Chronic Prenatal Hypoxia Induces Epigenetic Programming of PKCε Gene Repression in Rat Hearts

Andrew J. Patterson,* Man Chen,* Qin Xue, Daliao Xiao, Lubo Zhang

Rationale: Epidemiological studies demonstrate a clear association of adverse intrauterine environment with an increased risk of ischemic heart disease in adulthood. Hypoxia is a common stress to the fetus and results in decreased protein kinase C epsilon (PKCε) expression in the heart and increased cardiac vulnerability to ischemia and reperfusion injury in adult offspring.

Objectives: The present study tested the hypothesis that fetal hypoxia-induced methylation of cytosine-phosphate-guanine dinucleotides at the PKCε promoter is repressive and contributes to PKCε gene repression in the heart of adult offspring.

Methods and Results: Hypoxic treatment of pregnant rats from days 15 to 21 of gestation resulted in significant decreases in PKCε protein and mRNA in fetal hearts. Similar results were obtained in ex vivo hypoxic treatment of isolated fetal hearts and rat embryonic ventricular myocyte cell line H9c2. Increased methylation of PKCε promoter at SP1 binding sites, −346 and −268, were demonstrated in both fetal hearts of maternal hypoxia and H9c2 cells treated with 1% O₂ for 24 hours. Whereas hypoxia had no significant effect on the binding affinity of SP1 to the unmethylated sites in H9c2 cells, hearts of fetuses and adult offspring, methylation of both SP1 sites reduced SP1 binding. The addition of 5-aza-2’-deoxycytidine blocked the hypoxia-induced increase in methylation of both SP1 binding sites and restored PKCε mRNA and protein to the control levels. In hearts of both fetuses and adult offspring, hypoxia-induced methylation of SP1 sites was significantly greater in males than in females, and decreased PKCε mRNA was seen only in males. In fetal hearts, there was significantly higher abundance of estrogen receptor α and β isoforms in females than in males. Both estrogen receptor α and β interacted with the SP1 binding sites in the fetal heart, which may explain the sex differences in SP1 methylation in the fetal heart. Additionally, selective activation of PKCε restored the hypoxia-induced cardiac vulnerability to ischemic injury in offspring.

Conclusions: The findings demonstrate a direct effect of hypoxia on epigenetic modification of DNA methylation and programming of cardiac PKCε gene repression in a sex-dependent manner, linking fetal hypoxia and pathophysiological consequences in the hearts of adult offspring.

Key Words: fetal heart ■ PKCε ■ hypoxia ■ epigenetics ■ DNA methylation

Heart disease is the leading cause of death in the United States. In addition to other risk factors, recent epidemiological and animal studies have shown a clear association of adverse intrauterine environment with an increased risk of hypertension and ischemic heart disease in adulthood.1–4 Hypoxia is a common form of intrauterine stress, and the fetus may experience prolonged hypoxic stress under a variety of conditions, including pregnancy at high altitude, pregnancy with anemia, placental insufficiency, cord compression, preeclampsia, heart, lung and kidney disease, or with hemoglobinopathy. Animal studies suggest a possible link between fetal hypoxia and increased risk of cardiovascular disease in offspring.5–13 Studies in rats have demonstrated that maternal hypoxia results in an increase in cardiac vulnerability to ischemia and reperfusion injury in male offspring.10,14,15 In addition, it has been demonstrated that downregulation of protein kinase C epsilon (PKCε) protein expression in the hearts of adult offspring is a mechanism for the increased heart susceptibility to ischemia and reperfusion injury in the animals exposed to hypoxia before birth.14 Among other mechanisms, PKCε plays a pivotal role of cardioprotection in heart ischemia and reperfusion injury.16–18 The study in a PKCε knock-out mouse model has demonstrated that PKCε expression is not required for cardiac function under normal physiological conditions, but PKCε activation is necessary for acute cardioprotection during...
cardiac ischemia and reperfusion.19 The finding that fetal hypoxia resulted in a decrease in PKCε protein expression in the heart of adult offspring10,14 suggests that an epigenetic mechanism may explain PKCε gene repression in the heart. Epigenetic mechanisms are essential for development and differentiation and allow an organism to respond to the environment through changes in gene expression patterns.20–22 DNA methylation is a chief mechanism for epigenetic modification of gene expression patterns and occurs at cytosines in the cytosine-phosphate-guanine (CpG) dinucleotide sequence.23 Methylation in promoter regions is generally associated with repression of transcription, leading to a long-term shutdown of the associated gene. Methylation of CpG islands in gene promoter regions alters chromatin structure and transcription. Similarly, methylation of CpG dinucleotides within transcription factor binding sites generally represses transcription.20,24,25 The present study tested the hypothesis that fetal hypoxia-induced methylation of CpG dinucleotides at the PKCε promoter contributes to PKCε gene repression in the heart of adult offspring. Herein, we present evidence that hypoxia has a direct effect on PKCε expression in the fetal heart and demonstrate in a cell model that hypoxia causes an increase in methylation of CpG dinucleotides at the proximal promoter region of PKCε gene, resulting in decreased binding of sequence-specific transcription factors to the promoter and reduced PKCε gene expression.

