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Maternal Cocaine Administration Causes an Epigenetic Modification of Protein Kinase Cε Gene Expression in Fetal Rat Heart

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ABSTRACT

Protein kinase Cε (PKCε) plays a pivotal role in cardioprotection during cardiac ischemia and reperfusion injury. Recent studies demonstrated that prenatal cocaine exposure caused a decrease in PKCε expression and increased heart susceptibility to ischemic injury in adult offspring, suggesting an in utero programming of PKCε gene expression pattern in the heart. The present investigation aimed to elucidate whether an epigenetic mechanism, DNA methylation, accounts for cocaine-mediated repression of the PKCε gene in the heart. Pregnant rats were administered either saline or cocaine intraperitoneally (15 mg/kg) twice daily from days 15 to 20 of gestational age, and term fetal hearts were studied. Cocaine treatment significantly decreased PKCε mRNA and protein levels in the heart. CpG dinucleotides found in cAMP response element-binding protein (CREB), CREB/c-Jun1, and CREB/c-Jun2 binding sites at the proximal promoter region of the PKCε gene were densely methylated and were not affected by cocaine. In contrast, methylation of CpGs in the activator protein 1 (AP-1) binding sites was low but was significantly increased by cocaine. Reporter gene assays showed that the AP-1 binding site played a strong stimulatory role of PKCε gene transcription. Methylation of the AP-1 binding sites significantly decreased AP-1 binding to the PKCε promoter. Supershift analyses implicated c-Jun homodimers binding to the AP-1 binding sites. Cocaine did not affect nuclear c-Jun levels or the binding of c-Jun to the unmethylated AP-1 binding sites. The results indicate a role for DNA methylation in cocaine-mediated PKCε gene repression in the developing heart and suggest an epigenetic mechanism affecting this gene linked with vulnerability of ischemic injury in the heart of adult offspring.

Acute ischemic injury and myocardial infarction resulting from coronary artery disease is a major cause of death among people in the Western world. One of the most provocative recent findings in modern medicine has been the suggestion from Barker and colleagues (1993) that ischemic heart disease in adulthood is associated with adverse intrauterine environment and epigenetic programming in fetal life. Cocaine abuse among women of childbearing age is prevalent in the United States and is associated with numerous adverse perinatal outcomes including cardiac dysfunctions (van de Bor et al., 1990; Lipshultz et al., 1991; Shaw et al., 1991; Norris and Hill, 1992; Wiggins, 1992; Mehta et al., 1993; Frassica et al., 1994; Mone et al., 2004). Although there have been many studies of the effects of cocaine on the adult heart, studies of cocaine on the fetal heart and its potential delayed pathophysiological effects on cardiac function in adult offspring are extremely limited. We have demonstrated recently in a rat model that maternal cocaine exposure from days 15 to 20 of gestational age increases apoptosis in the term fetal heart (Zhang et al., 1999; Xiao et al., 2000, 2001; Li et al., 2005). In addition, prenatal cocaine exposure resulted in cardiac remodeling with myocyte hypertrophy in the left ventricle of postnatal rats and increased heart susceptibility to ischemia and reperfusion injury in adult offspring (Bae and Zhang, 2005b; Bae et al., 2005).

During ischemic disease, the heart can benefit from endog-

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ABBREVIATIONS: PKCε, protein kinase Cε; AP-1, activator protein 1; CREB, cAMP-response element binding protein; NFκB, nuclear factor κB; Egr-1, early growth response-1; PCR, polymerase chain reaction; MSP, methylation-specific polymerase chain reaction; Q-MSP, quantitative methylation-specific polymerase chain reaction; ChIP, chromatin immunoprecipitation; RT-PCR, reverse transcription-polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; bp, base pair(s).

enous protective mechanisms. It has been demonstrated in both cultured cardiomyocytes and the intact heart of experimental animal models that PKCε plays a pivotal role of cardioprotection during cardiac ischemia and reperfusion injury (Chen et al., 2001; Murriel and Mochly-Rosen, 2003; Gray et al., 2004). The cardioprotective effect of PKCε is proposed to be mediated by inhibition of apoptosis and hence reduction of myocardial infarction after ischemia and reperfusion (Heidkamp et al., 2001; Murriel and Mochly-Rosen, 2003). Recent studies in a PKCε knockout mouse model demonstrated that PKCε expression is not required for cardiac function under normal physiological conditions, but PKCε activation is necessary and sufficient for short-term cardioprotection during cardiac ischemia and reperfusion (Gray et al., 2004). We have shown in rats that a period of prenatal cocaine exposure significantly decreased PKCε expression later in the left ventricle of adult offspring (Bae et al., 2005). The pathophysiological significance of decreased PKCε levels in the heart was demonstrated by the findings that inhibition of PKCε by a PKCε translocation inhibition peptide mimic the effect of prenatal cocaine treatment and significantly increased ischemia and reperfusion injury in adult hearts (Bae and Zhang, 2005a).

These previous studies have suggested that prenatal cocaine exposure causes in utero programming of PKCε gene expression pattern, leading to a down-regulation of PKCε gene expression in the adult heart. However, the molecular mechanisms of epigenetic modification in PKCε gene expression involved in fetal programming are not understood. DNA methylation is a common mechanism for epigenetic modification of gene expression pattern and occurs at cytosines of the dinucleotide sequence CpG (Jones and Takai, 2001; Jaenisch and Bird, 2003). Methylation in promoter regions is generally associated with repression of transcription, leading to long-term shutdown of the associated gene (Jones and Takai, 2001; Jaenisch and Bird, 2003; Alikhani-Koopaei et al., 2004). Although methylation of CpG islands in gene promoter regions alters chromatin structure and transcription, methylation of CpG dinucleotides within transcription factor binding sites also affects transcription. Herein, we present evidence that CpG methylation of the activator protein 1 (AP-1) binding site at the promoter region of PKCε gene is a molecular mechanism in epigenetic programming of PKCε gene expression pattern involved in the adverse effects of cocaine on heart development.

Materials and Methods

Experimental Animals and Cocaine Treatment. Time-dated pregnant Sprague-Dawley rats were purchased from Charles River Laboratories (Portage, MI) and were randomly divided into two groups: 1) saline control; and 2) cocaine 15 mg/kg i.p. twice daily from days 15 to 20 of gestational age, as described previously (Bae et al., 2005). Previous studies have shown that long-term daily doses of cocaine from 10 to 40 mg/kg during rat gestation produces plasma cocaine levels in the human use range (Spear et al., 1989; Javaid and Davis, 1993), suggesting a comparable model in the present study. Isolated term (21 days) fetal hearts were studied. Procedures and protocols 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.

