

Prenatal Hypoxia Causes a Sex-Dependent Increase in Heart Susceptibility to Ischemia and Reperfusion Injury in Adult Male Offspring: Role of Protein Kinase C ϵ

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Received March 6, 2009; accepted May 22, 2009

ABSTRACT

The present study tested the hypothesis that protein kinase C (PKC) ϵ plays a key role in the sex dichotomy of heart susceptibility to ischemia and reperfusion injury in adult offspring resulting from prenatal hypoxic exposure. Time-dated pregnant rats were divided between normoxic and hypoxic (10.5% O₂ on days 15–21 of gestation) groups. Hearts of 3-month-old progeny were subjected to ischemia and reperfusion (I/R) injury in a Langendorff preparation. Preischemic values of left ventricle (LV) function were the same between control and hypoxic animals. Prenatal hypoxia significantly decreased postischemic recovery of LV function and increased cardiac enzyme release and infarct size in adult male, but not female, rats. This was associated with significant decreases in PKC ϵ and phospho-PKC ϵ levels in the LV of the male, but not female, rats. The

PKC ϵ translocation inhibitor peptide (PKC ϵ -TIP) significantly decreased phospho-PKC ϵ in control male rats to the levels found in the hypoxic animals and abolished the difference in I/R injury observed between the control and hypoxic rats. In females, PKC ϵ -TIP inhibited PKC ϵ phosphorylation and decreased postischemic recovery of LV function equally well in both control and hypoxic animals. PKC ϵ -TIP had no effect on PKC δ activation in either male or female hearts. The results demonstrated that prenatal hypoxia caused an increase in heart susceptibility to ischemia and reperfusion injury in offspring in a sex-dependent manner, which was due to fetal programming of PKC ϵ gene repression resulting in a down-regulation of PKC ϵ function in the heart of adult male offspring.

Human epidemiological studies have shown a clear association of adverse intrauterine environment and an increased risk of ischemic heart disease in later adult life (Barker et al., 1989, 1993). Of all the stresses to which the fetus is subjected, perhaps the most important and clinically relevant is that of hypoxia. The fetus may experience prolonged hypoxic stress under many different conditions, including pregnancy at high altitude; pregnancy with anemia; placental insufficiency; cord compression; preeclampsia; and heart, lung, and kidney disease; or with hemoglobinopathy. There is clear evidence of a link between hypoxia and fetal intrauterine growth restriction. Human studies at altitude suggest that hypoxia per se, independent of maternal nutrition, causes fetal growth restriction, resulting in low birth weight and altered body shape at birth (Giussani et al., 2001). In addition,

chronic hypoxia suppresses fetal cardiac function, alters cardiac gene expression pattern, and increases heart-to-body weight ratio (Zhang, 2005).

Animal studies have suggested a possible link between prenatal hypoxia and increased risk of cardiovascular disease in offspring (Zhang, 2005). Studies in a pregnant rat model demonstrated that maternal hypoxia caused an increase in hypoxia-inducible factor-1 α expression and apoptosis in the fetal heart and resulted in a premature exit from the cell cycle of cardiomyocytes and myocyte hypertrophy (Bae et al., 2003). In addition, prenatal hypoxia resulted in an increase in heart susceptibility to ischemia and reperfusion injury in adult male offspring (Li et al., 2003). In animal models of intrauterine malnutrition, sex dimorphism in manifestation of the severity of cardiovascular dysfunction in adult offspring has been observed, although the results were conflicting (do Carmo Pinho Franco et al., 2003; McMillen and Robinson, 2005). Differential sex effects on cardiac programming were also demonstrated in rats. Prenatal cocaine treatment increased heart vulnerability to ischemia and

This work was supported in part by the National Institutes of Health [Grants HL83966, HL82779].

Article, publication date, and citation information can be found at <http://jpet.aspetjournals.org>.
doi:10.1124/jpet.109.153239.

ABBREVIATIONS: PKC, protein kinase C; LV, left ventricle; dP/dt_{max}, maximal rate of contraction; dP/dt_{min}, maximal rate of relaxation; LVEDP, left ventricle end diastolic pressure; TIP, translocation inhibitor peptide; LVDP, left ventricle developed pressure; HR, heart rate; LDH, lactate dehydrogenase; RACK, receptors for activated C-kinase.

reperfusion injury only in male adult offspring (Bae et al., 2005). In contrast, fetal nicotine exposure resulted in increased heart susceptibility to ischemia injury in both male and female offspring (Lawrence et al., 2008). These findings suggest a stimuli specificity of fetal programming of sex-dependent cardiac dysfunction in adult offspring. It is unknown whether and to what extent the sex dichotomy exists in manifestation of the severity of heart ischemic vulnerability in adult offspring resulting from prenatal hypoxic exposure.

In addition, the mechanisms whereby fetal hypoxia causes an increase in the vulnerability of ischemic injury in the heart of adult offspring are not clear. Among other mechanisms, protein kinase C (PKC) ϵ plays a pivotal role of cardioprotection during cardiac ischemia and reperfusion injury (Murriel and Mochly-Rosen, 2003). Here, we present evidence that prenatal hypoxia exposure causes an increase in heart susceptibility to ischemia and reperfusion injury in a sex-dependent manner, which is due to fetal programming of PKC ϵ gene repression resulting in a down-regulation of PKC ϵ function in the heart of adult male offspring.

