

SUPPLEMENT MATERIAL

Chronic prenatal hypoxia induces epigenetic programming of PKC ϵ gene repression in rat hearts

by

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Short title: Hypoxia and PKC ϵ gene repression

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Supplemental Material

Expanded Materials and Methods

Experimental animals.

Time-dated pregnant Sprague-Dawley rats were purchased from Charles River Laboratories (Portage, MI), and were randomly divided into two groups: 1) normoxic control; and 2) hypoxic treatment of 10.5% O₂ from day 15 to 21 of gestation, as described previously.¹ Hearts were isolated from near-term (21 d) fetuses and 3 months old offspring. To isolate hearts, rats were anesthetized with 75 mg/kg ketamine and 5-mg/kg xylazine injected intramuscularly. To study the direct effect of hypoxia on the fetal heart, hearts were isolated from day 17 fetal rats and cultured in M199 media (Hyclone, Logan, UT) supplemented with 10% FBS and 1% penicillin/streptomycin at 37 °C in 95% air/5% CO₂, as reported previously.² Hearts were given 24 hours of recovery time before being placed in a hypoxic chamber with 1% O₂ for 48 hours. All procedures and protocols were approved by the Institutional Animal Care and Use Committee guidelines.

Cell culture

Rat embryonic ventricular myocyte cell line H9c2 was obtained from ATCC (Rockville, MD, USA). Cells were maintained in DMEM and supplemented with 10% FBS and 1% penicillin/streptomycin at 37 °C in 95% air/5% CO₂. Cells were grown and sub-cultured in 6-well plates with experiments performed between 70-80% confluent. For hypoxic studies, cells were transferred to the hypoxic chamber and maintained at 1%, 3%, or 10.5% O₂, respectively, for 24 hours.

Western blot analysis

Hearts or H9c2 cells were homogenized in a lysis buffer containing 150 mM NaCl, 50 mM Tris.HCl, 10 mM EDTA, 0.1% Tween-20, 0.1% β-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, and 5 μg/ml aprotinin, pH 7.4 and allowed to incubate for 1 hour on ice. Homogenates were then centrifuged at 4°C for 10 minutes at 10,000 g, and supernatants collected. Nuclear extracts were prepared from hearts and H9c2 cells using NXTRACT CellLytic Nuclear Extraction Kit (Sigma). Protein concentrations were measured using a protein assay kit (Bio-Rad, Hercules, CA). Samples with equal amounts of protein were loaded onto 12% polyacrylamide gel with 0.1% SDS and separated by electrophoresis at 100 V for 90 minutes. Proteins were then transferred onto nitrocellulose membranes. Nonspecific binding sites were blocked for 1 hour at room temperature in a Tris-buffered saline solution containing 5% dry-milk. The membranes were then probed with primary antibodies against PKCε, estrogen α and β subtype receptors (Santa Cruz Biotechnology; Santa Cruz, CA), SP1 (Active Motif; Carlsbad, CA), GAPDH (Millipore, Temecula, CA), as described previously.³ Beta2-microglobulin (B2M) antibody (Santa Cruz Biotechnology) was used to normalize loading.⁴ After washing, membranes were incubated with secondary horseradish peroxidase-conjugated antibodies. Proteins were visualized with enhanced chemiluminescence reagents, and blots were exposed to Hyperfilm. The results were analyzed with the Kodak ID image analysis software.

Real-time RT-PCR

RNA was extracted from hearts or H9c2 cells using TRIzol protocol (Invitrogen, Carlsbad, USA). PKCε mRNA abundance was determined by real-time RT-PCR using Icycler Thermal cycler (Bio-Rad, Hercules, CA), as described previously.³ The primers for PKCε are 5'-GCGAAGCCCCTAAGACAAT-3' (forward) and 5'-CACCCCAGATGAAATCCCTAC-3' (reverse). Real-time RT-PCR was performed in a final volume of 25 μl. Each PCR reaction mixture consisted of 600 nM of primers, 33 units of M-MLV reverse transcriptase (Promega, Madison, WI), and iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) containing 0.625 unit Taq polymerase, 400 μM each of dATP, dCTP, dGTP, and dTTP, 100 mM KCl, 16.6 mM ammonium sulfate, 40 mM Tris-HCl, 6 mM MgSO₄, SYBR Green I, 20 nM fluorescing

and stabilizers. We used the following RT-PCR protocol: 42 °C for 30 minutes, 95 °C for 15 minutes, followed by 40 cycles of 95 °C for 15 seconds, 60 °C for 20 seconds, 72 °C for 10 seconds. B2M was used as an internal reference and serial dilutions of the positive control was performed on each plate to create a standard curve. PCR was performed in triplicate, and threshold cycle numbers were averaged.