Western Blot. Protein levels of PKCε and phospho-PKCε^{Ser729} in hearts were determined by Western blot as described previously (Bae et al., 2005). In brief, hearts were homogenized in 5 volumes of ice-cold lysis buffer composed of 20 mM HEPES, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, and 10 μg/ml leupeptin followed by incubation on ice for 30 min. The homogenates were ultrasonicated and centrifuged at 20,000*g* for 30 min at 4°C. To determine nuclear c-Jun levels, nuclear extracts were prepared from hearts using CellLytic nuclear extraction kit from Sigma (St. Louis, MO). Samples with equal protein were loaded onto a 12% SDS-polyacrylamide gel and separated by electrophoresis at 100 V for 1 h. Proteins were then transferred onto Immobilon-P membrane and were probed with the primary antibodies of PKCε, c-Jun (Santa Cruz Biotechnology, Santa Cruz, CA), and phospho-PKCε ^{Ser729} (Upstate Biotechnology, Lake Placid, NY). After incubation with horseradish peroxidase-conjugated secondary antibody (Amersham, Little Chalfont, Buckinghamshire, UK), proteins were visualized with an enhanced chemiluminescence reagent, and the blots were exposed to Hyperfilm. Results were quantified with the Kodak electrophoresis documentation and analysis system and Kodak ID image analysis software (Eastman Kodak, Rochester, NY).

To determine the distribution of PKCε in cytosolic and particulate fractions in hearts, the homogenates were centrifuged at 100,000*g* for 30 min at 4°C, and the supernatants were collected and used as the cytosolic fraction (Zhang et al., 2006). The pellets were resuspended in the homogenization buffer containing 1% Triton X-100 by stirring overnight at 4°C, diluted with the buffer to a final concentration of 0.2% Triton X-100, and then centrifuged at 100,000*g* for 30 min at 4°C. The supernatants were collected and referred to as the particulate fraction. Protein concentrations were determined with a protein assay kit (Bio-Rad Laboratories, Hercules, CA). Immunoreactive bands for PKCε in cytosolic and particulate fractions were determined by Western blotting using the specific PKCε antibody as described above. The ratio of particulate/cytosolic PKCε was used to determine the relative activity of PKCε.

Real-Time RT-PCR. RNA was extracted from hearts using TRIzol reagents (Invitrogen, Carlsbad, CA). PKCε mRNA levels were determined by real-time RT-PCR using an Icycler Thermal cycler (Bio-Rad Laboratories). Specific PKCε primers were 5′-GCGAAGCCCCTAAGACAAT-3′ (forward) and 5′-CACCCCAGATGAAATCCCTAC-3′ (reverse). Real-time RT-PCR was performed in a final volume of 25 μl. Each reaction mixture consisted of 600 nM concentrations of primers, 33 units of Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI), and iQ SYBR Green Supermix (Bio-Rad Laboratories) containing 0.625 unit Taq polymerase, 400 μM concentration each of dATP, dCTP, dGTP, and dTTP, 100 mM KCl, 16.6 mM ammonium sulfate, 40 mM Tris-HCl, 6 mM $MgSO_4$, SYBR Green I, 20 nM fluorescein, and stabilizers. RT-PCR was performed under the following conditions: 42°C for 30 min, 95°C for 15 min, followed by 50 cycles of 95°C for 20 s, and 52°C for 1 min. GAPDH was used as an internal reference, and serial dilutions of the positive control were 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 using a GenElute Mammalian Genomic DNA Mini-Prep kit (Sigma), denatured with 2 N NaOH at 42°C for 15 min, and treated with sodium bisulfite at 55°C for 16 h, as described previously (Herman et al., 1996). DNA was purified with a Wizard DNA cleanup system (Promega) and resuspended in 120 μl of H_2O. The bisulfite-modified DNA was used as a template for real-time fluorogenic methylation-specific PCR (MSP) (Fackler et al., 2004; Hoque et al., 2006). Bisulfite treatment of DNA converted cytosines to uracils. However, methylated cytosines at CpG dinucleotides were not converted. Specific primers were designed to amplify the target regions of interest with unmethylated CpG dinucleotides by detecting uracils and methylated CpG dinucleotides by detecting cytosines. Primers and probes designed for detecting methylated and unmethylated promoter regions of interest are listed in Table 1. All oligonucleotide

primers and probes were synthesized by IDT (Coralville, IA). GAPDH was used as an internal reference gene. The conversion efficiency in the present study achieved 99.94%, as determined by measuring the conversion of cytosines to uracils of the GAPDH gene in the fetal heart. Real-time MSP was performed using the iQ SYBR Green Supermix with iCycler real-time PCR system (Bio-Rad Laboratories). Data are presented as the percentage of methylation of the regions of interest (methylated CpG/methylated CpG + unmethylated CpG × 100).

Electrophoretic Mobility Shift Assays. Nuclear extracts were prepared from hearts as described above. The unmethylated (5′-TCAAGGGCTGACGTAGTAAATATCC-3′) and methylated (5′-TCAAGGGCTGAC^{m}GTAGTAAATATCC-3′) AP-1 binding sequences from rat PKCε promoter region were synthesized by IDT. Electrophoretic mobility shift assays were performed as described previously (Soto et al., 1999; van Riggelen et al., 2005). In brief, after annealing with their respective reverse strands, the double-strand oligonucleotides were labeled with 3000 Ci/mmol [γ-^{32}P]ATP (MP Biomedicals, Irvine, CA) by T4 polynucleotide kinase (New England Biolabs, Ipswich, MA) and purified with a 15% polyacrylamide gel. Binding reactions were performed in 20 μl containing 10% glycerol, 12 mM HEPES, pH 7.9, 4 mM Tris-HCl, pH 7.9, 60 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 0.6 mg/ml bovine serum albumin, 2 μg of poly(dI-dC), and either 2 μg of nuclear extracts or 0.2 μg of purified AP-1 (c-Jun homodimer) (Promega). In competition studies, unlabeled oligonucleotide probes were added in 100-fold molar excess before the addition of the ^{32}P-labeled probes. For supershift assays, 2 μg of c-Jun, c-Fos, and Fra-1 antibodies (Santa Cruz Biotechnology), respectively, was added and further incubated for 1 h at 4°C. Protein-DNA complexes were resolved with 5.5% nondenaturing polyacrylamide gels (29:1 cross-linking ratio). Dried gels were exposed to Kodak XAR film for autoradiography.

