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

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Abstract

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 in rats.

Objectives—The present study tested the hypothesis that fetal hypoxia-induced methylation of CpG 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 day 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% O_2 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 (ERα) and β (ERβ) isoforms in females than in males. Both ERα and ERβ interacted with the SP1 binding sites in the fetal heart, which may explain the gender differences in SP1 methylation in the fetal heart. Additionally, selective activation of PKCε restored the hypoxia-induced cardiac vulnerability to ischemic injury in offspring.

Conclusion—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.

Keywords

Fetal heart; PKCε; hypoxia; epigenetics; DNA methylation

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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 down-regulation 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 offspring[10,14] 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 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.

Experimental animals

Pregnant rats were randomly divided into two groups: 1) normoxic control; and 2) hypoxic treatment of 10.5% O_2 from day 15 to 21 of gestation.[14] Hearts were isolated from near-term (21 day) fetuses and 3 months 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% CO_2 and 1% O_2 for 48 hours, as reported previously.[24]

Cell culture

H9c2 cells were grown and sub-cultured and experiments were performed at 70– 80% confluent. For hypoxic studies, cells were treated with 1%, 3%, or 10.5% O_2, respectively, for 24 hours.

Western blot analysis

Protein abundance of PKCε, specificity protein 1 (SP1), alpha (ERα) and beta (ERβ) subtypes of estrogen receptors in H9c2 cells and hearts of fetuses and adult offspring were measured with Western blot analysis, and were normalized to beta2-microglobulin (B2M), as described previously.[25, 26]

Real-time RT-PCR

PKCε mRNA abundance in H9c2 cells and hearts of fetuses and adult offspring was determined by real-time RT-PCR and was normalized to B2M.[25, 26]

Quantitative methylation-specific PCR

DNA collected from H9c2 cells and hearts of fetuses and adult offspring was treated with sodium bisulfite at 55 °C for 16 hours. 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 our previous sequencing of rat PKCε promoter.[25]

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were collected from H9c2 cells and hearts of fetuses and adult offspring. EMSA was performed using the oligonucleotide probes with either CpG or ^{m}CpG in the two SP1 binding sites (–346 and –268) at rat PKCε promoter region, as described previously.[25]

Chromatin immunoprecipitation (ChIP)

Chromatin extracts were prepared from H9c2 cells and fetal hearts. ChIP assays were performed for the two SP1 binding sites at the PKCε promoter in DNA sequences pulled-down by SP1, ERα, and ERβ antibodies.[25]

Hearts subjected to ischemia and reperfusion

Isolated hearts from 3 months old male offspring 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) in a Langendorff preparation, as previously described.[14, 27] Post-ischemic recovery of left ventricular function and lactate dehydrogenase (LDH) release were determined.[14]

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.

Results

Effect of hypoxia on PKC protein and mRNA in fetal cardiomyocytes

Maternal hypoxia resulted in significant decreases in both protein and mRNA abundance of PKCε in fetal rat hearts (Figure 1A). Similar findings were obtained in isolated fetal hearts treated *ex vivo* with 1% O_2 (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% O_2, but not 3% or 10.5% O_2, significantly decreased PKCε mRNA and protein levels as compared to 21% O_2 (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.[25] Treatment of H9c2 cells with 1% O_2 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 −168, as compared to 21% O_2 control (Figure 2). Hypoxia did not change significantly the methylation status of class B basic helix-loop-helix protein 2 (Stra13), peroxisome proliferator-activated receptor gamma (PPARG), 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 two 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 down-regulation. 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 two 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 PKCε expression

To determine the causal role of CpG dinucleotide methylation in the down-regulation of PKCε expression, we exposed H9c2 cells to 1% O_2 in the absence or presence of increasing concentrations of the DNA methylation inhibitor 5-aza-2'-deoxycytodine. As shown in Figure 4, 5-aza-2'-deoxycytodine produced a concentration-dependent inhibition of hypoxia-induced decrease in PKCε mRNA and 30 μM 5-aza-2'-deoxycytodine restored the mRNA to normoxic levels. This was accompanied by restoration of PKCε protein (Figure

4). The inhibition of hypoxia-induced methylation of the two SP1 binding sites by 5-aza-2'-deoxycytodine was demonstrated (Figure 5A). To confirm that hypoxia-induced methylation alters SP1 binding to the PKCε 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-deoxycytine, there were no significant differences in SP1 binding to the either sites between hypoxic and normoxic samples (Figure 5B).