Methods
An expanded Methods section is available in the online Data Supplement at http://circres.ahajournals.org.

Experimental Animals
Pregnant rats were randomly divided into 2 groups: (1) normoxic control and (2) hypoxic treatment of 10.5% O2 from days 15 to 21 of gestation.14 Hearts were isolated from near-term (21-day) fetuses and 3-month-old offspring. To study the direct effect of hypoxia on the fetal heart, hearts were isolated from day 17 fetal rats and cultured at 37°C in 95% air/5% CO2 and 1% O2 for 48 hours. Postischemic recovery of left ventricular function and lactate dehydrogenase release were determined.25 Treatment of H9c2 cells with 1% O2 for 24 hours resulted in a decrease in PKCε expression in fetal rat hearts (Figure 1A). Similar findings were obtained in isolated fetal hearts treated ex vivo with 1% O2 (Figure 1A), demonstrating a direct effect of hypoxia on PKCε expression in the heart. Further study demonstrated in rat embryonic ventricular myocyte cell line H9c2 that 1% O2 but not 3% or 10.5% O2 significantly decreased PKCε mRNA and protein levels as compared with 21% O2 (Figure 1B).

Methylation of SP1 Binding Sites at the PKC Promoter
Eight putative transcription binding sites containing CpG dinucleotides at rat PKCε promoter were previously identified.24 Treatment of H9c2 cells with 1% O2 for 24 hours resulted in a significant increase in methylation of SP1 binding sites at −346 and −268 but decreased methylation of metal regulatory transcription factor 1 (MTF1) binding site at −539.
Hypoxia did not change significantly the methylation status of class B basic helix-loop-helix protein 2, peroxisome proliferator-activated receptor-γ, E2F transcription factor (E2F), early growth response protein 1 (Egr1), and MTF1 at −603. Consistent with the findings in H9c2 cells, maternal hypoxic treatment revealed a similar pattern of increased methylation of the 2 SP1 binding sites, −346 and −268, at the PKCε promoter in the fetal hearts (Figure 2). In addition, in vivo hypoxia resulted in an increase in methylation of the putative Egr1 binding site in the fetal heart (Figure 2).

**Methylation Inhibits SP1 Binding**

H9c2 cells were used to further determine the role of methylation in PKCε downregulation. Given the previous finding that deletions of the regions containing the putative Egr1 site and MTF1 site at −168 had no significant effects on the PKCε promoter activity,25 our further investigation focused on the 2 SP1 binding sites. SP1 binding and the functional significance of the SP1 binding sites in the regulation of PKCε gene activity was demonstrated previously.25 To determine if methylation of the SP1 sites inhibits SP1 binding from nuclear extracts of H9c2 cells, EMSA was performed with methylated and unmethylated oligonucleotide probes containing the SP1 sites at −346 and −268. As shown in Figure 3,
nuclear extracts from H9c2 cells bound and shifted the double-stranded unmethylated SP1 oligonucleotides at both sites but failed to cause a shift of the methylated SP1 oligonucleotides. This is consistent with the previous findings in fetal rat hearts showing a loss of binding of the nuclear extracts to the methylated SP1 oligonucleotides.24