Materials and Methods

Experimental Animals and Hypoxic Exposure. Time-dated pregnant Sprague-Dawley rats were purchased from Charles River Laboratories (Portage, MI) and were randomly divided into the normoxic control group and continuous hypoxic exposure group (10.5% oxygen) from day 15 to day 21 of gestation. Given that fetal rat heart is relatively resistant to oxygen deprivation in the first half of gestation and grows markedly along with the body mass in late gestation, the present study examined the effects of fetal hypoxia on the heart from gestation days 15 to 21, a period comparable with the third trimester of gestation in humans. Hypoxia was induced by a mixture of nitrogen gas and air as described previously (Li et al., 2003). Previous studies showed that an ambient oxygen level of 10.5% lowered maternal arterial oxygen tension to ~50 mm Hg (Rhee et al., 1997). The normoxic control group was housed identically with room air flowing through chambers. Water and food were provided as desired. All procedures and protocols used in the present study were approved by the Institutional Animal Care and Use Committee of Loma Linda University and followed the guidelines in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Hearts Subjected to Ischemia and Reperfusion. At 3 months of age, male and female progeny, raised in normoxic conditions after birth, were anesthetized by intramuscular injection of ketamine (75 mg/kg) and xylazine (5 mg/kg). Hearts were excised rapidly and retrogradely perfused via the aorta in a modified Langendorff apparatus under constant pressure (70 mm Hg) with gassed (95% O₂, 5% CO₂) Krebs-Henseleit buffer at 37°C, as described previously (Li et al., 2003; Bae and Zhang, 2005). A pressure transducer connected to a saline-filled balloon inserted into the left ventricle (LV) was used to assess ventricular function by measuring the ventricular pressure (mm Hg) and its first derivative (dp/dt). LV end diastolic pressure (LVEDP) was set at approximately 5 mm Hg. After baseline recording, hearts were perfused for 20 min in the absence or presence of 5 μ M PKC ϵ translocation inhibitor peptide (PKC ϵ -TIP; EAVSLKPT) (Calbiochem, San Diego, CA) or 5 μ M of a scrambled PKC ϵ -TIP (LSETKPAV) (Calbiochem) as a negative control, followed by subjecting to 20 min of global ischemia and 30 min of reperfusion, an approach used in many previous studies in a Langendorff preparation (Inagaki et al., 2003; Gray et al., 2004; Pierre et al., 2007). Previous studies with prolonged reperfusion from 60 to 180 min showed that myocardial infarction and left ventricular recovery reached a plateau at approximately 30 min of reperfusion (Li et al.,

2003, 2004; Bae and Zhang, 2005; Pierre et al., 2007). Whereas membrane permeability is likely to be low for the inhibitor peptide, several studies, both in vivo (Przyklenk et al., 2003) and in Langendorff preparations (Bae and Zhang, 2005; Pierre et al., 2007; Li et al., 2008; Meyer et al., 2009), have demonstrated that 5 μ M PKC ϵ -TIP (~1000-fold higher concentration than its intracellular effective dose) inhibits PKC ϵ -mediated cardioprotection in rat hearts. The specific effect of PKC ϵ -TIP on PKC ϵ activation has been demonstrated in rat hearts in a Langendorff preparation showing that 5 μ M PKC ϵ -TIP inhibits preconditioning-mediated PKC ϵ translocation in the heart (Pierre et al., 2007). The specific effect of PKC ϵ -TIP is further supported by the findings of the lack of effect with a scrambled PKC ϵ -TIP (Przyklenk et al., 2003). Taken together, these studies have demonstrated that PKC ϵ -TIP can cross cardiomyocyte plasma membrane in a Langendorff preparation despite its limited permeability and exert its effects on intracellular PKC ϵ . LV functional parameters, LV developed pressure (LVDP), heart rate (HR), dp/dt_{max}, dp/dt_{min}, and LVEDP were continuously recorded with an online computer. Pulmonary artery effluent was collected as an index of coronary flow.

Myocardial Infarct Size. Myocardial infarct size was measured as described previously (Bae and Zhang, 2005). In brief, at the end of reperfusion, left ventricles were collected, cut into four slices, incubated with 1% triphenyltetrazolium chloride solution for 15 min at 37°C, and immersed in formalin for 30 min. Each slice was then photographed (digital camera DC290; Eastman Kodak, Rochester, NY) separately, and the areas of myocardial infarction in each slice were analyzed by computerized planimetry (Image-Pro Plus; Media-Cybernetics, Inc., Bethesda, MD), corrected for the tissue weight, summed for each heart, and expressed as a percentage of the total left ventricle weight.

Lactate Dehydrogenase Activity Measurement. LDH activity was measured as described previously (Pierre et al., 2007). In brief, coronary effluent was collected for 30 s just before the onset of ischemia and at 0, 1, 2, 3, 4, 5, 10, 15, 20, and 30 min of reperfusion. LDH activity was measured using a standard assay (TOX 7 kit; Sigma-Aldrich, St. Louis, MO), following the manufacturer's directions, and activity is expressed as area under curve.

