ONLINE DATA SUPPLEMENT

Fetal Nicotine Exposure Causes PKCε Gene Repression by Promoter Methylation in Rat Hearts

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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). Nicotine was administrated through osmotic minipumps implanted s.c. as described previously.[1] In brief, on the 4th day of pregnancy, rats were anesthetized with ketamine and xylazine, and an incision was made on the back to insert osmotic minipumps (type 2ML4; Alza, Palo Alto, CA). The incision was closed with four sutures. Half of the pregnant rats were implanted with the minipumps containing nicotine at a concentration of 102 mg/ml, and the other half were implanted with the minipumps containing only saline, which served as the vehicle control. The flow rate of the minipumps was 60 μl/day, which delivered a dose of 2.1 mg of nicotine free-base per day. In rats of an average of 350 g body weight, this corresponds to a dose rate of 4 μg/kg/min, which closely resembles doses occurring in moderate to heavy human smokers [2,3]. As previously reported,[1] nicotine treatment did not affect the litter size and the length of gestation, and all of the pregnancies reached full term. On day 21 of pregnancy, some rats were euthanized, and fetal hearts were isolated. Each litter was counted as an individual sample. Other rats were allowed to give birth naturally. Pups born to the dams were kept with their mothers until weaning. At weaning, male and female pups were separated and transferred to cages where they were housed in groups of two. Male and female offspring were killed at 3 months old, and hearts were isolated. For *ex vivo* studies of the direct effect of nicotine, the pregnant rats were euthanized and the fetuses were removed on day 17 of pregnancy. Fetal hearts were then extracted and cultured in H199 media containing 10% fetal bovine serum and 1% penicillin/streptomycin, as previously reported.[4] The cultured hearts were allowed to acclimatize to the media for 24 hours. Treatment of nicotine or norepinephrine at 1 μM or 10 μM concentrations was added to the media after 24 hours for additional 48 hours. Media was changed at 24 hours intervals. All procedures and protocols used in the present study were approved by the Institutional Animal Care and Use Committee of Loma Linda University and followed the guidelines by the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

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% CO_2. Cells were grown and subcultured in 6-well plates with experiments performed between 70-80% confluent. For norepinephrine studies, cells were treated with 10 μM norepinephrine in the absence or presence of 5-aza-2'-deoxycytidine, prazosin, or propranolol, respectively, for 48 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 were collected. Nuclear extracts were prepared from hearts using NXTRACT

CelLytic Nuclear Extraction Kit (Sigma, St. Louis, MO). Protein concentrations were measured using a protein assay kit (Bio-Rad, Hercules, CA). Samples with equal amounts of protein were loaded onto 7.5% 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, PKCδ or Egr-1 (Santa Cruze Biotechnology; Santa Cruz, CA) as described previously.[4,5] Actin antibody (Sigma, St. Louis, MO) was used to normalize the loading. 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 and PKCδ mRNA abundance was determined by real-time RT-PCR using Icycler Thermal cycler (Bio-Rad, Hercules, CA), as described previously.[4,5] The primers for PKCε are 5'-GCGAAGCCCCTAAGACAAT- 3' (forward) and 5'-CACCCCAGATGAAATCCCTAC-3' (reverse), and the primers for PKCδ are 5'-ACAGAAGAAGCCCACCAT- 3' (forward) and 5'-GAACTCAGCCTTGCCGTT-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 MMLV 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 20 seconds, 52 °C for 1 minute. GAPDH 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, St. Louis, MO), denatured with 2 N NaOH at 42 °C for 15 minutes and treated with sodium bisulfite using an iCycler Thermal cycler with a 4-cycle program of 95 ºC for 5 minutes and 55 ºC for 4 hours. DNA was purified with a Wizard DNA clean up system (Promega, Madison, WI) and resuspended in H_2O. 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.[5] Real-time MSP was performed using the iQ SYBR Green Supermix with iCycler real-time PCR system (Bio-Rad, Hercules, CA).