Quantitative methylation-specific PCR.

DNA was isolated from hearts or H9c2 cells using a GenElute Mammalian Genomic DNA Mini-Prep kit (Sigma), denatured with 2 N NaOH at 42 °C for 15 minutes, and treated with sodium bisulfite at 55 °C for 16 hours, as previously described.³ DNA was purified with a Wizard DNA clean up system (Promega) and resuspended in 120 µl of H₂O. Bisulfite-treated DNA was used as a template for real-time fluorogenic methylation-specific PCR (MSP) using primers created to amplify promoter binding sites containing possible methylation sites based on the previous sequencing of rat PKCε promoter.³ GAPDH was used as an internal reference gene. Real-time MSP was performed using the iQ SYBR Green Supermix with iCycler real-time PCR system (Bio-Rad).

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were collected from hearts or H9c2 cells using NXTRACT CellLytic Nuclear Extraction Kit (Sigma). The oligonucleotide probes with CpG and ^mCpG at the two SP1 binding sites (-346 and -268) in rat PKC promoter region were labeled and subjected to gel shift assays using the Biotin 3' end labeling kit and LightShift Chemiluminescent EMSA Kit (Pierce Biotechnology, Rockford, IL), as previously described.³ Briefly, single stranded oligos were incubated with Terminal Deoxynucleotidyl Transferase (TdT) and Biotin-11-dUTP in binding mixture for 30 minutes at 37 °C. The TdT adds a biotin labeled dUTP to the 3'-end of the oligonucleotides. The oligos were extracted using chloroform and isoamyl alcohol to remove the enzyme and unincorporated biotin-11-dUTP. Dot blots were performed to ensure the oligos were labeled equally. Combining sense and antisense oligos and exposing to 95°C for 5 minutes was done to anneal complementary oligos. The labeled oligonucleotides were then incubated with or without nuclear extracts in the binding buffer (from LightShift kit). Binding reactions were performed in 20 µl containing 50 fmol oligonucleotides probes, 1× binding buffer, 1 µg of poly(dI-dC), and 5 µg of nuclear extracts. For competitions studies, increasing concentrations of non-labeled oligonucleotides were added to binding reactions. The samples were then run on a native 5% polyacrylamide gel. The contents of the gel were then transferred to a nylon membrane (Pierce) and crosslinked to the membrane using a UV crosslinker (125 mJoules/cm²). Membranes were blocked and then visualized using the reagents provided in the LightShift kit.

Chromatin immunoprecipitation (ChIP)

Chromatin extracts were prepared from hearts or H9c2 cells. ChIP assays were performed using the ChIP-IT kit (Active Motif), as previously described.³ Briefly, cells were exposed to 1% formaldehyde for 10 minutes to crosslink and maintain DNA/protein interactions. After the reactions were stopped with glycine, cells were washed, chromatin isolated and the DNA sheared into medium fragments (100 – 1000 base pairs) using a sonicator. ChIP reactions were performed using SP1 antibody or ERα and ERβ antibodies to precipitate the transcription factor/DNA complex. Crosslinking was then reversed using a salt solution and proteins digested with proteinase K. Two sets of primers flanking the two SP1 binding sites at -346 and -268 were used: 5'-accatttctctcgacatgc-3' (forward) and 5'-agatttcaaccggatcctc-3' (reverse); 5'-agaggatccgggttgaatc-3' (forward) and 5'-ctcacctaccttccgaaca-3' (reverse). PCR amplification products were visualized on 2% agarose gel stained with ethidium bromide. To quantify PCR amplification, 45 cycles of real-time PCR were carried out with 3 minutes initial denaturation followed by 95 °C for 30 seconds, 59 °C for 30 seconds, and 72 °C for 30 seconds, using the iQ SYBR Green Supermix with iCycler real-time PCR system (Bio-Rad).