Western Blot Analysis. At the end of reperfusion, left ventricles were isolated, and protein levels of PKC ϵ , phospho-PKC ϵ , PKC δ , and phospho-PKC δ were determined by Western blot analysis. In brief, tissues 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 μ g/ml leupeptin, and 5 μ g/ml aprotinin, pH 7.4. Homogenates were then centrifuged at 4°C for 10 min at 10,000g, and supernatants were collected. Proteins were measured using a protein assay kit from Bio-Rad Laboratories (Hercules, CA). Samples with equal proteins were loaded on to 7.5% polyacrylamide gel with 0.1% SDS and were separated by electrophoresis at 100 V for 2 h. Proteins were then transferred to nitrocellulose membrane and incubated with primary antibodies for PKC ϵ , PKC δ (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), phospho-PKC ϵ , and phospho-PKC δ (Millipore, Billerica, MA), respectively. After washing, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). Proteins were visualized with enhanced chemiluminescence reagents, and blots were exposed to Hyperfilm (GE Healthcare). Results were quantified with the electrophoresis documentation and analysis system and Kodak ID image analysis software (Eastman Kodak). To minimize any confounding influence of variability among gels, an internal control was loaded in each gel and band intensities were normalized to actin and the internal control.

Statistical Analysis. Data were expressed as means \pm S.E.M. Experimental number (*n*) represents offspring from different dams. Statistical significance ($P < 0.05$) was determined by two-way analysis of variance followed by Newman-Keuls post hoc testing.

Results

Body Weight and Baseline Cardiac Function. Neither male nor female adult offspring showed significant difference in the body mass or heart weight between control and prenatally hypoxic groups (Table 1). Previous studies in the same rat model demonstrated that chronic maternal hypoxia resulted in a significant decrease in fetal body weight and birth weight but an increase in the heart-to-body weight ratio (Bae et al., 2003; Li et al., 2003; Williams et al., 2005). The present finding of no significant difference in body weight of adult offspring between control animals and those exposed to hypoxia before birth is in agreement with our previous findings (Li et al., 2003, 2004), but not with those of others who showed a decrease in body weight of adult offspring in the hypoxic-treated animals (Xu et al., 2006). In the latter study, the hypoxia treatment was interrupted daily, and the hypoxic rats were exposed to air with normal oxygen level for approximately an hour. Whether this daily short period of reoxygenation during the hypoxic treatment has an additional effect on growth postnatally remains to be determined. In addition, in the present study LVDP, HR, dP/dt_{max} , dP/dt_{min} , and coronary flow rate at the baseline were not significantly different among the groups in either male or female offspring (Table 1).

Postischemic Recovery of LV Function in Male Hearts. Global ischemia for 20 min caused a persistent impairment in LV function in all groups. As shown in Fig. 1, compared with the control group, there were significant decreases in postischemic recovery of LVDP, dP/dt_{max} , and dP/dt_{min} in the hypoxic group. Recovery of HR and coronary flow was not significantly different between the control and hypoxic groups (data not shown). Inhibition of PKC ϵ with PKC ϵ -TIP resulted in significant decreases in postischemic recovery of LVDP, dP/dt_{max} , and dP/dt_{min} in the heart of control animals (Fig. 1). In contrast, the scrambled PKC ϵ -TIP had no significant effects (Fig. 1). Unlike control animals, PKC ϵ -TIP had no effect on postischemic recovery of LV function in the heart of hypoxic animals (Fig. 1). In the presence of PKC ϵ -TIP, there was no difference in postischemic recovery of LV function between the control and hypoxic animals, which was the same as that in the heart of hypoxic animals in the absence of PKC ϵ -TIP (Fig. 1).

Postischemic Recovery of LV Function in Female Hearts. In contrast to the findings in male offspring, prenatal hypoxia showed no effect on postischemic recovery of

LVDP, dP/dt_{max} , and dP/dt_{min} in female offspring (Fig. 2). PKC ϵ -TIP significantly decreased postischemic recovery of LV function in the hearts of both control and hypoxic groups (Fig. 2). There was no difference in postischemic recovery of LV function between the control and hypoxic animals either in the absence or presence of PKC ϵ -TIP.

Myocardial Infarction and LDH Release. In male animals, ischemia- and reperfusion-induced increase in LVEDP was significantly higher in the hearts of hypoxic group compared with that in the control group (Fig. 3A). This was consistent with the significant increases in myocardial infarct size (Fig. 3B) and LDH release (Fig. 3C) in the hearts of hypoxic animals. PKC ϵ -TIP significantly increased LVEDP (Fig. 3A), myocardial infarct size (Fig. 3B), and LDH release (Fig. 3C) in the control group. In contrast, the scrambled PKC ϵ -TIP had no significant effects (Fig. 3). Unlike control animals, PKC ϵ -TIP had no effects on LVEDP, myocardial infarct size, and LDH release in the hypoxic group (Fig. 3). In the presence of PKC ϵ -TIP, there were no differences in LVEDP, myocardial infarct size, and LDH release between the control and hypoxic groups, which were the same as those found in the hearts of hypoxic group animals in the absence of PKC ϵ -TIP (Fig. 3). In contrast, in females there were no significant differences in ischemia and reperfusion-induced increase in LVEDP, myocardial infarct size, and LDH release between the control and hypoxic groups (Fig. 4). PKC ϵ -TIP increased LVEDP (Fig. 4A), myocardial infarct size (Fig. 4B), and LDH release (Fig. 4C) to the same extent in both the control and hypoxic groups.

