranes were hybridized overnight at 42 ºC with ^{32}P-labeled DNA restriction fragments. Membranes were washed at high stringency in a solution containing 3 mmol/L sodium citrate, 30 mmol/L sodium chloride, and 0.1 % SDS at 65ºC and were exposed to X-ray film. Equal loading of RNA on gels was confirmed by staining 28S and 18S ribosomal RNA with ethidium bromide. RNA blots shown are representative of at least three experiments. Membranes were hybridized with DNA fragments specifying rat ICER, mouse heme oxygenase 1 (HO1), rat sGC α1 (sGCα1) and β1 (sGCβ1), human tissue plasminogen activator (tPA), rat hexokinase 2 (HK2), metallothionein 1 (MT1), γ-glutamylcysteine synthetase heavy chain (GCS:hc), γ-glutamylcysteine synthetase light chain (GCS:lc), p21 $^{(Waf1/Cip1)}$, endothelin 1 (ET1), angiotensin II receptor (AT2R), and transforming growth factor-β3 (TGFβ3).

Measurement of gene expression using quantitative RT-PCR

RNA was extracted from cells using Trizol (Invitrogen, Carlsbad, CA), and cDNA was synthesized using MMLV-RT (Invitrogen). Real-time amplification of transcripts was detected using a Mastercycler ep

Realplex (Eppendorf, Hamburg, Germany). The relative expression of target transcripts was normalized to levels of *18S* rRNA. Changes in the relative gene expression normalized to levels of 18S rRNA were determined using the relative CT method.

Immunoblot

RPaSMC in 12-well plates were washed with ice-cold PBS and harvested in 100 µl RIPA buffer/well with EDTA containing 1 µl/100 µl buffer each of phosphatase inhibitor cocktail 1 and protease inhibitor and phosphatase inhibitor cocktail 2. Fifty µl of the cell extracts were added to 10 µl Laemmli' s SDS 6x buffer, boiled 5 min at 100˚C, and kept on ice before being subjected 10% SDS-PAGE. Proteins were transferred electrophoretically to Amersham Hybond^{TM}–P membranes, blocked with 5% nonfat dry milk in 1xTBS buffer for 1 hour, and reacted with primary antibodies directed against total CREB, P-CREB, and ICER. Bound antibodies were detected using anti-rabbit IgG linked to HRP (1:20.000). Blots were developed with Luminogen TMPS-3 detection reagent A and B and exposed to Kodak BioMax XAR films.

Nitrite and nitrate measurements

Nitrite and nitrate plasma levels were determined using the Nitrate/Nitrite Fluorometric Assay Kit (catalog No. 780051, Cayman Chemical Company) according to the manufacturer`s protocol.

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Supplemental Table 1: Microarray results of altered gene expression in rat pulmonary artery smooth muscle cells exposed to GSNO

	GSNO	Array 1	Array 2	Array 1+2
Up-regulated	1h	111	130	65
	2h	187	177	101
	4h	264	289	138
Down-regulated	1h	63	100	18
	2h	98	186	50
	4h	290	402	129

DNA microarray techniques measured expression levels of 8,798 Affymetrix gene entries in two independent isolates of RPaSMC that were untreated or exposed to GSNO (100 μmol/L) for 1, 2, and 4h. The absolute numbers of up-regulated and down-regulated gene entries were filtered by criteria requiring that a gene should have an Affymetrix detection call of *present* after GSNO stimulation in the up-regulated group or before GSNO stimulation in the down-regulated group with at least a two-fold expression change compared to control.

Supplemental Table 2: Selected genes known to be activated by cAMP/PKA/CREB

Gen locus	Name of the gene	mRNA sequence (Rattus norvegicus)	Increase (Fold)	Reference
S66024	Inducible cAMP early repressor	NM_017334.1	140	[3]
AI179610	Heme oxygenase 1	NM_012580.2	58	[4]
D26393	Hexokinase-2	NM_012735.1	50	[5, 6]
M26744	Interleukin-6	NM_012589.1	45	[7-9]
J05181	γ-glutamylcysteine sythetase (heavy chain)	NM_012815.2	25	[10]
	γ-glutamylcysteine synthetase (light chain)	NM_017305.2	5	[10]
GI58853	Neuron-derived orphan receptor 1	NM_017352	20	[11]
X13722	LDL Receptor	NM_175762.2	12	[12]
U09540	CYP1B1 P450	NM_012940.1	10	[13, 14]
	Metallothionein 2A	NM_001137564.1	9	[15]
U90121	Thrombomodulin		7	[16]
M23697	Tissue plasminogen activator	NM_013151.2	6	[17]
S77528	CCAAT/enhancer binding protein (C/EBP)	NM_024125.4	5	[18, 19]

This table shows a selection of genes up-regulated in RPaSMC incubated with GSNO for 4 h, all of which contain CRE promoter sites and are activated by cAMP/PKA/CREB signaling (see References). These observations highlight a link between NO-mediated gene regulation and activation of cAMP/PKA/CREB/CRE signaling in RPaSMC.