Effect of Hypoxia on SP1 Abundance and Binding Affinity

Western blots revealed that hypoxia caused a significant increase in nuclear SP1 abundance in H9c2 cells (Online Figure I). In contrast, there was no significant difference in SP1 abundance either in fetal hearts between control and maternal hypoxic treatments or in the hearts of both male and female offspring between the control and prenatally hypoxic animals (Online Figure I). The binding affinity of SP1 to the unmethylated SP1 binding sites was determined in competition studies performed in pooled nuclear extracts with the increasing ratio of unlabeled/labeled oligonucleotides encompassing the SP1 sites at \(-346\) and \(-268\), respectively. Hypoxia had no significant effect on the binding of nuclear extracts to either SP1 sites at \(-346\) or \(-268\) in H9c2 cells, fetal hearts, or the hearts of both male and female offspring (Online Figure II).

Inhibition of DNA Methylation Restored PKCe Expression

To determine the causal role of CpG dinucleotide methylation in the downregulation of PKCe expression, we exposed H9c2 cells to 1% O2 in the absence or presence of increasing concentrations of the DNA methylation inhibitor 5-aza-2'-deoxycytidine. As shown in Figure 4, 5-aza-2'-deoxycytidine produced a concentration-dependent inhibition of hypoxia-induced decrease in PKCe mRNA and 30 \(\mu\)mol/L 5-aza-2'-deoxycytidine restored the mRNA to normoxic levels. This was accompanied by restoration of PKCe protein (Figure 4). The inhibition of hypoxia-induced methylation of the 2 SP1 binding sites by 5-aza-2'-deoxycytidine was demonstrated (Figure 5A). To confirm that hypoxia-induced methylation alters SP1 binding to the PKCe promoter in the context of intact chromatin, ChIP assays were performed using a SP1 antibody. Figure 5B shows that hypoxia caused significant decreases in the SP1 binding to both SP1 sites at \(-346\) and \(-268\), respectively, in H9c2 cells. However, in the presence of 5-aza-2-deoxycytidine, there were no significant differences in SP1 binding to either sites between hypoxic and normoxic samples (Figure 5B).

Sex Differences in Hypoxia-Induced Changes in Methylation and PKCe Expression

We further investigated the potential sex differences in hypoxia-induced methylation of the SP1 binding sites and PKCe transcription. Maternal hypoxia caused a minimal but significant increase in methylation at both SP1 binding sites in the hearts of female fetuses but induced significantly greater methylation in male fetal hearts (Figure 6A). PKCe mRNA was significantly decreased in the hearts of male but not female fetuses (Figure 6B). The similar pattern of sex differences in the hypoxia-induced SP1 methylation and PKCe transcription was demonstrated in the hearts of male and female offspring (Figure 6A and 6B). The sex difference observed in the fetal hearts was intriguing given that male and female fetuses were probably exposed to the similar concen-
trations of steroid hormones in utero. However, both ERα/H9251 and ERβ/H9252 abundance was significantly higher in the hearts of female, as compared with male fetuses (Figure 7A). Chromatin immunoprecipitation assay demonstrated the PCR products of the two SP1 binding sites in the DNA sequences pulled down by both ERα/H9251 and ERβ/H9252 antibodies in the fetal hearts (Figure 7B), suggesting an interaction between estrogen receptors and the SP1 binding sites at the PKCε promoter.

PKCε Activation Restored Hypoxia-Induced Cardiac Vulnerability to Ischemic Injury

The cause-and-effect evidence of the functional importance of PKCε in hypoxia-mediated, sex-dependent programming of increased heart vulnerability to ischemia and reperfusion injury in adult male offspring has been previously demonstrated by selective inhibition of PKCε with a PKCε translocation inhibitor peptide.14 To further demonstrate that decreased PKCε is an important factor in the hypoxia-induced increase in cardiac ischemic susceptibility in male offspring, additional studies were performed in the hearts in a Langendorff preparation using a selective PKCε activator peptide ψ-εRACK obtained from KAI Pharmaceuticals.16,27–29 There were no significant differences in left ventricular developed pressure, heart rate, dP/dt max, and coronary flow rate at the baseline among all groups (Online Table I). In the absence of ψ-εRACK, fetal hypoxia caused a significant decrease in postischemic recovery of left ventricular function, as shown in the previous studies,27,29 and abolished the hypoxic effects (Figure 8 and Online Figure III).