Western Blot. In male animals, there was a significant decrease in PKC ϵ protein levels in the LV of hypoxic group animals compared with that in the control group (Fig. 5A). This was accompanied by a significant decrease in phospho-PKC ϵ in the hypoxic group (Fig. 5B). PKC ϵ -TIP had no effect on PKC ϵ levels in the LV in either control or hypoxic groups (Fig. 5A). It significantly decreased phospho-PKC ϵ in the LV in the control group (Fig. 5B). In contrast, it had no further effect on decreased phospho-PKC ϵ in the hypoxic group (Fig. 5B). In the presence of PKC ϵ -TIP, there was no significant difference in phospho-PKC ϵ levels between the control and hypoxic groups, which were the same as that found in the hypoxic group in the absence of PKC ϵ -TIP (Fig. 5B). Similar to PKC ϵ , prenatal hypoxia

TABLE 1
Body and heart weight and preischemic left ventricle functional parameters
n = 5 to 11 rats.

	BW	HW	HR	LVEDP	LVDP	dP/dt_{min}	dP/dt_{max}	CF
	<i>g</i>		<i>bpm</i>	<i>mm Hg</i>		<i>mm Hg/s</i>		<i>ml/min</i>
Male								
C	492 ± 11	1.3 ± 0.1	263 ± 6.9	5.2 ± 0.2	100.5 ± 2.8	3739 ± 126	2158 ± 78	12.3 ± 0.4
C + S-TIP	495 ± 13	1.3 ± 0.1	265 ± 8.2	5.1 ± 0.2	103.6 ± 2.4	3850 ± 58	2143 ± 41	12.6 ± 0.2
C + TIP	507 ± 14	1.3 ± 0.1	259 ± 5.4	5.5 ± 0.3	95.8 ± 1.6	3766 ± 98	2078 ± 57	12.8 ± 0.7
H	515 ± 9	1.3 ± 0.0	249 ± 5.2	5.3 ± 0.4	104.1 ± 4.7	3768 ± 143	2058 ± 21	12.4 ± 0.2
H + TIP	518 ± 6	1.3 ± 0.0	260 ± 5.6	5.7 ± 0.2	99.6 ± 2.5	3617 ± 156	1967 ± 66	13.2 ± 0.4
Female								
C	297 ± 8	0.9 ± 0.0	250 ± 6.2	5.5 ± 0.3	97.6 ± 4.4	2867 ± 72	1776 ± 71	8.9 ± 0.5
C + TIP	298 ± 7	0.8 ± 0.0	247 ± 2.4	5.4 ± 0.2	90.8 ± 2.7	2846 ± 157	1624 ± 68	8.8 ± 0.3
H	305 ± 2	0.9 ± 0.0	259 ± 10.4	5.6 ± 0.4	93.5 ± 3.0	2945 ± 139	1775 ± 109	9.3 ± 0.9
H + TIP	296 ± 3	0.8 ± 0.0	249 ± 4.2	5.5 ± 0.2	97.2 ± 5.9	3081 ± 93	1863 ± 136	8.9 ± 0.3

BW, body weight; C, control; H, hypoxia; S-TIP, scrambled PKC ϵ -TIP; TIP, PKC ϵ -TIP; HW, heart weight; CF, coronary flow.

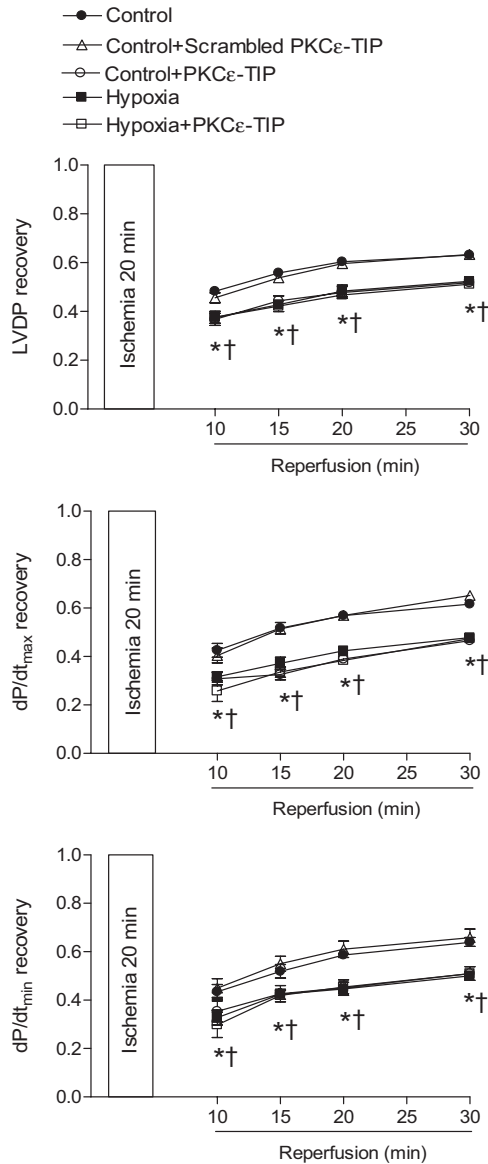


Fig. 1. Effect of prenatal hypoxia on postischemic recovery of left ventricle function in male offspring. Hearts were isolated from 3-month-old male offspring that were exposed to normoxia (control) or hypoxia before birth and that were pretreated in the absence or presence of 5 μ M PKC ϵ -TIP or scrambled PKC ϵ -TIP for 20 min before subjecting them to 20 min of ischemia and 30 min of reperfusion in a Langendorff preparation. Postischemic recovery of left ventricle function during reperfusion was measured relative to the preischemic values. Data are means \pm S.E.M. *, $P < 0.05$, +PKC ϵ -TIP versus -PKC ϵ -TIP; †, $P < 0.05$, hypoxia versus control. $n = 5$ to 11 rats.