Sex differences in hypoxia-induced changes in methylation and PKCε expression

We further investigated the potential sex differences in hypoxia-induced methylation of the SP1 binding sites and PKCε 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). PKCε 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 PKCε transcription was demonstrated in the hearts of male and female offspring (Figure 6A, B). The sex difference observed in the fetal hearts was intriguing given that male and female fetuses were likely exposed to the similar concentrations of steroid hormones *in utero*. However, both ERα and ERβ 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 and ERβ 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 ventricle developed pressure (LVDP), heart rate (HR), dP/dt_{max}, dP/dt_{min} 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 post-ischemic recovery of LVDP and increases in left ventricle end diastolic pressure (LVEDP) and lactate dehydrogenase (LDH) release (Figure 8), as well as decreases in the recovery of dP/dt_{max} and dP/dt_{min} (Online Figure III), as previously reported.[14] ψ-εRACK increased post-ischemic 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 two 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 in a sex-dependent manner.[10,14,15] Particularly, the decreased expression of cardioprotective genes, namely PKCε in hearts of adult male offspring are of significant interest.[14] Acute hypoxia and reactive oxygen species increase the activity of PKCε in the heart, which plays an important role in cardioprotection during ischemia and reperfusion injury.[30] The present study demonstrated that chronic hypoxia during gestation down-regulated 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.[14, 31] The present study demonstrated that a selective PKCε activator peptide[16, 27−29] 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.[19] 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[10,14] 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.[32] The similar results of isolated hearts treated *ex vivo* with hypoxia indicate that hypoxia is necessary and sufficient to impact 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 down-regulation 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[33] 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.[34−38] H9c2 cells retain many electrophysical properties found in freshly isolated cardiomyocytes,[33] albeit they do not spontaneously contract and are capable of continuous growth.[39] In the present study, the hypoxia-induced decrease in PKCε expression observed in fetal hearts *in vivo* and *ex vivo* mirrored that found in H9c2 cells, suggesting a congruent underlying mechanism for each model and providing a comparable model of H9c2 cells in the study of PKCε gene regulation. Similar to the present finding, it has been reported that PKCε protein expression is decreased in H9c2 cells exposed to 2 and 8 hours of hypoxia in the presence of low and high glucose.[40, 41]

Another question pertained to normal physiological PKCε expression in fetal heart cells. 21% O_2 (147 torr) is generally considered normal for adult cells. Because fetal partial oxygen pressures are closer to 20 torr,[42] 21% O_2 is hyperoxic for fetal cardiomyocytes. This may alter underlying gene expression patterns since normal oxygen levels in fetal hearts are significantly lower. Interestingly, we found that exposure to 21%, 10.5% and 3% O_2 had no significantly different effects on PKCε expression in H9c2 cells. While the potential effect of 'hyperoxia' on fetal cardiomyocytes remains to be further investigated, the finding that 1% O_2 down-regulated PKCε expression suggests modification in gene expression patterns as a mode of adaptation to oxygen insufficiency in cardiomyocytes. This is in agreement with many previous studies in which hypoxic effects were investigated in H9c2 cells under 1% O_2.[37,38,43,44]

The finding of similar pattern of increased methylation of the two SP1 binding sites at the PKCε 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 PKCε promoter activity.[25] In contrast, deletion of the confirmed binding sites for both SP1 (−346) and SP1 (−268) significantly decreased the PKCε 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 PKCε promoter-luciferase constructs selectively at SP1 binding sites at −268 and −346, demonstrated that mutation of C^mG at either SP1 site −268 or −346 alone had no significant effect on the promoter activity, but mutation of C^mG at the both 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 PKCε 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 PKCε gene repression.