Supplemental Figure Legends

Supplemental Figure 1. Scatter plot of genes influenced by GSNO in RPaSMC. Changes of mRNA expression levels of genes in RPaSMC in response to 4 hours incubation with GSNO were determined via Affymetrix microarray. Cells were solubilized, RNA was extracted and mRNA levels analyzed by Affymetrix microarray as described in the method section. The data shows the results of 2 independent experiments. A logarithmic scatter plot of gene expression was generated, upregulated genes are labeled in green, downregulated genes are labeled in blue. In addition to affymetric analysis, the red dots denote those genes whose transcripts were confirmed either via mRNA or immunoblots. The box highlights the gene encoding ICER, which was strongly upregulated by GSNO..

Supplemental Figure 2. RNA blot hybridization of RPaSMC exposed to GSNO.
RNA was extracted from untreated RPaSMC and RPaSMC exposed to GSNO for 2h. RNA blot were hybridized with DNA probes specifying heme-oxygenase 1 (HO1), hexokinase 2 (HK2), tissue plasminogen activator (tPA), metallothionein 1 (MT1), γ-glutamylcysteine synthetase heavy (GCS:hc) and light chain (GCS:lc), p21 $^{(Waf1/Cip1)}$, soluble guanylate cyclase subunit α1 (sGCα1) and β1 (sGCβ1), endothelin1 (ET1), transforming growth factor-β3 (TGFβ3) and angiotensin II receptor (AT2R). Equal loading of RNA on gels was confirmed by staining 28S ribosomal RNA with ethidium bromide. RNA blots shown are representative of at least three different experiments..

Supplemental Figure 3A-C. GSNO regulates critical biological pathways. Microarray results for untreated RPaSMCs and RPaSMCs exposed to GSNO for 4h were imported into GenMAPP (http://www.genmapp.org) and used to illustrate biological pathways with up- or down-regulated

gene expression. Critical molecular mediators of oxidative stress (A), glutathione metabolism (B), and cellular apoptosis (C) are regulated by GSNO.

Supplemental Figure 4. Inhaled nitric oxide (NO) increases pulmonary ICER protein levels in vivo. **A**. Immunoblots of ICER protein levels from lungs of WT mice breathing air supplemented with or without NO gas (80 ppm) for 2h are shown. **B**: Analysis of the immunoblots shown in A: *n*=5 mice in each group; mean±SD; *p<0.05 lungs of WT mice breathing inhaled NO vs without breathing NO.

Supplemental Figure 5. Plasma nitrite and nitrate levels are similar in WT and sGCα1$^{-/-}$ mice breathing NO. WT mice and mice deficient for the soluble guanylate cyclase α1 subunit (sGCα1$^{-/-}$) breathed air supplemented without or with nitric oxide (iNO, 80 ppm) for 2h, and plasma nitrite and nitrate levels were measured. Breathing NO increased plasma nitrite and nitrate levels in WT and sGCα1$^{-/-}$mice. *n*=5 mice in each group, mean±SD; *p<0.05 air supplemented with NO vs without NO.

Supplemental Figure 6. The PKA inhibitor H89 inhibits the ability of GSNO to induce ICER gene expression. RPaSMC were preincubated with H89 (10 µmol/L) for 30 min and then stimulated with and without 100 µmol/L GSNO for 2h. RNA was extracted, and relative ICER mRNA levels were quantitated using qRT-PCR. *n*=3 per experimental group; mean±SD; *p<0.05 GSNO vs untreated RPaSMC, #p<0.05 H89 with GSNO vs GSNO alone.

Supplemental Figure 7. PKG activators do not induce CRE-dependent gene transcription in RPaSMC. RPaSMC were transfected with CRE-luciferase reporter plasmid. Luciferase activities were measured in RPaSMC stimulated with cGMP analogues, Sp-8-pCPT-cGMP (100

μmol/L) or Sp-8-Br-cGMP (100 μmol/L), or the cAMP analogue, 8-Br-cAMP (1 mmol/L), for 4 h (*p=0.001 8-Br-cAMP vs control).

Supplemental Figure 8. **A**: Forskolin-mediated ICER induction is Ca^{2+}-dependent. RPaSMC were pretreated with the intracellular Ca^{2+} chelator, BAPTA-AM (50 μmol/L), for 30 minutes and stimulated with the forskolin (FK). Intracellular Ca^{2+} chelation abrogated forskolin-mediated induction of ICER gene expression. *p=0.0053 FK vs control, †p=0.0055 FK with BAPTA-AM vs FK only. **B**: RPaSMC were pretreated with the SOCC inhibitor, SKF-96365 (50 μmol/L), for 30 minutes and stimulated with the adenylate-cyclase activator, forskolin. SOCC inhibition abrogated forskolin-mediated induction of ICER gene expression. *p=0.0003 FK vs control, #p=0.0002 SKF-96365 with FK vs FK alone.