TABLE 1
Sequences of Q-MSP primers

Primer Name	Q-MSP Primer Sequence (5′→3′)
PKCεAP-1	
FM	gatgtgtttaagggttgac
RM	ctacacacgaaaatccg
FUM	gatgtgtttaagggttgat
RUM	cctacacacaaaaatcca
PKCεCREB	
FM	tgttaataatgttcggttttatac
RM	cgtaataatataaaataacactcacg
FUM	gtatgttaataatgtttggttttatat
RUM	acataataatataaaataacactcaca
PKCεCREB/cJun1	
FM	tatgttaataatgttcggttttatac
RM	cgtataaaattctcaaaaatattaacg
FUM	gtatgttaataatgtttggttttatat
RUM	acatataaaattctcaaaaatattaaca
PKCεCREB/cJun2	
FM	ggtgaaaataaatatagttgatttttatc
RM	atttcctacgaatacccg
FUM	ggtgaaaataaatatagttgatttttatt
RUM	cttaatttcctacaaataccca
PKCδAP-1	
FM	ttttatgcgtttgattgatcgagc
RM	ccgccgccaaacctaccg
FUM	ttttatgtgtttgattgattgagt
RUM	ccaccaccaaacctacca
PKCδNFκB	
FM	gcgtaagtagttggggaagtttcgtc
RM	acctcgcctaccaataacacg
FUM	gtgtaagtagttggggaagttttgtt
RUM	acctcacctaccaataacaca
PKCδEgr-1	
FM	tttcgtgggcggagtttcgaaggggc
RM	cccaaataccgaccccgatcccg
FUM	ttttgtgggtggagttttgaaggggt
RUM	cacccaaataccaaccccaatccca
GAPDH	
FM	tttggttattagggttgttttttt
RM	taataaccaacttcccattctc
FUM	cctggttaccagggctgc
RUM	gggtagagtcatactggaacatg

FM, forward orientation, methylated; RM, reverse orientation, methylated; FUM, forward orientation, unmethylated; RUM, reverse orientation, methylated.

Chromatin Immunoprecipitation Assays. Hearts were minced and fixed with 1% formaldehyde. Cross-linking was stopped by the addition of glycine to a final concentration of 125 mM. Chromatin extracts were prepared and sonicated to produce DNA fragments between 100 and 500 bp in length (Nguyen et al., 2001). Chromatin immunoprecipitation (ChIP) assays were performed using the ChIP-IT kit from Active Motif (Carlsbad, CA) according to manufacturer's protocol using 2 μg of anti-c-Jun antibody. Both positive control and negative controls provided by the kit were performed along with the experimental samples. Antibody-pulled chromatin extracts were used as templates for PCR and DNA from an aliquot of nonprecipitated lysates was used as template for total input. Two different sets of primers flanking the AP-1 element at −361 of the rat PKCε promoter were used: 5′-ATGCTCTTGTGGTGTGTGGA-3′ (forward) and 5′-TTACTACGTCAGCCCTTGAGC-3′ (reverse); 5′-AGATGTGCTCAAGGGCTGA-3′ (forward) and 5′-CAGGAACACCATAGAGGGTCA-3′ (reverse), which yielded products of 115 and 95 bp in length, respectively. PCR amplification products were visualized on 1% agarose gel stained with ethidium bromide. To quantify PCR amplification, 45 cycles of real-time PCR were carried out with 3-min initial denaturation followed by 95°C for 30 s, 54°C for 30 s, and 72°C for 30 s, using the iQ SYBR Green Supermix with iCycler real-time PCR system (Bio-Rad Laboratories).

Cell Transfection and Reporter Gene Assays. A 2040-bp fragment of rat PKCε promoter region spanning −2000 to +40 bp relative to the transcriptional start site of the ATG codon (A as +1) from the rat genome data bank was cloned by PCR and inserted into the KpnI/HindIII sites of the luciferase reporter gene plasmid, pGL2 (Promega) to yield the full-length promoter-reporter plasmid denoted as pPKCε 2k-*Luc*. Two 5′-deletion mutants were also studied, including 437 (−398 to +40) and 400 (−361 to +40) base pairs of PKCε gene 5′ flanking sequence. The resulting plasmid pPKCε 398-*Luc* contained the AP-1 binding site, and pPKCε 361-*Luc* lacked the AP-1 binding site. All promoter construct sequences were confirmed with DNA sequencing analyses. Cell transfection was performed using a rat embryonic heart-derived myogenic cell line H9C2 (Kaneda et al., 2005). H9C2 cells were obtained from American Type Culture Collection (Manassas, VA) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 22 mM glucose. High-glucose medium increased the expression of PKCε in H9C2 cells (Kim et al., 2003). H9C2 cells were seeded in six-well plates (2×10^6 cells/plate) and transiently cotransfected with 1 μg of promoter/reporter vector along with 0.1 μg of internal control pRL-SV40 vector using Tfx-20 transfection reagents for eukaryotic cells (Promega) after manufacturer's instructions. After 48 h, firefly and *Renilla reniformis* luciferase activities in cell extracts were measured in a luminometer using a dual-luciferase reporter assay system (Promega). The truncated promoter activities were then calculated by normalizing the firefly luciferase activities to *R. reniformis* luciferase activity.

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

Results

Cocaine Suppresses PKCε Expression. To determine whether PKCε expression in the fetal heart was altered by maternal cocaine administration, PKCε protein and mRNA levels in fetal hearts were determined by Western blot and

quantitative real time RT-PCR, respectively. As shown in Fig. 1, there were significant decreases in both protein and mRNA levels of PKCε in the fetal hearts of the maternal cocaine administration group compared with those in the control hearts. In accordance, there was also a significant decrease in the levels of phospho-PKCε in the cocaine-treated hearts (Fig. 2A). To further demonstrate the effect of cocaine on PKCε activity, we determined the levels of PKCε in cytosolic and particulate fractions in the hearts. PKC translocation from soluble to particulate compartments has been used as an indicator of its enzymatic and physiological activation. As shown in Fig. 2B, the cocaine treatment significantly decreased the ratio of particulate/cytosolic distribution of PKCε in the fetal hearts.

Cocaine Increases the Methylation of AP-1 Binding Sites in the PKCε Promoter. Given the previous finding of a persistent decrease in PKCε protein levels in the heart of adult offspring of prenatal cocaine exposure (Bae et al., 2005), we reasoned that cocaine-mediated reduction of PKCε expression in the fetal heart was associated with a permanent suppression of the PKCε gene. We then determined the effect of cocaine exposure from days 15 to 20 of gestation on DNA methylation in the promoter region of PKCε gene in the hearts of term fetuses. Analysis of a 2000-bp DNA fragment upstream of the transcription starting site of the rat PKCε gene identified four putative transcription factor binding sites containing CpG dinucleotides in their putative core binding sequences: AP-1, cAMP-response element binding protein (CREB), CREB/c-Jun binding site 1, and CREB/c-Jun binding site 2 (Fig. 3A). To define the methylation status of CpG dinucleotides in these binding sites, we performed quantitative methylation-specific PCR (Q-MSP) after bisulfite treatment of DNA isolated from fetal hearts. The bisulfite conversion efficiency in the present study achieved 99.94%, as determined by measuring the conversion of cytosines to uracils of the GAPDH gene in the fetal heart. Primers for detecting methylation status of CpG dinucleotides in the four putative transcription factor binding sites are listed in Table 1. Figure 3A shows the methylation pattern of CpG dinucleotides in these binding sites of the PKCε gene in the control and cocaine-treated fetal hearts. The methylation levels were relatively low in the AP-1 binding sites compared with those in CREB, CREB/c-Jun 1, and CREB/c-Jun 2 binding sites in control hearts. In hearts treated with cocaine, there was a significant increase in the methylation levels of the AP-1 binding sites (Fig. 3A). In contrast, the methylation levels in CREB, CREB/c-Jun 1, and CREB/c-Jun 2 binding sites in the heart were not significantly changed by the cocaine treatment (Fig. 3A).