The causal effect of increased methylation in hypoxia-induced PKCε gene repression was further demonstrated with a DNA methylation inhibitor 5-aza-2'-deoxycytodine in the present study. 5-Aza-2'-deoxycytodine 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'-deoxycytodine concentration-dependently inhibited hypoxia-induced down-regulation of PKCε mRNA expression. This is caused by inhibition of the hypoxia-induced methylation of both SP1 binding sites resulting in recovered SP1 binding to the PKCε promoter in the intact chromatin. Whereas the study of 5-aza-2'-deoxycytodine 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 μM,[50] which may reflect cell-type specific response to 5-aza-2-deoxycytidine and derivatives. The ability of 5-aza-2-deoxycytidine to restore PKCε 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 PKCε 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 PKCε gene repression resulting in downregulation of PKCε function in the heart of adult male offspring.[14] In the present study, no significant differences were found in SP1 methylation and PKCε 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 PKCε 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 PKCε 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 PKCε promoter in intact chromatin in the fetal heart suggests a possible mechanism for the increased protection of SP1 binding sites and PKCε 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 α 1 (RAR-1) gene by the formation of an ER-SP1 complex on the SP1 sites in the RAR-1 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 gender 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 PKCε transcription demonstrated in the hearts of male and female offspring supports the notion that fetal hypoxia-induced methylation of CpG dinucleotides at the PKCε promoter contributes to PKCε 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 subtle epigenetic modifications of gene expression pattern in fetal programming of cardiac function in response to adverse intrauterine 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.

Novelty and Significance

What Is Known?

- Adverse intrauterine environment increases the risk of hypertension and ischemic heart disease in adulthood.

- Protein kinase C epsilon (PKCε) plays a pivotal role of cardioprotection against ischemia and reperfusion injury.

- Fetal hypoxia decreases PKCε gene expression in the heart and increases cardiac vulnerability to ischemic injury in adult male offspring in rats.

What New Information Does This Article Contribute?

- Fetal hypoxia causes an epigenetic modification of increased DNA methylation patterns within the PKCε gene promoter, resulting in a repression of PKCε gene expression in the heart of male rats.

- Decreased PKCε protein expression results in increased cardiac vulnerability to ischemic injury in male offspring.

- The sex-dependent difference in epigenetic modification of PKCε gene expression patterns occurs during fetal development, as a result of the different expression patterns of estrogen receptors in male and female fetal hearts.

Intrauterine stress is associated with an increasde risk of ischemic heart disease in later life. Previous studies in rats showed that fetal hypoxia results in decreased PKCε expression and increased cardiac ischemic injury in male offspring. In this study, we found that hypoxia caused increased DNA methylation within the PKCε gene promoter,

resulting in a repression of PKCε gene expression in the hearts of male fetal rats. This epigenetic modification pattern persisted into adulthood. We further demonstrated a causal role of decreased PKCε 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 pathophysiological conditions in adults.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Non-standard Abbreviations and Acronyms

PKCε	protein kinase C epsilon
CpG	cytosine-phosphate-guanine
ERα	β, estrogen receptor alpha, beta
SP1	specificity protein 1
MTF1	metal regulatory transcription factor 1
Stra13	class B basic helix-loop-helix protein 2
PPARG	peroxisome proliferator-activated receptor gamma
E2F	E2F transcription factor
Egr1	early growth response protein 1
EMSA	electrophoretic mobility shift assay
ChIP	chromatin immunoprecipitation
LVDP	left ventricle developed pressure
LVEDP	left ventricle end diastolic pressure
LDH	lactose dehydrogenase