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were collected from hearts using NXTRACT CelLytic Nuclear Extraction Kit (Sigma, St. Louis, MO). The oligonucleotide probes with CpG and ^{m}CpG in the Egr-1 binding site (-1008) at rat PKCε promoter region were labeled and subjected to gel shift assays using the Biotin 3′ end labeling kit and Light-Shift Chemiluminescent EMSA Kit (Pierce Biotechnology, Rockford, IL), as previously described.[4,6] Briefly, single stranded oligos were incubated with

Terminal Deoxynucleotidyl Transferase (TdT) and Biotin-11-dUTP in binding mixture for 30 minutes at 37 ºC. 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 ºC 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 Light-Shift kit). Binding reactions were performed in 20 µl containing 50 fmol oligonucleotieds probes, 1× binding buffer, 1 µg of poly (dIdC), 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 Biotechnology, Rockford, IL) 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, Carlsbad, CA), as previously described.[4,6] Briefly, tissues were exposed to 1% formaldehyde for 10 minutes to crosslink and maintain DNA/protein interactions. After the reactions were stopped with glycine, tissues were washed, chromatin isolated and the DNA sheared into medium fragments (100 - 1000 base pairs) using a sonicator. ChIP reactions were performed using an Egr-1 antibody to precipitate the transcription factor/DNA complex. Crosslinking was then reversed using a salt solution and proteins digested with proteinase K. A set of primers flanking the Egr-1 binding site was used: 5′-GATCCGAGGAGCACAGAC-3′ (forward) and 5′-GTGAGCCGAGCAGAAAAC-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, Hercules, CA).

Reporter gene assay

A 1941-bp fragment of rat PKCε promoter region spanning -1941 to -1 bp relative to the transcriptional start site of the PKCε gene was amplified by PCR and inserted into the pDrive Cloning Vector (Qiagen, Valencia, CA). The KpnI/HindIII fragment flanking the PKCε promoter region was then inserted into the luciferase reporter gene plasmid, pGL3 (Promega, Madison, WI) to yield the full-length promoter-reporter plasmid denoted as pPKCε1941. Two 5'-truncation mutants as well as one site-specific deletion mutation were constructed, as described previously.[4,5] For truncation mutation, 1163 (pPKCε1163), 826 (pPKCε826) base pairs of 5' upstream of PKCε gene were inserted in front of luciferase reporter gene. For site-specific deletion mutations, transcription factor Egr-1 (CTCCCCCGCGCG) at -1008 was deleted from the pPKCε1941 (pPKCε1941(-Egr-1_{1008})). All promoter constructs sequences were confirmed with DNA sequencing analyses. Cell transfection was performed using a rat embryonic heart-derived myogenic cell line H9c2.[4,5] H9c2 cells were obtained from American Type Culture Collection (Rockville, MD) and maintained in DMEM supplemented with 10% fetal bovine

serum and 22 mM glucose. High-glucose medium increased the expression of PKCε in H9c2 cells. H9c2 cells were seeded in six-well plates (2 x 10^6 cells/plate) and transiently cotransfected with 1 μg of promoter/reporter vector along with 0.05 μg of internal control pRL-SV40 vector using Tfx-20 transfection reagents for eukaryotic cells (Promega, Madison, WI) following manufacturer's instructions. After 48 hours, firefly and Renilla reniformis luciferase activities in cell extracts were measured in a luminometer using a dual-luciferase reporter assay system (Promega, Madison, WI), as described previously.[4,5] The truncated promoter activities were then calculated by normalizing the firefly luciferase activities to R. reniformis luciferase activity.