Hearts subjected to ischemia and reperfusion

Hearts of 3 months old male offspring were isolated and retrogradely perfused *via* the aorta in a modified

Langendorff apparatus, as previously described.¹ After the baseline recording, hearts were subjected to 20 minutes of global ischemia, followed by 30 minutes of reperfusion in the absence or presence of a PKC ϵ activator peptide ψ - ϵ RACK (0.5 μ M, KAE-1, KAI Pharmaceuticals) for 10 minutes before ischemia and throughout the period of ischemia and reperfusion, as previously described.⁵ Left ventricular developed pressure (LVDP), heart rate (HR), dP/dt_{max} , dP/dt_{min} , and LV end diastolic pressure (LVEDP) were continuously recorded. Lactate dehydrogenase (LDH) activity was measured in coronary effluent collected at 30 seconds before the onset of ischemia, and at 0, 1, 2, 3, 4, 5, 10, 15, 20, and 30 minutes of reperfusion. LDH activity was measured using a standard TOX 7 assay kit (Sigma) and expressed as area under curve (AUC).

Statistical analysis

Data are expressed as mean \pm SEM. Statistical significance ($P < 0.05$) was determined by analysis of variance (ANOVA) followed by Neuman-Keuls post hoc testing or Student's t test, where appropriate.

References

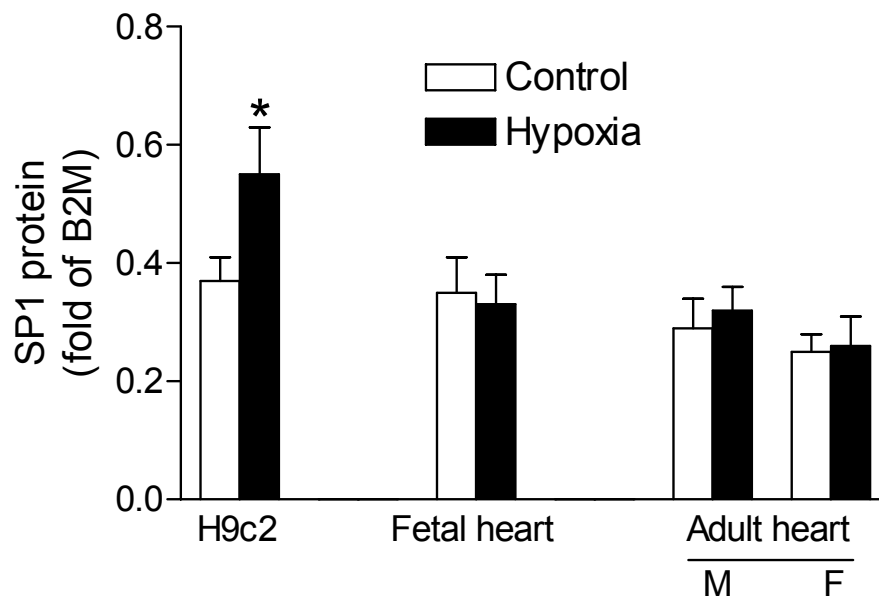
1. Xue Q, Zhang L. Prenatal hypoxia causes a sex-dependent increase in heart susceptibility to ischemia and reperfusion injury in adult male offspring: Role of PKC ϵ . *J Pharmacol Exp Ther*. 2009;330:624-632.
2. Meyer K, Zhang H, Zhang L. Direct effect of cocaine on epigenetic regulation of PKC ϵ gene repression in the fetal rat heart. *J Mol Cell Cardiol*. 2009;47:504-511.
3. Zhang H, Meyer KD, Zhang L. Fetal exposure to cocaine causes programming of Prkce gene repression in the left ventricle of adult rat offspring. *Biol Reprod*. 2009;80:440-448.
4. Foldager CB, Munir S, Ulrik-Vinther M, Søballe K, Bünger C, Lind M. Validation of suitable house keeping genes for hypoxia-cultured human chondrocytes. *BMC Mol Biol*. 2009;10:94.
5. Inagaki K, Hahn HS, Dorn GW, Mochly-Rosen D. Additive protection of the ischemic heart ex vivo by combined treatment with delta-protein kinase C inhibitor and epsilon-protein kinase C activator. *Circulation*. 2003;108:869-875.