also decreased PKC δ protein levels in the LV of hypoxic group compared with that in the control group (Fig. 5C). However, phospho-PKC δ was not significantly different between the control and hypoxic groups (Fig. 5D). Unlike its inhibitory effect on phospho-PKC ϵ in the control hearts, PKC ϵ -TIP did not affect phospho-PKC δ levels in either control or hypoxic groups (Fig. 5D). In females, there were no significant differences in PKC ϵ , phospho-PKC ϵ , PKC δ , and phospho-PKC δ levels in the LV between the control and hypoxic groups (Fig. 6). PKC ϵ -TIP decreased phospho-PKC ϵ in the LV to the same extent in both the control and hypoxic groups (Fig. 6B).

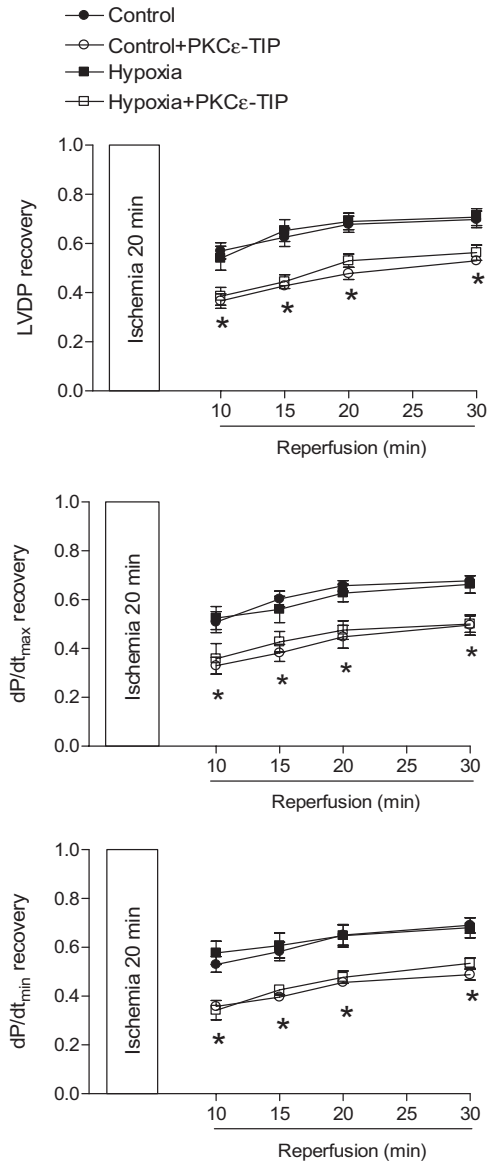


Fig. 2. Effect of prenatal hypoxia on postischemic recovery of left ventricle function in female offspring. Hearts were isolated from 3-month-old female offspring that were exposed to normoxia (control) or hypoxia before birth and that were pretreated in the absence or presence of 5 μ M PKC ϵ -TIP for 20 min before subjecting them to 20 min of ischemia and 30 min of reperfusion in a Langendorff preparation. Postischemic recovery of left ventricle function during reperfusion was measured relative to the preischemic values. Data are means \pm S.E.M. *, $P < 0.05$, +PKC ϵ -TIP versus -PKC ϵ -TIP. $n = 5$ to 6 rats.

Discussion

The present study clearly demonstrated a sex dichotomy in manifestation of increased cardiac vulnerability to ischemia and reperfusion injury in adult offspring resulting from fetal hypoxia. This is consistent with the previous finding that maternal cocaine administration during pregnancy increased heart susceptibility to ischemic injury only in male offspring (Bae et al., 2005). In contrast, prenatal nicotine exposure resulted in a significant decrease in postischemic recovery of left ventricular function in both male and female hearts, with the detrimental effects in female hearts being more pronounced (Lawrence et al., 2008). These findings suggest differential sex mechanisms of in utero cardiac programming