High-performance liquid chromatography (HPLC)

Fetal rat hearts were dissected, weighed and homogenized in a glass homogenizer with 250 μl of 0.1 N perchlonc acid per mg of wet tissue at 300 rpm for 1 minute, followed by centrifugation at 2000 g for 5 min. A 300 μl sample was taken and extracted according to the method described previously.[7] Samples of eluted amines (100 μl) were injected into a HPLC with electrochemical detection consisting of a LC-4B amperometric detector, PM A30 dual piston pump and LC-22A temperature controller (Bioanalytical System Inc., West Lafaytte, IN). Parameters used were as follows: flow rate, 1.5 ml/min and detector attenuation, 2 nAt. Reverse phase columns (C18) were obtained from Phenomenex Inc (Palos Verdes, CA). Mobile phase consisted of 0.1 M sodium acetate, 0.02 M citric acid, 0.2 mM sodium EDTA, 150 mg of sodium octyl sulfate and 30 ml of methanol per 1L of solution. Norepinephrine content was determined with the following formula: ng of norepinephrine in 100 μl = Peak height of norepinephrine/Peak height of norepinephrine standard x 3 ng of norepinephrine/100 μl x Peak height DHBA standard/Peak height DHBA internal standard (norepinephrine and DHBA standard were 3 ng).

Statistical analysis

Data are expressed as mean ± 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.

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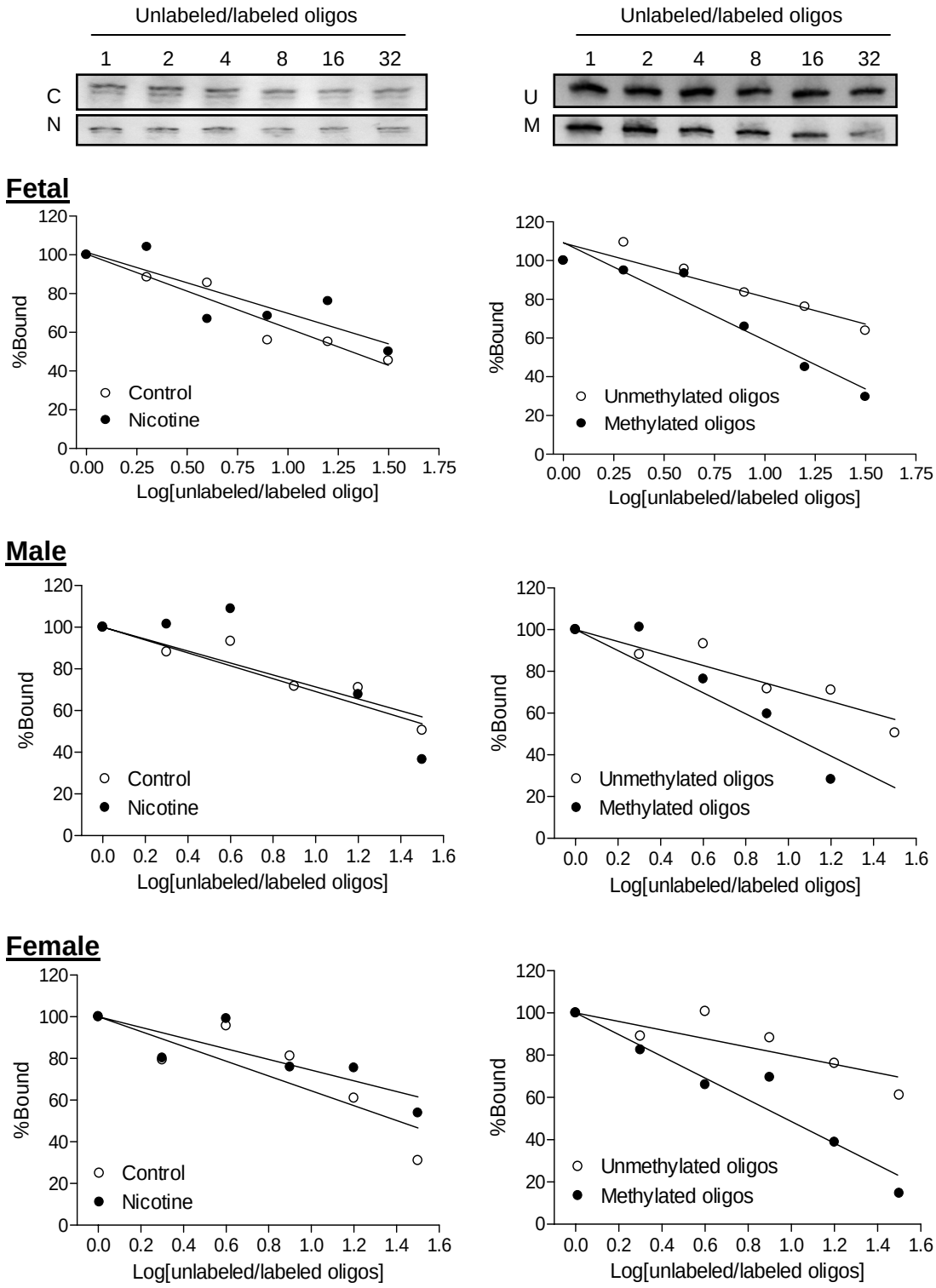