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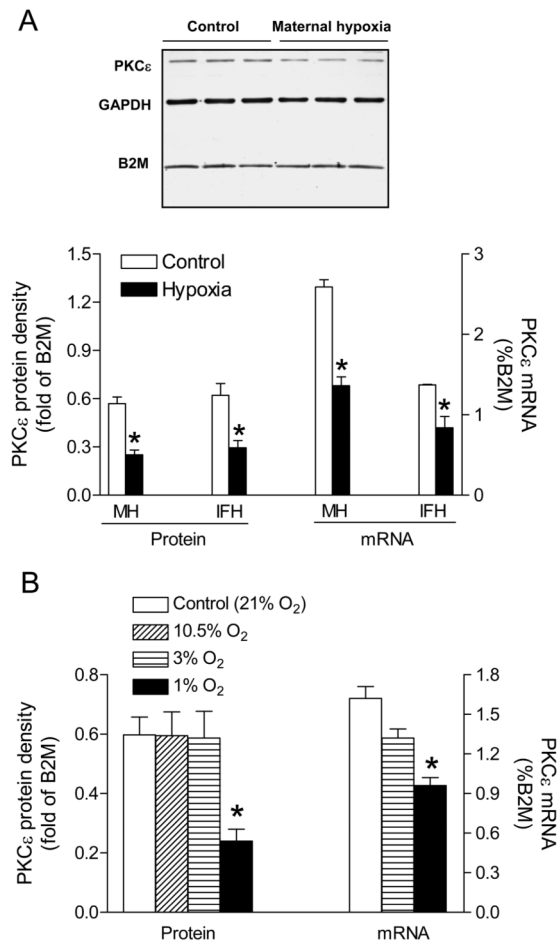
Figure 1. Effect of hypoxia on PKCε protein and mRNA

(**A**) Fetal hearts of maternal hypoxia (MH) and isolated fetal hearts (IFH) treated with 1% (hypoxia) and 21% O_2 (control) for 48 hours. (**B**) H9c2 cells treated with 1%, 3%, 10.5%, and 21% O_2 for 24 hours. Data are means ± SEM. * $P < 0.05$ *vs*. control (n = 5–15)
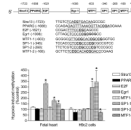
Figure 2. Effect of hypoxia on methylation of the PKCε promoter
Methylation patterns were determined in fetal hearts of maternal hypoxia and H9c2 cells treated with 1% O_2 *vs*. 21% O_2 for 24 hours. Data are means ± SEM. * P < 0.05 *vs*. control (n = 5–10)

SP1 (-346)

SP1 shift ➤

UM-oligo   M-oligo

SP1 (-268)