Online Table I. Pre-ischemic left ventricle functional parameters

	HR (bpm)	LVEDP (mmHg)	LVDP (mmHg)	dP/dt _{max} (mmHg/s)	dP/dt _{min} (mmHg/s)	CF (ml/min)
Control	258±6	4.9±0.2	108±1	3575±75	2297±89	11.8±0.3
Hypoxia	257±2	5.1±0.3	112±2	3460±128	2228±98	11.6±0.5
Control+ψ-εRACK	260±8	5.1±0.2	106±3	3573±120	23138±70	12.2±0.6
Hypoxia+ψ-εRACK	258±5	4.7±0.2	107±2	3536±112	2183±69	12.4±0.7

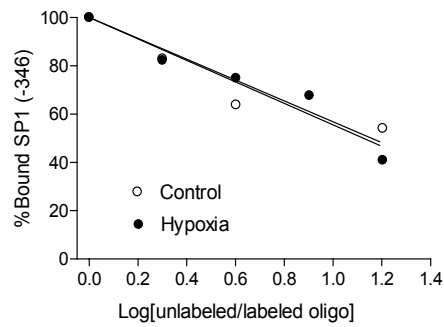
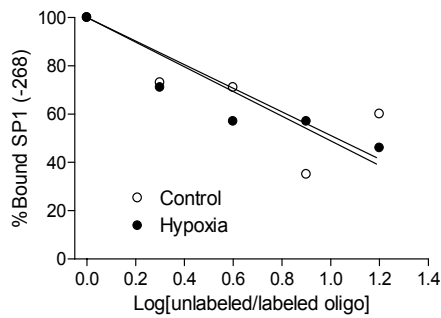
Hearts were isolated from 3-month-old male offspring that had been exposed to normoxia (control) or hypoxia before birth, and were treated in the absence or presence of the PKCε activator ψ-εRACK (0.5 μM) for 10 min before subjecting to 20 min of ischemia and 30 min of reperfusion in a Langendorff preparation. HR, heart rate; LVDP, left ventricular developed pressure; LVEDP, left ventricular end diastolic pressure; dP/dt_{max}, maximal rate of contraction; dP/dt_{min}, maximal rate of relaxation; CF, coronary flow. n = 5.

Figures and Legends

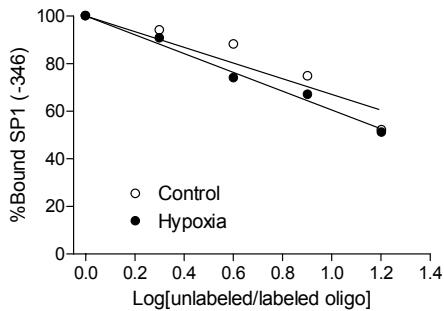
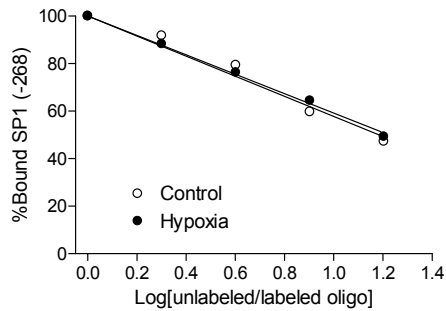


Online Figure I. Effect of hypoxia on SP1 abundance. Pregnant rats were treated with hypoxia, and hearts were isolated from near-term fetuses and 3 months old offspring. M, male; F, female. H9c2 cells were treated with 1% O₂ (hypoxia) vs. 21% O₂ (control) for 24 hours. SP1 protein abundance was determined in nuclear extracts. Data are mean \pm SEM. * P < 0.05 vs. control (n = 5-8).

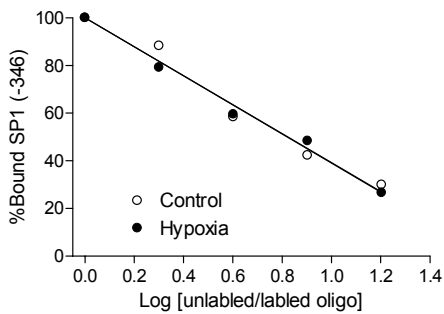
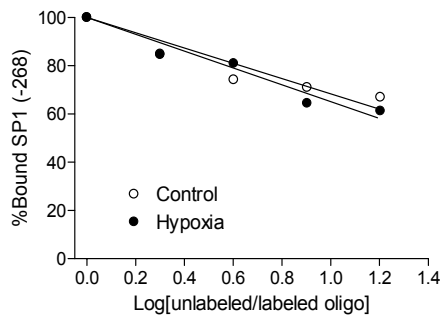
H9c2 cells