Discussion

The present study demonstrates that hypoxia has a direct effect on fetal cardiomyocytes causing a reduction in PKCε protein and mRNA. This is correlated with increased methylation of CpG dinucleotides in the 2 SP1 binding sites at the proximal promoter region of PKCε gene, resulting in decreased binding of SP1 to the promoter. The causal effect of methylation in the hypoxia-induced PKCε gene repression is demonstrated through the use of a DNA methylation inhibitor, which blocks the methylation and restores PKCε mRNA and protein expression to normal.
Previous studies demonstrated the long-term adverse effect of maternal hypoxia on cardiac vulnerability to ischemia and reperfusion injury in adult 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 \( \psi \)-rRACK (0.5 \( \mu \)mol/L) for 10 minutes before subjecting to 20 minutes of ischemia and 30 minutes of reperfusion in a Langendorff preparation. Postischemic recovery of left ventricular developed pressure (LVDP) and end-diastolic pressure (LVEDP) were determined. Lactate dehydrogenase (LDH) release over 30 minutes of reperfusion was measured as area under the curve (AUC). Data are means±SEM. *\( P<0.05 \), hypoxia vs control; †\( P<0.05 \), \( \psi \)-rRACK vs \( -\psi \)-rRACK, \( n=5 \).

Figure 8. Effect of PKCε activation on cardiac ischemia and reperfusion injury. Hearts were isolated from 3-month-old male offspring that had been exposed to normoxia (control) or hypoxia during ischemia and reperfusion injury. Previous studies demonstrated that chronic hypoxia during gestation downregulated PKCε expression in the developing heart through an epigenetic modification. This suggests differential regulations of acute hypoxia and chronic hypoxia on PKCε activity and gene expression in the heart. The causal role of reduced PKCε in sex-dependent programming of increased heart vulnerability to ischemia and reperfusion injury in adult male offspring has been demonstrated by selective inhibition of PKCε with a PKCε translocation inhibitor peptide. The present study demonstrated that a selective PKCε activator peptide restored the hypoxia-induced cardiac vulnerability to ischemic injury, providing further evidence of cause-and-effect role of decreased PKCε in the hypoxia-mediated, sex-dependent programming of increased heart ischemic vulnerability in male offspring. This is consistent with other studies showing that PKCε expression is necessary for acute cardioprotection during cardiac ischemia and reperfusion. The present finding of decreased PKCε protein and mRNA in fetal hearts caused by maternal hypoxia suggests that the reduction in PKCε expression observed in adult rats originates in utero. Previous studies in the same animal model demonstrated that maternal hypoxia increased hypoxia-inducible factor 1α (HIF-1α) protein in fetal hearts, suggesting tissue hypoxia in the fetal heart. The similar results of isolated hearts treated ex vivo with hypoxia indicate that hypoxia is necessary and sufficient to affect the PKCε expression in fetal hearts. Whether this decreased PKCε expression in the heart is a protective mechanism that increases fetal survival is not clear at present. However, the finding that the downregulation of PKCε resulted in increased heart ischemic vulnerability in offspring would suggest that it is a maladaptive response.

The present study used the embryonic rat ventricular myocyte cell line H9c2 to investigate the underlying mechanisms. H9c2 cell line has been widely used in a variety of myocardiocyte studies, including those investigating apoptosis, differentiation and ischemia, and reperfusion injury. H9c2 cells retain many electrophysical properties found in freshly isolated cardiomyocytes, albeit they do not spontaneously contract and are capable of continued growth. The present study used the embryonic rat ventricular myocyte cell line H9c2 to investigate the underlying mechanisms. H9c2 cell line has been widely used in a variety of myocardiocyte studies, including those investigating apoptosis, differentiation and ischemia, and reperfusion injury. H9c2 cells retain many electrophysical properties found in freshly isolated cardiomyocytes, albeit they do not spontaneously contract and are capable of continued growth.
insufficiency in cardiomyocytes. This is in agreement with many previous studies in which hypoxic effects were investigated in H9c2 cells under 1% O₂.37,38,43,44