To determine whether the cocaine-mediated increase in the methylation levels in the AP-1 binding sites was specific to the PKCε gene, we determined CpG methylation of the PKCδ gene in the fetal heart. Three transcription factor binding sites AP-1, nuclear factor κB (NFκB), and early growth response-1 (Egr-1), which contain CpG dinucleotides in their putative binding sequences, were identified in the promoter region of the rat PKCδ gene (Fig. 3B). The methylation pattern of CpG dinucleotides in these binding sites in the control and cocaine-treated fetal hearts are shown in Fig. 3B. Compared with the PKCε gene, the methylation levels in

Fig. 1. Effect of cocaine on PKCε protein and mRNA levels in fetal hearts. Time-dated pregnant Sprague-Dawley rats received either saline as control or cocaine (30 mg/kg/day) from days 15 to 20 of gestational age, and fetal hearts were obtained at day 21. PKCε protein and mRNA levels in the hearts were determined with Western blot and quantitative real-time RT-PCR, respectively. Data are mean ± S.E.M., $n = 5$. *, $P < 0.05$ versus control.

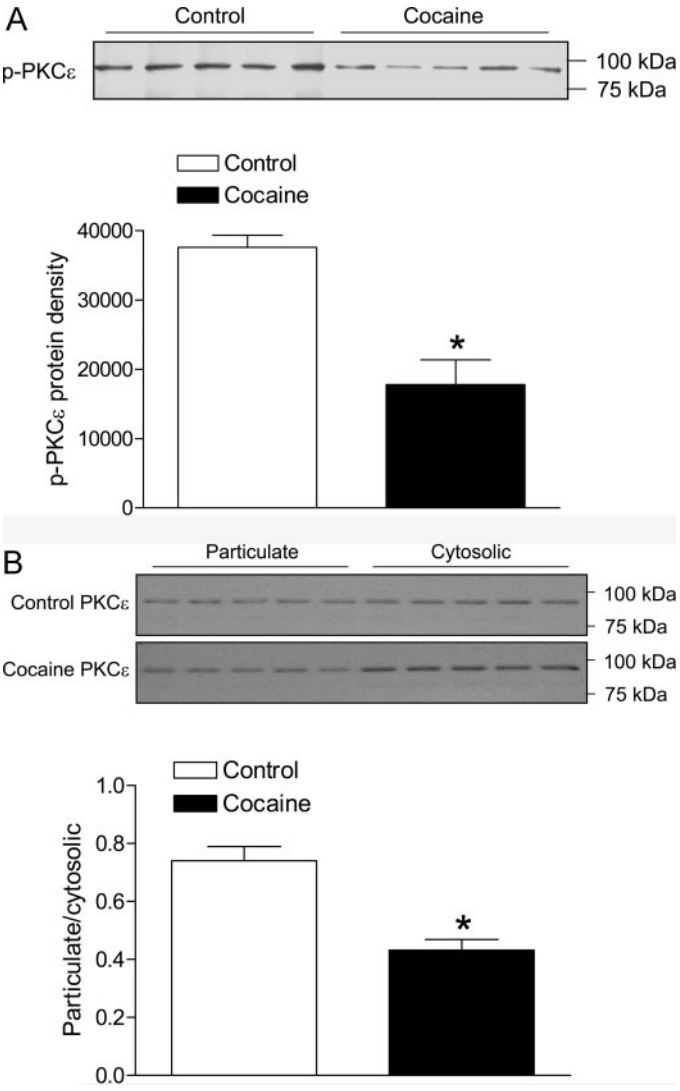


Fig. 2. Effect of cocaine on phospho-PKCε^{Ser729} and subcellular distribution of PKCε in fetal hearts. Time-dated pregnant Sprague-Dawley rats received either saline as control or cocaine (30 mg/kg/day) from days 15 to 20 of gestational age, and fetal hearts were obtained at day 21. A, protein levels of phospho-PKCε^{Ser729} were determined with Western blot. B, cytosolic and particulate fractions were prepared from the hearts as described under *Materials and Methods*. PKCε levels were determined with Western blot analysis and expressed as the ratio of particulate/cytosolic fractions. Data are mean ± S.E.M., $n = 5$. *, $P < 0.05$ versus control.

the transcription factor binding sites in the PKCδ gene were significantly lower and were less than 2% (Fig. 3B). Cocaine treatment showed no significant effect on the methylation levels of the transcription factor binding sites in the PKCδ gene (Fig. 3B).