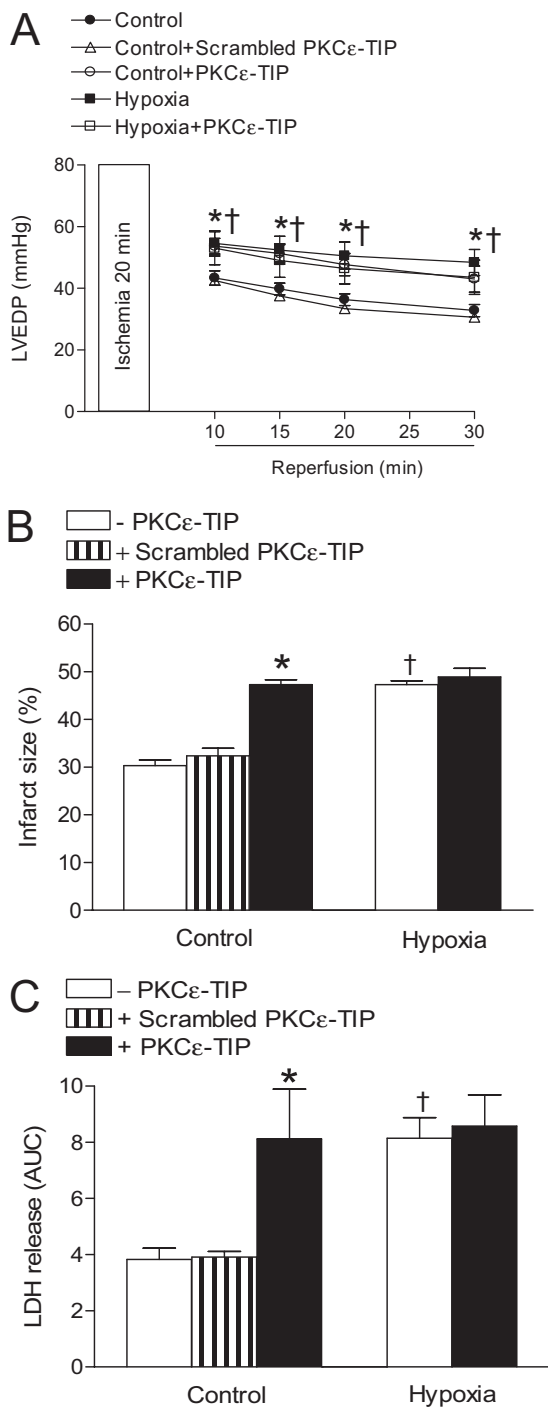


Fig. 3. Effect of prenatal hypoxia on ischemia and reperfusion injury of left ventricle in male offspring. Hearts were isolated from 3-month-old male offspring that were exposed to normoxia (control) or hypoxia before birth and that were pretreated in the absence or presence of 5 μ M PKC ϵ -TIP or scrambled PKC ϵ -TIP for 20 min before subjecting them to 20 min of ischemia and 30 min of reperfusion in a Langendorff preparation. A, LVEDP was measured during reperfusion. B, infarct size of the left ventricle was measured at the end of reperfusion. C, LDH release over 30 min of reperfusion was measured as area under curve. Data are means \pm S.E.M. *, $P < 0.05$, +PKC ϵ -TIP versus -PKC ϵ -TIP; †, $P < 0.05$, hypoxia versus control. $n = 5$ to 11 rats.

caused by adverse intrauterine environments. In addition, unlike its effect on the heart, fetal nicotine exposure significantly increased the vascular contractility in male but not female adult offspring (Xiao et al., 2007), suggesting further

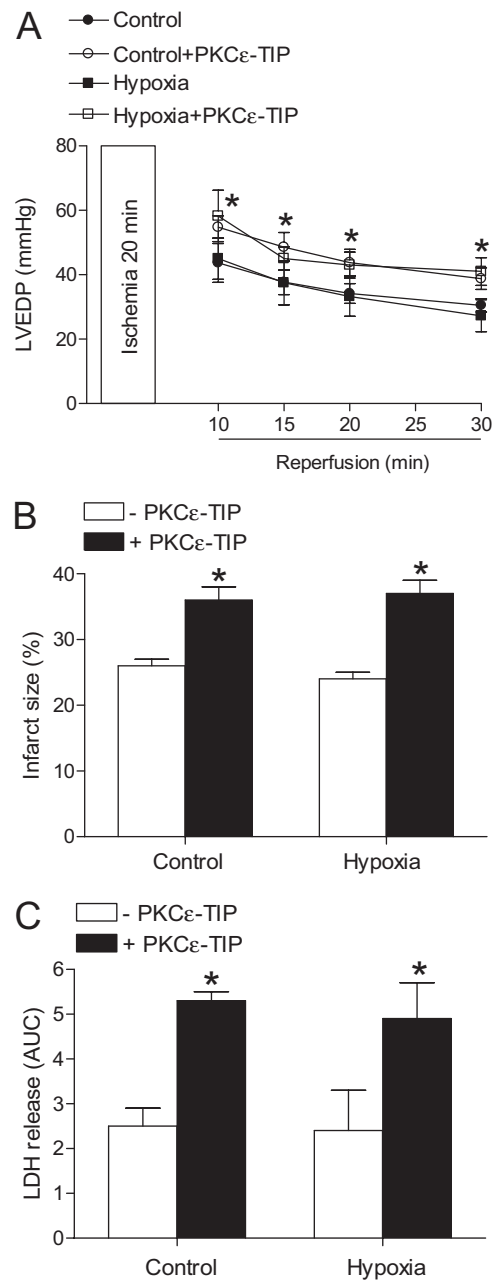


Fig. 4. Effect of prenatal hypoxia on ischemia and reperfusion injury of left ventricle in female offspring. Hearts were isolated from 3-month-old female offspring that were exposed to normoxia (control) or hypoxia before birth and that were pretreated in the absence or presence of 5 μ M PKC ϵ -TIP for 20 min before subjecting them to 20 min of ischemia and 30 min of reperfusion in a Langendorff preparation. A, LVEDP was measured during reperfusion. B, infarct size of the left ventricle was measured at the end of reperfusion. C, LDH release over 30 min of reperfusion was measured as area under curve. Data are means \pm S.E.M. *, $P < 0.05$, +PKC ϵ -TIP versus -PKC ϵ -TIP. $n = 5$ to 6 rats.

an organ specificity, tissue specificity, or both of sex-dependent programming induced by intrauterine insults. The sex dichotomy in fetal programming of adult disease has been well demonstrated in several animal models showing that female offspring are generally less sensitive in manifestation of cardiovascular disease caused by adverse prenatal stimuli (do Carmo Pinho Franco et al., 2003). It has been shown in animal models that female hearts have greater resistance to ischemia- and reperfusion-mediated injury in the Langen-