The finding of similar pattern of increased methylation of the 2 SP1 binding sites at the PKCe promoter between H9c2 cells and the fetal hearts of maternal hypoxic treatment provides further evidence supporting the model of H9c2 cells in the present study. Whereas the differences in methylation patterns in the putative Egr1 and MTF1 (−168) binding sites observed in fetal hearts and H9c2 cells are intriguing, the functional significance of this finding is not clear given that deletions of the regions containing the Egr1 and MTF1 (−168) sites had no significant effects on the PKCe promoter activity.25 In contrast, deletion of the confirmed binding sites for both SP1 (−346) and SP1 (−268) significantly decreased the PKCe promoter activity in H9c2 cells.25 In the present study, we demonstrated by EMSA that methylation of CpG dinucleotides at the core of both SP1 binding sites abolished SP1 binding in H9c2 cells. Previous studies with site-directed methylation of PKCe promoter-luciferase constructs selectively at SP1 binding sites at −268 and −346 demonstrated that mutation of C/G at either SP1 site −268 or −346 alone had no significant effect on the promoter activity, but mutation of C/G at both the SP1 binding sites significantly reduced the promoter activity in H9c2 cells.24 Although increases in nuclear SP1 protein abundance in hypoxic H9c2 cells may serve as a compensatory mechanism, this increase is relatively ineffective because increased methylation of SP1 binding sites caused an even more significant decrease in SP1 binding to the PKCe promoter in the intact chromatin as demonstrated by ChIP assays. The finding that SP1 abundance was not significantly affected by hypoxia in fetal hearts, as well as in the hearts of either male or female adult offspring, together with the finding that hypoxia had no significant effect on SP1 binding affinity in H9c2 cells, fetal and adult hearts, reinforces a primary role of methylation in programming of PKCe gene repression.

The causal effect of increased methylation in hypoxia-induced PKCe gene repression was further demonstrated with a DNA methylation inhibitor 5-aza-2'-deoxycytidine in the present study. 5-Aza-2'-deoxycytidine binds to DNA methyltransferase 1, causing its depletion and preventing DNA methylation,45 and has been widely used to inhibit DNA methylation.46-49 In the present study, we demonstrated that 5-aza-2'-deoxycytidine concentration-dependently inhibited hypoxia-induced downregulation of PKCe mRNA expression. This is caused by inhibition of the hypoxia-induced methylation of both SP1 binding sites resulting in recovered SP1 binding to the PKCe promoter in the intact chromatin. Whereas the study of 5-aza-2'-deoxycytidine in H9c2 cells is limited, relatively high concentrations of 5-aza-cytidine (a close derivative of 5-aza-2-deoxycytidine) have been shown to rescue luciferase activity in transfected H9c2 cells in excess of 250 μmol/L,50 which may reflect cell-type specific response to 5-aza-2-deoxycytidine and derivatives. The ability of 5-aza-2-deoxycytidine to restore PKCe mRNA and protein expression in cardiomyocytes in the presence of a stressor is consistent with earlier studies that showed that 5-aza-2-deoxycytidine blocked cocaine-mediated repression of PKCe expression in fetal rat hearts.24