Figure S1. Effect of methylation on Egr-1 binding. **Left panels**. Pooled nuclear extracts from hearts of fetuses, male and female offspring from control (C) and nicotine (N)-treated groups were incubated with unmethylated oligonucleotides containing Egr-1 consensus sequence. **Right panels**. Pooled nuclear extracts from hearts of fetuses, male and female offspring were incubated with unmethylated (U) and methylated (M) oligonucleotides containing Egr-1 consensus sequence.

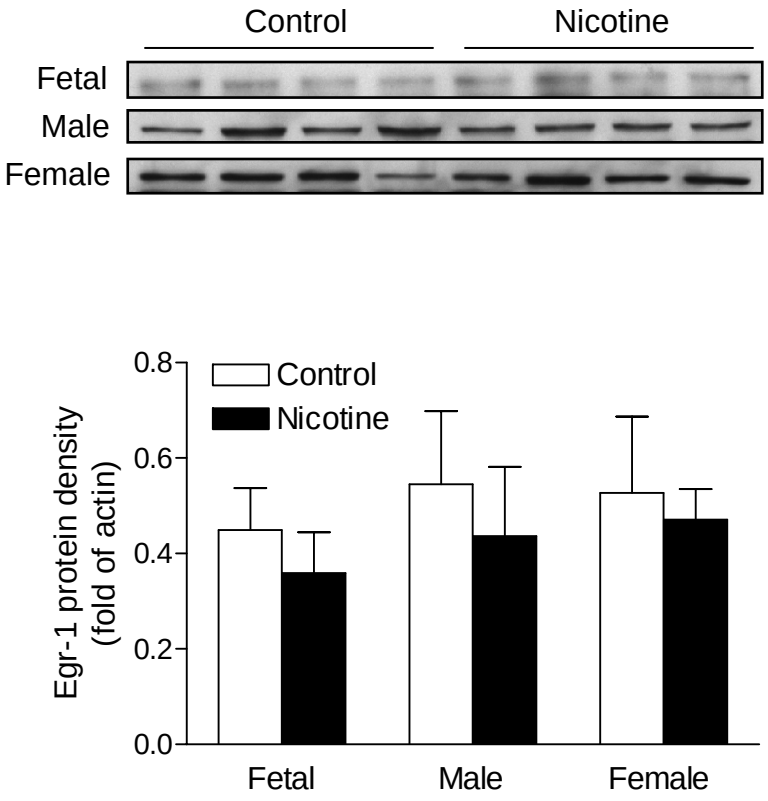

Figure S2. Effect of nicotine on Egr-1 abundance. Pregnant rats were treated with saline (control) or nicotine, and hearts were isolated from near-term fetuses and 3 months old offspring. Egr-1 protein abundance was determined in nuclear extracts. Data are mean ± SEM. (n = 5).