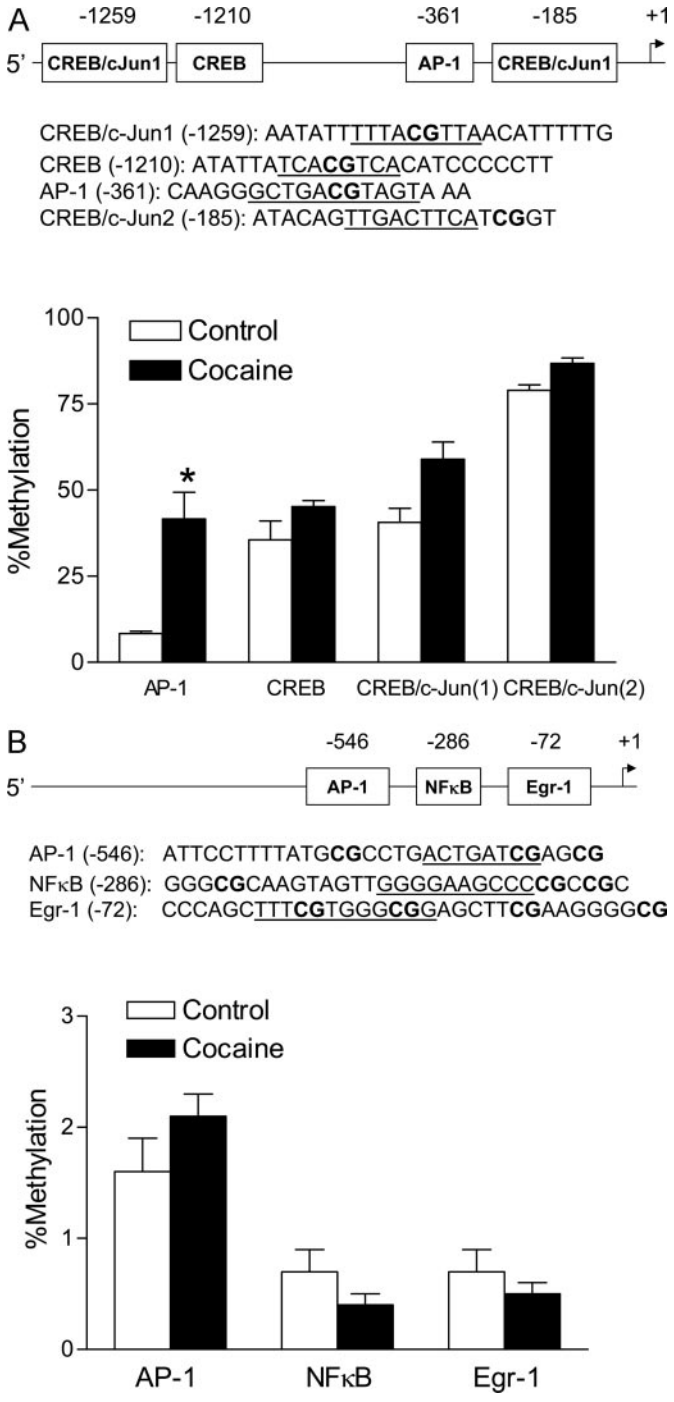


Fig. 3. Effect of cocaine on methylation of CpG dinucleotides at transcription factor binding sites of the PKCε and PKCδ promoters in fetal hearts. Time-dated pregnant Sprague-Dawley rats received either saline as control or cocaine (30 mg/kg/day) from days 15 to 20 of gestational age, and fetal hearts were obtained at day 21. DNA was extracted from hearts and treated with sodium bisulfite. The modified DNA was used as templates for real-time fluorogenic MSP in the PKCε (A) and PKCδ (B) promoters. Primers for the promoter regions of interest are listed in Table 1. 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. Data are presented as the percentage of methylation of the regions of interest (methylated CpG/methylated CpG + unmethylated CpG × 100). Values are mean ± S.E.M., $n = 5$. *, $P < 0.05$ versus control.

Involvement of the AP-1 Binding Sites in PKCε Promoter Activation. Although PKC has been demonstrated to be important in regulating the expression of many other genes, studies of promoter elements in the regulation of PKC gene expression are extremely limited. To determine whether the AP-1 binding site is involved in the regulation of PKCε promoter activity, the activities of 5′-deletion mutants were evaluated in the rat embryonic ventricular myocyte cell line H9C2. The levels of basal full-length promoter activity obtained with pPKCε 2k-*Luc* was at least 15-fold greater than the vector alone in the high-glucose medium. Basal promoter activity decreased by 42% with deletion from −2000 to −398, which contains the AP-1 binding site, and it decreased 92% with deletion from −2000 to −361, which lacks the AP-1 binding site (Fig. 4), indicating a strong stimulatory role of AP-1 binding in PKCε promoter activity.

To further evaluate the nuclear proteins binding to the consensus AP-1 element of the PKCε promoter at −361, electrophoretic mobility shift assays were performed. Incubation of nuclear extracts from the rat heart with a double-stranded oligonucleotide probe encompassing the putative AP-1 site at −361 resulted in the appearance of one major DNA protein complex (Fig. 5A). Addition of c-Jun antibody caused supershifting of the −361 AP-1 DNA-protein complex, whereas two other antisera directed to c-Fos and Fra-1 did not (Fig. 5A). These results suggest that c-Jun homodimers bind the AP-1 site in the rat heart. Figure 5B shows no significant difference in the binding of AP-1/c-Jun to the double-stranded unmethylated AP-1 oligonucleotides in nuclear extracts between the control and cocaine-treated fetal

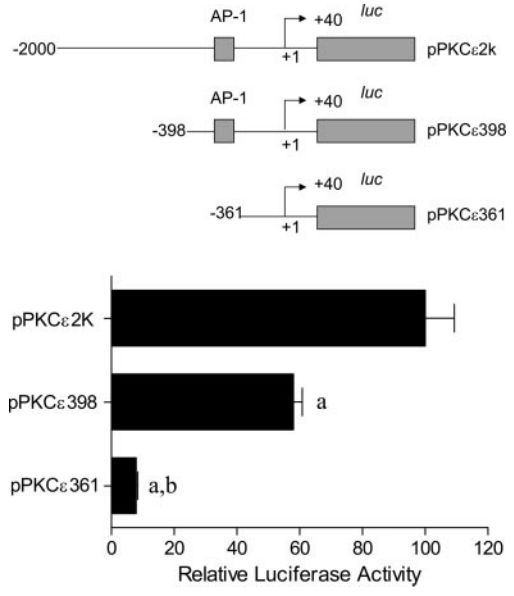
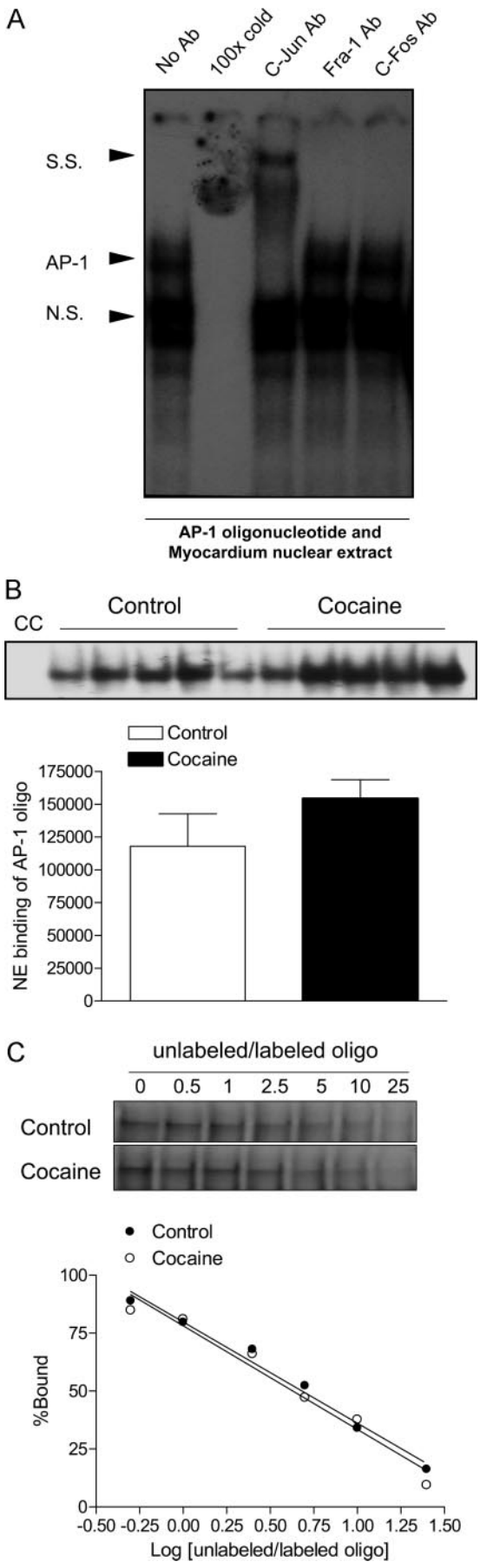