Previous studies demonstrated that prenatal hypoxia caused an increase in heart susceptibility to ischemia and reperfusion injury in a sex-dependent manner, which was due to fetal programming of PKCe gene repression resulting in downregulation of PKCe function in the heart of adult male offspring.14 In the present study, no significant differences were found in SP1 methylation and PKCe mRNA abundance between male and female control groups in both fetuses and offspring. This is consistent with the previous findings of no significant difference in PKCe protein abundance in the heart between control males and females.14,51 However, the sex differences in hypoxia-induced changes in methylation of the SP1 binding sites and PKCe transcription were demonstrated in both fetal and offspring hearts. The finding that the hypoxia-induced methylation was significantly greater in the hearts of male fetuses is intriguing given that male and female fetuses are likely exposed to the similar concentrations of steroid hormones in utero. Whereas the mechanisms are not clear at present, the sex difference observed may be caused in part by the greater expression of ERα and ERβ in the hearts of female fetuses. The finding that both ERα and ERβ interacted with the SP1 binding sites at the PKCe promoter in intact chromatin in the fetal heart suggests a possible mechanism for the increased protection of SP1 binding sites and PKCe transcription in the female hearts in response to hypoxic stress. It has been demonstrated that both ERα and ERβ can activate transcription of the retinoic acid receptor α1 gene by the formation of an ER-SP1 complex on the SP1 sites in the retinoic acid receptor α1 promoter.52 It is not clear at present whether the presence of phytoestrogens in the diet of soy-based chows used in the present study might contribute to the sex difference, though the pregnancy is a stage of high estrogen concentrations. This remains as an intriguing area for the future investigation using a casein-based diet. The similar pattern of sex differences in the hypoxia-induced SP1 methylation and PKCe transcription demonstrated in the hearts of male and female offspring supports the notion that fetal hypoxia-induced methylation of CpG dinucleotides at the PKCe promoter contributes to PKCe gene repression in the heart of adult offspring.

The present investigation provides evidence of a novel mechanism of methylation in non-CpG island, sequence-specific transcription factor binding sites in suble epigenetic modifications of gene expression pattern in fetal programming of cardiac function in response to adverse intratuterine environment. Although it may be difficult to translate the present findings directly into the humans, the possibility that fetal hypoxia may result in programming of a specific gene in the offspring with a consequence of increased cardiac vulnerability provides a mechanistic understanding worthy of investigation in humans. This is because hypoxia is one of the most important and clinically relevant stresses to the fetus and because large epidemiological studies indicate a link between in utero adverse stimuli during gestation and an increased risk of ischemic heart disease in the adulthood.
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Disclosures
None.

References
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Intrauterine stress is associated with an increased risk of ischemic heart disease in later life. Previous studies in rats showed that fetal hypoxia results in decreased PKCe expression and increased cardiac ischemic injury in male offspring. In this study, we found that hypoxia caused increased DNA methylation within the PKCe gene promoter, resulting in a repression of PKCe gene expression in the hearts of male fetal rats. This epigenetic modification pattern persisted into adulthood. We further demonstrated a causal role of decreased PKCe expression in increased heart ischemic vulnerability. Higher levels of estrogen receptors were found in the female fetal hearts, which may protect females from epigenetic modifications caused by intrauterine stress. This study is the first to show that hypoxia alters the epigenetic signature of a cardioprotective gene in utero, and that this modification can persist throughout life, leading to increased susceptibility to ischemia and reperfusion injury in the heart. Together, these findings provide a novel mechanism for hypoxia-induced programming of cardiovascular disease. Because hypoxia is a common stress to the fetus, this study may lead to further inquiries into the molecular adaptations in other tissues that promote pathophysiologic conditions in adults.
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SUPPLEMENT MATERIAL

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

by

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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 isolated 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 Cellytic 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 was 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.³ 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 i cycler Thermal cycler (Bio-Rad, Hercules, CA), as described previously.³ The primers for PKCε are 5′-GCGAAGCCCTAAGACAAT-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 6mCpG 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′-accatttctctctggctgc-3′ (forward) and 5′-gatttcctgccttgcaatgcct-3′ (reverse); 5′-agaggatcgcgggtgaga-3′ (forward) and 5′-ctctctcatctggagcaaa-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. 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 PKCε activator peptide ψ-εRACK (0.5 μM, KAE-1, KAI Pharmaceuticals) for 10 minutes before ischemia and throughout the period of ischemia and reperfusion, as previously described. 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 ± 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**
Online Table I. Pre-ischemic left ventricle functional parameters

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

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.
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. Date are mean ± SEM. * P < 0.05 vs. control (n = 5-8).
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 PKCε 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 PKCε activator ψ-εRACK (0.5 μM) 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\text{max} and dP/dt\text{min} were determined. Data are mean ± SEM. * P < 0.05, hypoxia vs. control; † P < 0.05, + ψ-εRACK vs. -ψ-εRACK. n = 5.