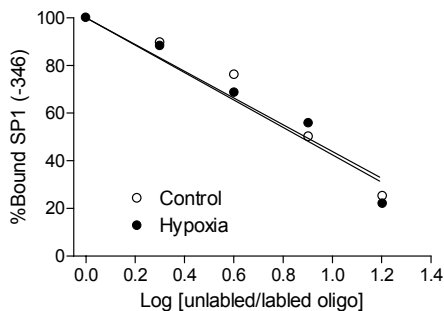
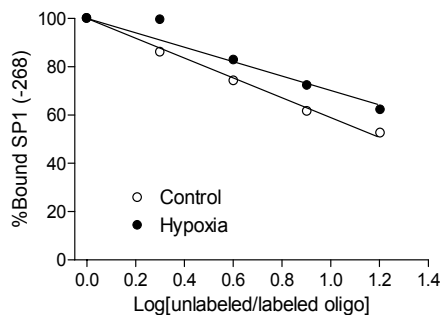
Fetal hearts



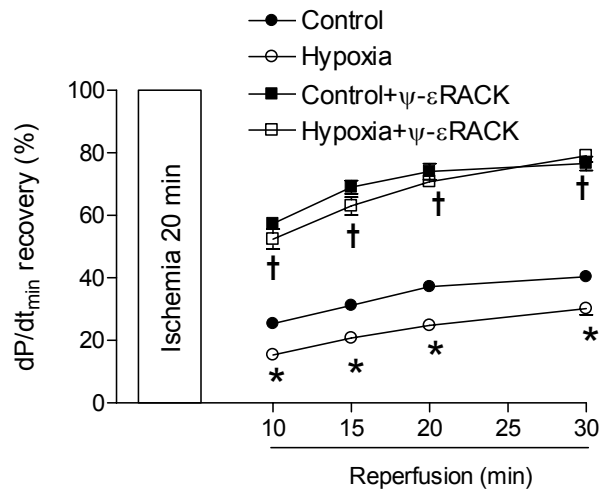
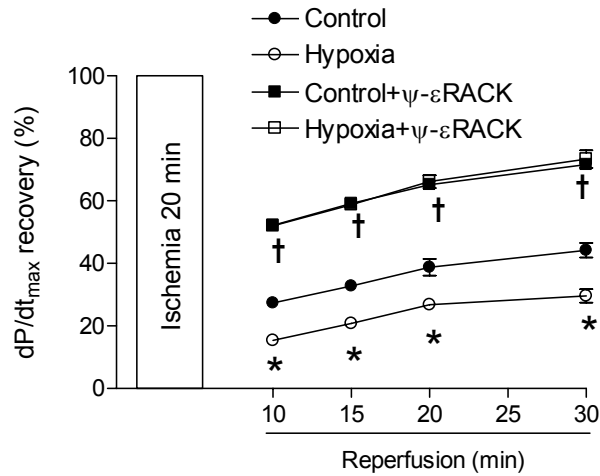
Adult male hearts



Adult female hearts



Online Figure II. Effect of hypoxia on SP1 binding affinity. Pregnant rats were treated with hypoxia, and hearts were isolated from near-term fetuses and 3 months old offspring. H9c2 cells were treated with 1% O₂ (hypoxia) vs. 21% O₂ (control) for 24 hours. Competition bindings were performed in nuclear extracts with oligonucleotides containing the consensus SP1 motifs at -346 and -268.



Online Figure III. Effect of PKC ϵ activation on cardiac ischemia and reperfusion injury. Hearts were isolated from 3 months old male offspring that had been exposed to normoxia (control) or hypoxia before birth, and were treated in the absence or presence of the PKC ϵ activator ψ - ϵ RACK (0.5 μ M) for 10 minutes before subjecting to 20 minutes of ischemia and 30 minutes of reperfusion in a Langendorff preparation. Post-ischemic recovery of dP/dt_{max} and dP/dt_{min} were determined. Data are mean \pm SEM. * P < 0.05, hypoxia vs. control; † P < 0.05, + ψ - ϵ RACK vs. - ψ - ϵ RACK. n = 5.