Fig. 4. Role of the AP-1 binding site at −361 in the PKCε gene promoter activity. A full-length (−2000 to +40 bp, pPKCε 2k-*Luc*) and two truncated (−398 to +40, pPKCε 398-*Luc*; −361 to +40, pPKCε 361-*Luc*) PKCε promoter-reporter gene constructs were transiently cotransfected with pRL-SV40-driven *R. reniformis* luciferase in a rat embryonic heart-derived myogenic cell line H9C2. After 48 h, firefly and *R. reniformis* luciferase activities in cell extracts were measured using a dual-luciferase reporter assay system (Promega). The truncated promoter activities were then calculated by normalizing the firefly luciferase activities to *R. reniformis* luciferase activity. Data are mean ± S.E.M., $n = 6$. a , $P <$ 0.05 versus pPKCε 2k-*Luc*; b, $P < 0.05$ versus pPKCε 398-*Luc*.



hearts. To confirm this finding, a competition study was performed with pooled nuclear extracts and increasing ratios of unlabeled oligonucleotides as an indication of AP-1 binding. As shown in Fig. 5C, the two competition curves obtained in the pooled nuclear extracts from the control and cocaine-treated fetal hearts, respectively, were superimposed.

Methylation of the AP-1 Binding Sites Inhibits AP-1 Binding. To determine whether methylation of the AP-1 binding site in the PKCε promoter inhibits AP-1 binding, we performed electrophoretic mobility shift assays with methylated and unmethylated oligonucleotide probes containing the AP-1 site at -361. As shown in Fig. 6, recombinant AP-1 composed of c-Jun homodimers bound and shifted the double-stranded unmethylated AP-1 oligonucleotides but not the double-stranded methylated AP-1 oligonucleotides. To verify whether cocaine-mediated increase in methylation of the AP-1 binding site can inhibit AP-1 binding to the PKCε promoter in vivo in the context of intact chromatin, we performed ChIP assays using an AP-1/c-Jun antibody. Figure 7A shows the binding of AP-1/c-Jun to the AP-1 element at -361 in the promoter region of the PKCε gene in intact chromatin in vivo in the rat heart. Quantitative real-time PCR shows a marked decrease of 75% in AP-1/c-Jun occupancy on the PKCε promoter in cocaine-treated fetal hearts compared with control hearts (Fig. 7B).

Cocaine Does Not Decrease AP-1/c-Jun Levels and Activity. To determine whether the cocaine-induced decrease in AP-1/c-Jun binding in fetal hearts was due to effects on AP-1/c-Jun abundance, Western blot analyses were performed using an AP-1/c-Jun antibody. Figure 8 shows no significant difference in AP-1/c-Jun levels in nuclear extracts between control and cocaine-treated fetal hearts.

Discussion

PKCε plays a pivotal role of cardioprotection during cardiac ischemia and reperfusion injury (Chen et al., 2001; Murriel and Mochly-Rosen, 2003; Gray et al., 2004). We have demonstrated previously that prenatal cocaine exposure results in decreased cardiac PKCε protein expression and increased heart susceptibility to ischemic and reperfusion injury in adult offspring (Bae and Zhang, 2005b; Bae et al., 2005). In the present study, we show that maternal administration of cocaine selectively increases methylation status of CpG dinucleotides in the Ap-1 binding sites of the PKCε

Fig. 5. Effect of cocaine on the binding of nuclear extracts to the AP-1 element of the PKCε promoter in fetal hearts. Time-dated pregnant Sprague-Dawley rats received either saline as control or cocaine (30 mg/kg/day) from days 15 to 20 of gestational age, and fetal hearts were obtained at day 21. Nuclear extracts from hearts were incubated with ^{32}P-labeled double-stranded oligonucleotide probes containing the PKCε gene consensus AP-1 binding motif at -361. A, supershift analyses of nuclear protein-DNA complexes of the AP-1 binding site. Nuclear extracts were incubated with ^{32}P-labeled AP-1 oligonucleotide probes in the absence or presence of three different antisera directed to c-Jun, Fra-1, and c-Fos, respectively. Competition reaction was performed with unlabeled competitor oligonucleotide at a 100-fold molar excess. Supershifted (S.S.) complex for c-Jun is identified with an arrowhead. B, nuclear extracts (NE) from control and cocaine-treated hearts were incubated with ^{32}P-labeled AP-1 oligonucleotide probes. Cold competition (CC) was performed with unlabeled competitor oligonucleotide at a 100-fold molar excess. Data are mean $\pm$ S.E.M., $n = 5$. C, pooled nuclear extracts from control and cocaine-treated hearts, respectively, were incubated with ^{32}P-labeled AP-1 oligonucleotide probes in the presence of 0-, 0.5-, 1-, 2.5-, 5-, 10-, and 25-folds of the unlabeled oligonucleotide.

gene promoter in fetal hearts. We further demonstrate that increased methylation of the AP-1 binding sites results in a significant decrease in AP-1 binding to the PKCε promoter, providing new insights into the epigenetic mechanisms of in utero programming of PKCε gene expression pattern in the developing heart.

Epigenetic mechanisms are essential for development and differentiation and allow an organism to respond to the environment through changes in gene expression (Reik et al., 2001; Jaenisch and Bird, 2003; Reik et al., 2003). DNA methylation is a chief mechanism in epigenetic modification of gene expression pattern. It is well-established that DNA methylation patterns can be altered in cancer, aging, and by diet (Issa et al., 1994; Jones and Laird, 1999; van den Veyver, 2002; Jaenisch and Bird, 2003). Despite its potential significance, studies of DNA methylation in fetal epigenetic programming caused by adverse intrauterine environment are extremely limited. By examining the 2000-bp DNA fragment upstream of the transcription starting site of the rat PKCε gene, we were able to locate the TATA box and several transcription factor binding sites that contain CpG dinucleotides in their putative core binding sequences. The presence of CpG dinucleotides in the promoter region suggests that the PKCε gene may be regulated at least in part through CpG methylation. Although cytosines are methylated in approximately 70% of CpGs of mammalian DNA, CpGs in the promoter/enhancer regions of many mammalian genes are not methylated. Methylation in promoter regions is generally associated with transcription repression, leading to long-term shutdown of the associated gene (Jones and Takai, 2001; Jaenisch and Bird, 2003; Alikhani-Koopaei et al., 2004). We have found that three of four transcription factor binding sites that contain CpG dinucleotides (i.e., CREB, CREB/c-Jun 1, and CREB/c-Jun 2) are heavily methylated in normal control fetal hearts, suggesting that these binding sites are less actively involved in the regulation of PKCε gene activity in the fetal heart. The findings that methylation levels of the AP-1 binding sites were relatively low in the control hearts and were significantly increased in cocaine-treated hearts suggest an important role of the AP-1 binding site in the regulation of PKCε gene expression in the fetal heart. This is supported by the finding that deletion of the AP-1 binding site significantly decreased the PKCε promoter activity, determined in transient transfection experiments in H9C2 cells with constructs containing truncated rat PKCε promoter fused to luciferase.