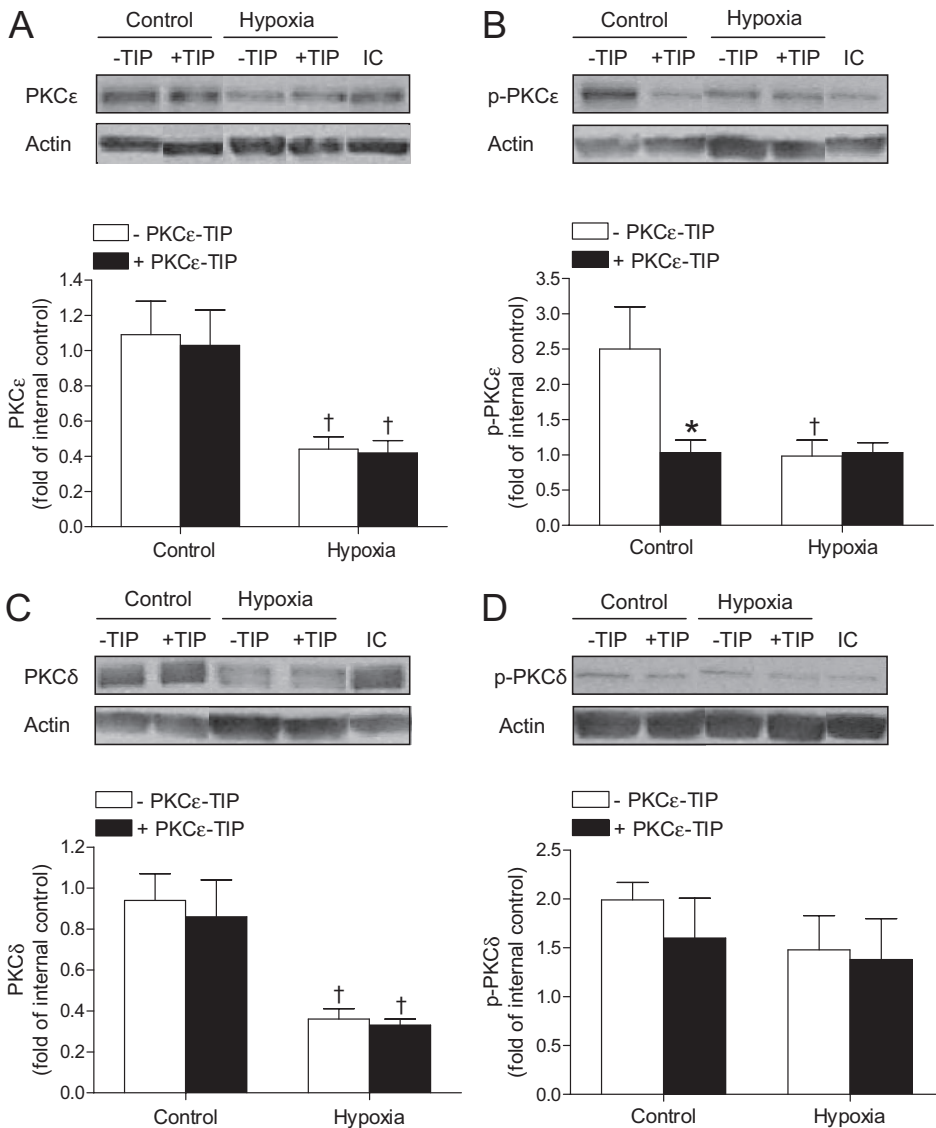


Fig. 5. Effect of prenatal hypoxia on PKCε and PKCδ protein abundance in left ventricle of male offspring. Hearts were isolated from 3-month-old male offspring that were exposed to normoxia (control) or hypoxia before birth and that were pretreated in the absence or presence of 5 μM PKCε-TIP (TIP) for 20 min before subjecting to 20 min of ischemia and 30 min of reperfusion in a Langendorff preparation. PKCε (A), phospho-PKCε (p-PKCε) (B), PKCδ (C), and phospho-PKCδ (p-PKCδ) (D) protein abundance in the left ventricle was determined with Western blot analyses and normalized to actin and an internal control (IC). Data are means ± S.E.M. *, $P < 0.05$, +PKCε-TIP versus -PKCε-TIP; †, $P < 0.05$, hypoxia versus control. $n = 5$ rats.

dorff preparation, with reduced myocardial infarct size (Bae and Zhang, 2005; Wang et al., 2005). In addition, cardiomyocytes from female hearts have been shown to be more resistant to ischemia and reperfusion injury (Ranki et al., 2001). Studies of ovariectomized rats and estrogen replacement have suggested that estrogen plays an important role in the cardioprotection of global ischemia and reperfusion injury in female hearts (Zhai et al., 2000). In addition, accumulating evidence suggests that, in addition to sex-defining steroids, differences exist between genetically male (XY) and female (XX) cells in determining an “ischemia-sensitive” phenotype (Hurn et al., 2005), which could be differentially programmed.