SP1 shift ➤
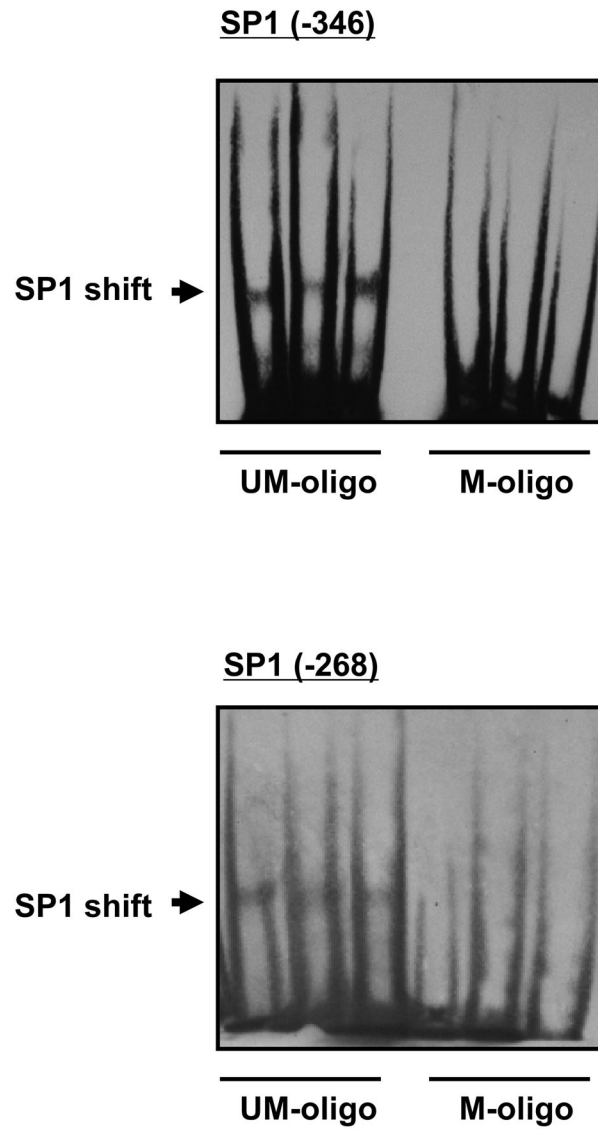
UM-oligo   M-oligo

Figure 3. Effect of methylation on SP1 binding
Nuclear extracts of H9c2 cells were incubated with unmethylated (UM-oligo) or methylated (M-oligo) oligonucleotides containing SP1 consensus sequence at sites −346 and −268.
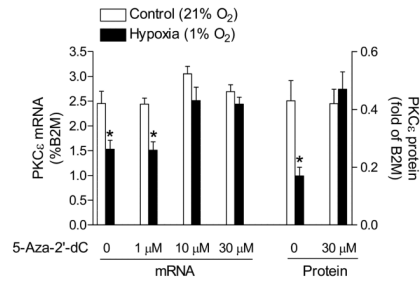
Figure 4. Effect of 5-aza-2'-deoxycytidine on PKCε mRNA and protein
H9c2 cells were treated with 1% O_2 and 21% O_2 for 24 hours in the absence or presence of increasing concentration of 5-aza-2'-deoxycytidine (5-Aza-2'-dC). Data are means ± SEM. * $P < 0.05$ *vs.* control (n = 4–17).
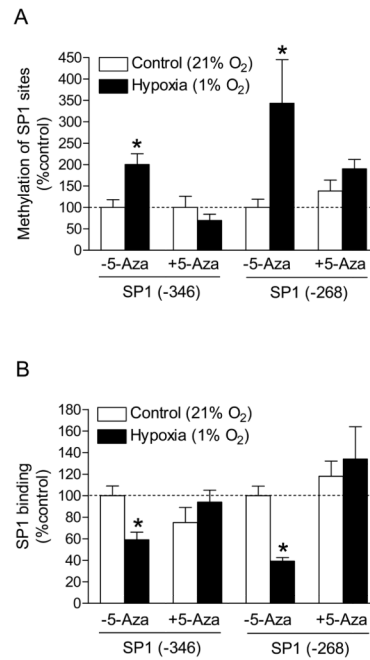
Figure 5. Effect of 5-aza-2'-deoxycytidine on methylation of SP1 sites and SP1 binding