The AP-1 family of transcription factors includes the Jun and Fos family members. c-Jun can form stable homo- and heterodimers with other members of the AP-1 family,

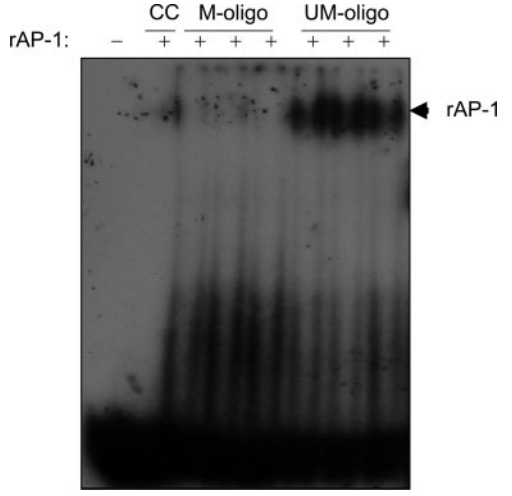
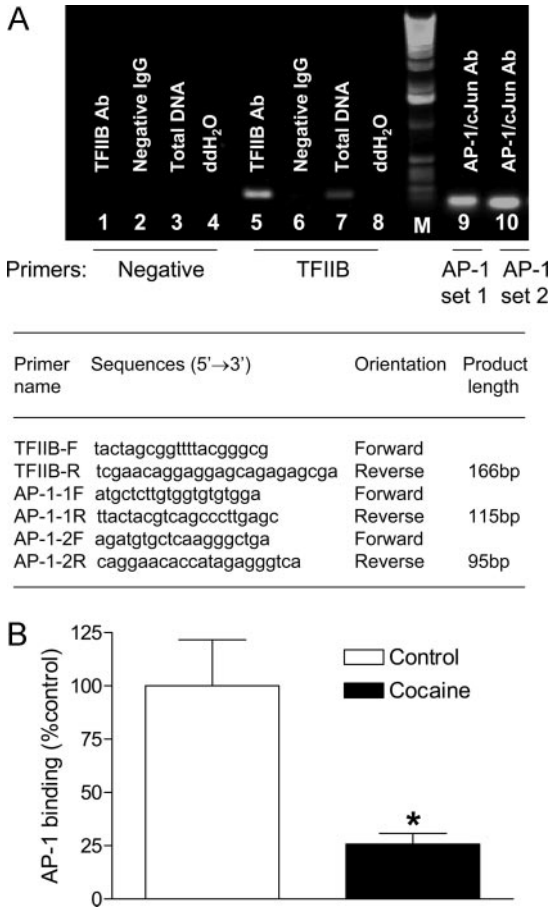


Fig. 6. Electrophoretic mobility shift assays with methylated and unmethylated AP-1 binding element. Recombinant AP-1/c-Jun homodimers (rAP-1) were incubated with ^{32}P-labeled double-stranded oligonucleotide probes containing either unmethylated (UM-oligo) or methylated (M-oligo) CpG dinucleotides at consensus AP-1 binding motif at −361 (5′-TCAAGGGCTGACGTAGTAAATATCC-3′ and 5′-TCAAGGGCTGAC^{m}G-TAGTAAATATCC-3′, respectively). Both unmethylated and methylated oligonucleotides were synthesized by IDT. Cold competition (CC) was performed with unlabeled competitor oligonucleotide at a 100-fold molar excess.



Primer name	Sequences (5'→3')	Orientation	Product length
TFIIB-F	tactagcggttttacgggcg	Forward	
TFIIB-R	tcgaacaggaggagcagagagcga	Reverse	166bp
AP-1-1F	atgctcttgtggtgtgtgga	Forward	
AP-1-1R	ttactacgtcagcccttgagc	Reverse	115bp
AP-1-2F	agatgtgctcaagggctga	Forward	
AP-1-2R	caggaacaccatagagggtca	Reverse	95bp

Fig. 7. Effect of cocaine on the binding of AP-1/c-Jun to the AP-1 element of the endogenous PKCε promoter at −361 in intact chromatin in vivo. Time-dated pregnant Sprague-Dawley rats received either saline as control or cocaine (30 mg/kg/day) from days 15 to 20 of gestational age, and fetal hearts were obtained at day 21. Chromatin extracts prepared from hearts were sonicated to produce DNA fragments between 100 and 500 bp in length. ChIP assays were performed using the ChIP-IT kit from Active Motif according to manufacturer's protocol using anti-c-Jun antibody. Antibody-pulled chromatin extracts were used as templates for PCR. Primers for the regions of interest are listed in A. A, lanes 1 to 4 show the results with the negative primers. Lanes 5 to 8 show the results of the positive primers flanking the TFIIB site of the constitutively active GAPDH promoter. PCR amplification of TFIIB antibody-pulled chromatin extracts is shown in lane 5. Lane 6 shows that the negative control IgG (provided by the kit) did not enrich specific DNA sequences. Lanes 9 and 10 show the PCR amplification of AP-1/c-Jun antibody-pulled chromatin extracts from hearts with two different sets of primers flanking the AP-1 element at −361 of the PKCε promoter (see Table 1 for sequences). B, AP-1/c-Jun antibody-pulled chromatin extracts were used as templates for quantitative real-time PCR using the set 1 AP-1 primers showed in A. Data are mean ± S.E.M., $n = 6$ to 8. *, $P < 0.05$ versus control.

whereas members of the Fos family do not homodimerize but can form stable heterodimers with Jun proteins (Shaulian and Karin, 2002). In the present study, we found that the antiserum to c-Jun caused supershifting of the protein-DNA complex resulting from binding of nuclear extracts from the heart with a double-stranded oligonucleotide probe containing the AP-1 element in the PKCε gene. In contrast, neither c-Fos nor Fra-1 antibodies induced supershifting of the AP-1 protein-DNA complex from nuclear proteins of the heart. These findings suggest that c-Jun homodimers are probably bound to the −361 AP-1 element in the PKCε gene in rat hearts.

The cocaine-mediated increase in methylation of the AP-1 binding site was associated with repressed PKCε gene expression, as demonstrated by the significant decrease in PKCε protein and mRNA levels in the cocaine-treated fetal hearts. The finding that the decrease in mRNA levels was much more pronounced than that in protein levels is interesting, and suggests a compensatory mechanism occurred at the translational level. Other possibilities of changes in mRNA and/or protein stability exist and warrant further investigation. Consistent with the decreased PKCε protein levels, the active form of phospho-PKCε was significantly reduced in the cocaine-treated hearts, suggesting a decrease in the PKCε activity. This is further supported by the finding that the ratio of particulate/cytosolic distribution of PKCε in the cocaine-treated fetal hearts was reduced. PKC translocation from soluble to particulate compartments has been well documented as an indicator of its enzymatic and physiological activation.