H9c2 cells were treated with 1% O_2 and 21% O_2 for 24 hours in the absence or presence of 30 μM 5-aza-2'-deoxycytidine (5-Aza). (**A**) Methylation of SP1 binding sites was determined by methylation-specific PCR. (**B**) SP1 binding was determined by ChIP assays. Data are means ± SEM. * P < 0.05 *vs*. control (n = 4–9).
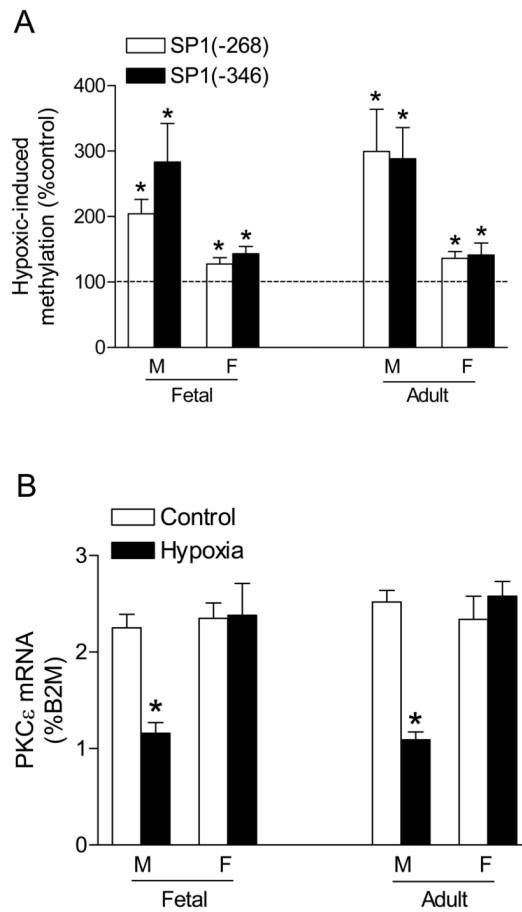
Figure 6. Effect of hypoxia on SP1 methylation and PKCε mRNA
Pregnant rats were treated with hypoxia, and hearts were isolated from near-term fetuses or
3 months old offspring. M, male; F, female. (**A**) Methylation of SP1 binding sites at the
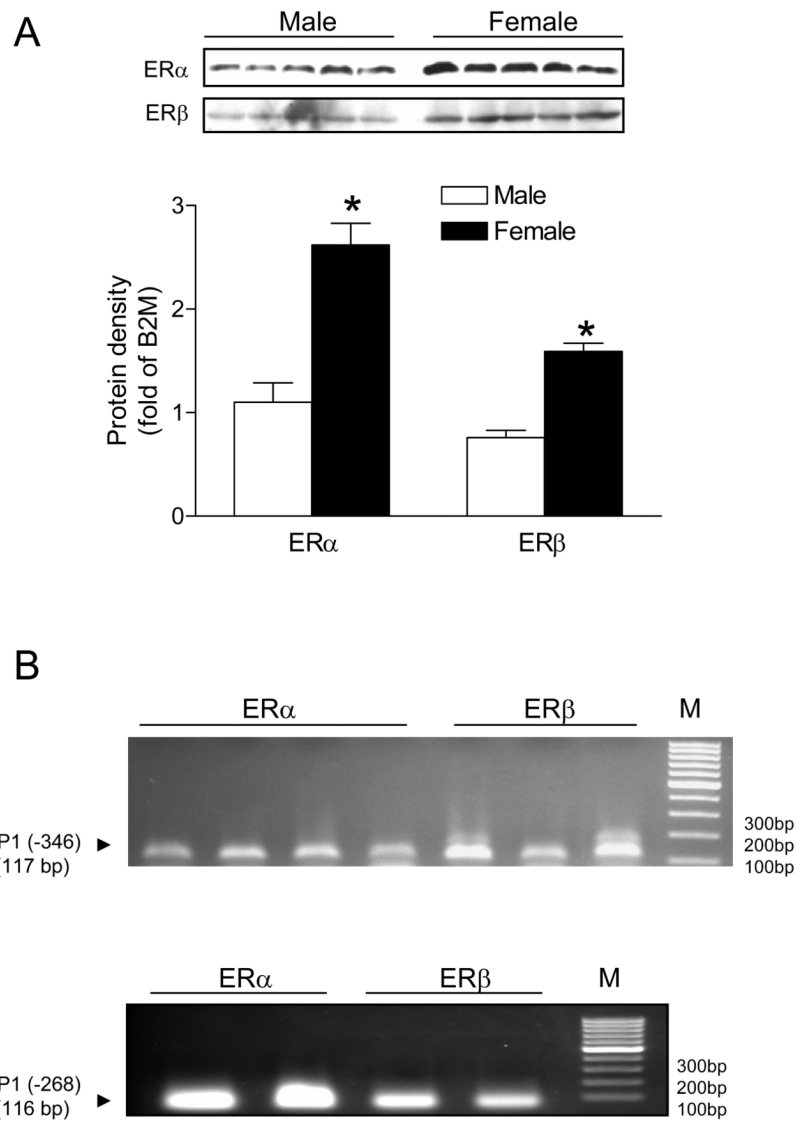PKCε promoter. (**B**) PKCε mRNA. Data are means ± SEM. * P < 0.05 *vs*. control (n = 5)