Previous studies demonstrated that transcriptional regulation by DNA methylation is typically observed in CpG islands located around the promoter region via the sequence-nonspecific and methylation-specific binding of inhibiting methyl-CpG binding proteins (Jones and Laird, 1999; Wade, 2001). Studies of CpG methylation in non-CpG islands and its effect on transcriptional regulation are relatively limited. It has been shown that DNA methylation can alter gene expression through changes in the binding affinity of sequence-specific transcription factors by altering the major groove structure of DNA to which the DNA binding proteins bind (Campanero et al., 2000; Dong et al., 2000; Zhu et al., 2003; Fujimoto et al., 2005). To determine whether methylation of the AP-1 binding sites in the PKCε promoter inhibits AP-1 binding, we performed electrophoretic mobility shift assays with methylated and unmethylated oligonucleotide probes containing the AP-1 site at −361. The finding that the methylated AP-1 probe with methylated-CpG dinucleotides at the core of the consensus AP-1 element in the PKCε promoter abolished the binding of AP-1/c-Jun homodimers indicates that CpG methylation in non-CpG islands and sequence-specific AP-1 binding site can directly inhibit the DNA binding of AP-1 complexes, resulting in down-regulation of PKCε gene expression in the heart. Although thymine methyls in the AP-1 binding site are critical for the AP-1 binding through their contacts with alanine residues in AP-1, methylation that occurred in wrong place—cytosines of CpG dinucleotides in the AP-1 site can block AP-1 binding (Dong et al., 2000; Rogstad et al., 2002; Fujimoto et al., 2005). Unlike the AP-1 binding sites, the fully methylated Sp1/Sp3 probe with methylated-CpG dinucleotides at the core and outside of the consensus Sp1/Sp3 element reduced but did not abolish Sp1/Sp3 binding (Alikhani-Koopaei et al., 2004). These results suggest that the effects of CpG methylation on transcription factor binding to sequence-specific transcription factor binding sites depend on the site and its context in relation to other regulatory elements within a gene promoter and to relative levels of transcription factors in a cell that bind to the site.

To determine whether the cocaine-mediated increase in methylation of the AP-1 binding sites can inhibit AP-1 binding to the PKCε promoter in vivo in the context of intact chromatin, we performed ChIP assays using an AP-1/c-Jun antibody. ChIP assays confirmed that cocaine treatment significantly decreased the recruitment of AP-1 complexes to the PKCε promoter in the fetal heart. The finding of the three-fold decrease in AP-1 binding in vivo in intact chromatin corresponded with the 3-fold increase in methylation of the AP-1 binding sites in the cocaine-treated hearts and with the loss of AP-1 binding to the methylated AP-1 binding sites. We further demonstrated that there were no significant differences in AP-1/c-Jun expression levels and no differences in the binding of AP-1/c-Jun to the unmethylated AP-1 oligonucleotides in nuclear extracts from control and cocaine-treated fetal hearts. Taken together, these results suggest that increased methylation of the AP-1 binding sites is necessary and sufficient for the decreased AP-1 binding to the PKCε promoter and thus to the decreased PKCε gene expression levels observed in cocaine-treated fetal hearts.

In contrast to PKCε that is cardioprotective, activation of PKCδ in adult myocardium mediates ischemic damage upon reperfusion (Chen et al., 2001; Murriel and Mochly-Rosen, 2003). In the present study, we found that the levels of CpG methylation in sequence-specific transcription factor binding sites of the PKCδ promoter were very low in the fetal heart. Previous studies showed a developmental regulation of PKCδ expression in the rat heart and a marked decline of PKCδ from fetal to adult hearts (Bogoyevitch et al., 1993; Rybin and Steinberg, 1994). In contrast to the findings in the PKCε promoter, cocaine treatment did not change methylation levels of the PKCδ promoter in the fetal heart. This finding suggests that the effect of cocaine is selective to the PKCε

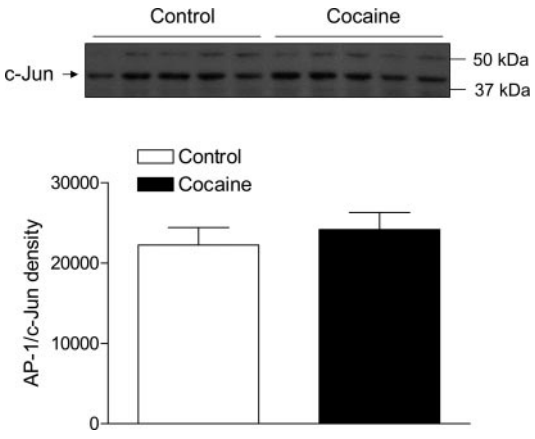


Fig. 8. Effect of cocaine on AP-1/c-Jun protein levels in nuclear extracts of fetal hearts. Time-dated pregnant Sprague-Dawley rats received either saline as control or cocaine (30 mg/kg/day) from days 15 to 20 of gestational age, and fetal hearts were obtained at day 21. AP-1/c-Jun protein levels in nuclear extracts prepared from control and cocaine-treated hearts, respectively, were determined by Western blot using a c-Jun antibody. Data are mean ± S.E.M., $n = 5$.

promoter in the heart. The mechanisms of the selective effect are not clear at present. One of the possible mechanisms is that high binding activities of the transcription factors to the PKCδ promoter in the fetal heart protect it from the access of methyltransferase.

Our investigation has demonstrated evidence of CpG methylation in non-CpG island, sequence-specific transcription factor binding sites of the PKCε promoter in the developing heart, providing a novel model to evaluate subtle epigenetic modifications of gene expression pattern in fetal programming in response to adverse intrauterine environment. Our collective observations indicate that an increase in methylation of a single CpG dinucleotide at the core of the consensus AP-1 element in the PKCε promoter is necessary and sufficient for decreased AP-1 binding to the PKCε promoter and repressed PKCε gene expression in the fetal heart. Because cardiac myocytes do not divide postnatally, the CpG methylation status of the PKCε gene promoter that occurred in term fetal hearts may not change significantly, which may explain the prolonged decrease in PKCε protein expression observed in the heart of adult offspring that were exposed to cocaine before birth (Bae et al., 2005). Future studies are needed to confirm that the increased methylation produced by cocaine persists and continues into adulthood. These findings seem to be clinically important, a contention supported by the observations that PKCε plays a pivotal role of cardioprotection during cardiac ischemia and reperfusion injury (Murriel and Mochly-Rosen, 2003; Bae and Zhang, 2005a), and heart susceptibility to ischemia and reperfusion injury is significantly increased in adult offspring that were exposed to cocaine before birth (Bae et al., 2005).

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