Figure 7. Estrogen receptors and SP1 binding sites in the fetal heart
Hearts were isolated from near-term fetuses. (**A**) Protein abundance of estrogen receptor alpha (ERα) and beta (ERβ) subtypes. Data are mean ± SEM. * $P < 0.05$ *vs*. male (n = 5). (**B**) PCR products of the SP1 binding sites (−346 and −268) following chromatin immunoprecipitation (ChIP) with ERα and ERβ antibodies, respectively, in the fetal heart. M, markers.
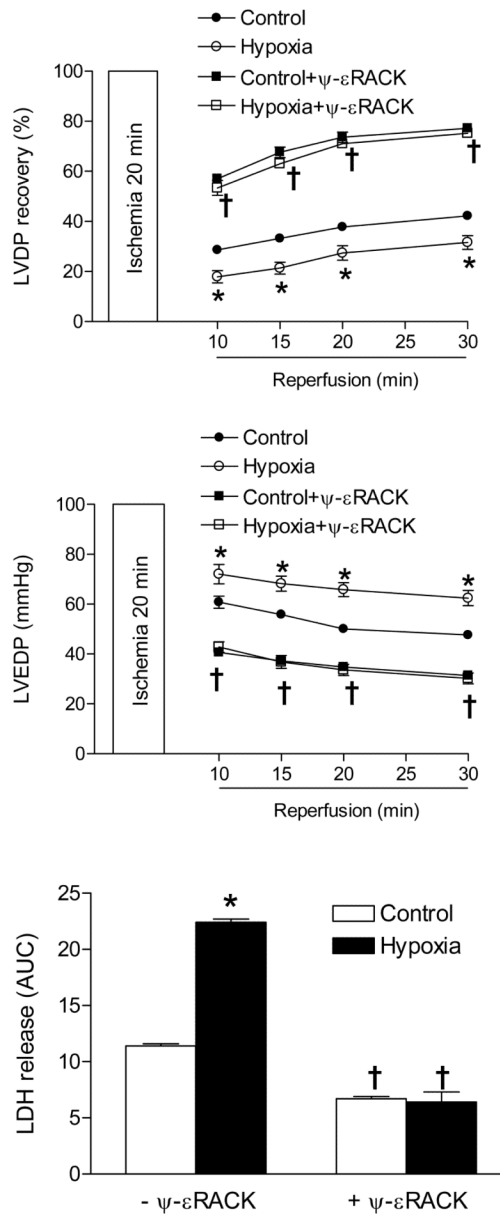
Figure 8. Effect of PKCε activation on cardiac ischemia and reperfusion injury
Hearts were isolated from 3-month-old male offspring that had been exposed to normoxia (control) or hypoxia before birth, and were treated in the absence or presence of the PKCε activator ψ-εRACK (0.5 μM) for 10 minutes before subjecting to 20 minutes of ischemia and 30 minutes of reperfusion in a Langendorff preparation. Post-ischemic recovery of 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 curve (AUC). Data are mean ± SEM. * $P < 0.05$, hypoxia vs. control; † $P < 0.05$, + ψ-εRACK vs. -ψ-εRACK. n = 5.