In the present study, the hypoxic-mediated decrease in PKCε and phospho-PKCε in the male heart was associated with an increase in heart vulnerability to ischemic injury. In contrast, the female heart showed a lack of change in PKCε and heart susceptibility to ischemia. Similar findings were obtained in a rat model of prenatal cocaine treatment (Bae et al., 2005). Unlike fetal hypoxia and cocaine treatments, maternal nicotine administration during pregnancy resulted in decreased PKCε protein expression in the heart of both male

and female offspring, which corresponded to the decreased postischemic recovery of left ventricular function in both male and female hearts (Lawrence et al., 2008). These findings demonstrate a stimuli specificity of sex-dependent programming of PKCε gene expression pattern in the heart and suggest a common mechanism of PKCε in cardiac programming in response to intrauterine adverse stimuli.

The role of PKCε in sex-dependent programming of heart vulnerability to ischemia and reperfusion injury in adult offspring was further demonstrated by selective inhibition of PKCε with PKCε-TIP. The activation of PKC isozymes is initiated by their translocation to the unique subcellular sites and binding to isozyme-specific anchoring proteins, receptors for activated C-kinase (RACKs). PKC isozyme-selective inhibitory peptides, containing isozyme-specific RACK-binding sites, have been demonstrated to inhibit translocation and phosphorylation of the corresponding PKC isozymes and consequently inhibit their isozyme-unique function (Dorn and Mochly-Rosen, 2002). PKCε-TIP selectively blocks binding of PKCε to its RACK at the intracellular concentration of 3 to 10 nM and has been widely used to study the role of PKCε in cardiac function (Zhou et al., 2002;

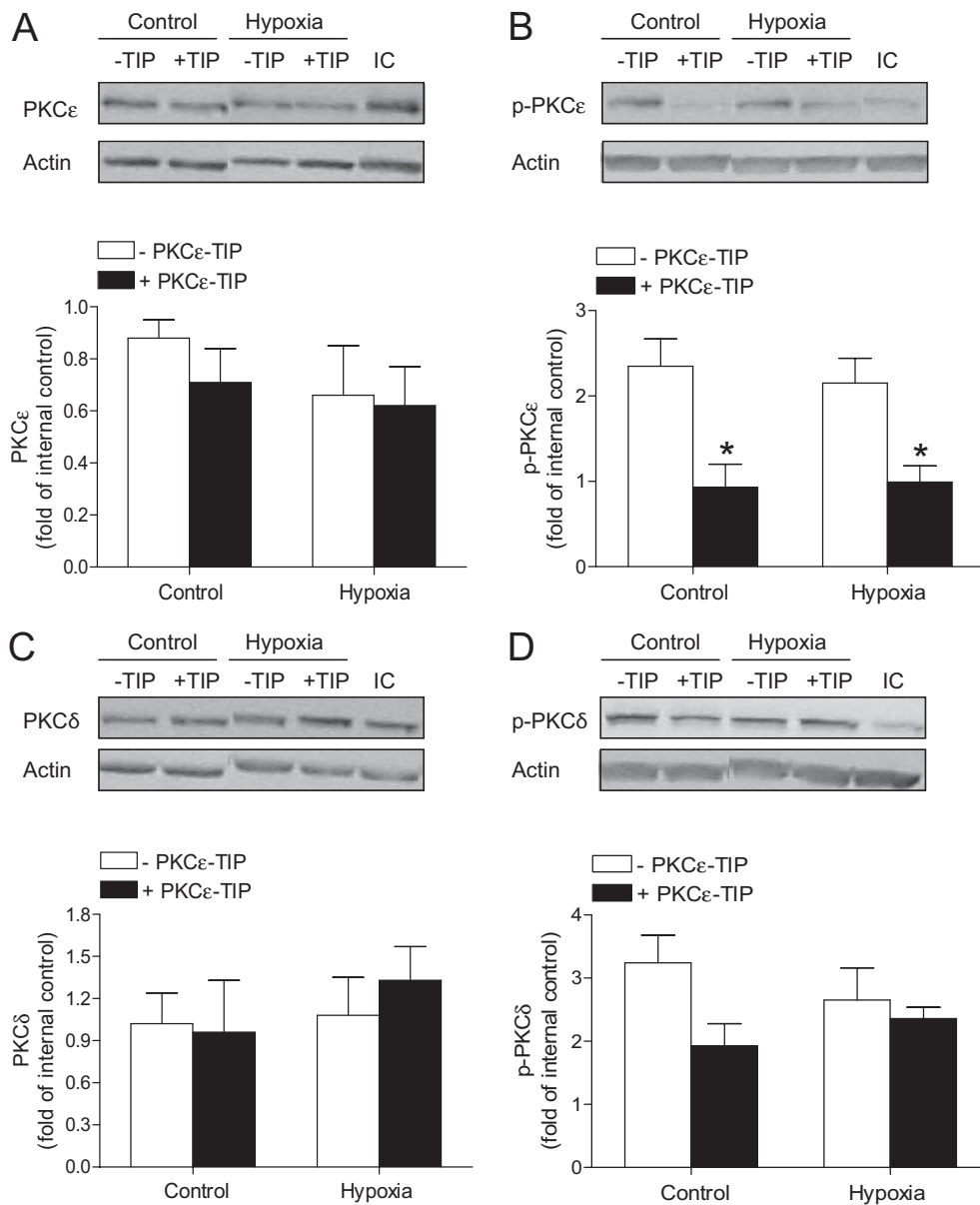


Fig. 6. Effect of prenatal hypoxia on PKC ϵ and PKC δ protein abundance in the left ventricle of female offspring. Hearts were isolated from 3-month-old female offspring that were exposed to normoxia (control) or hypoxia before birth and that were pretreated in the absence or presence of 5 μ M PKC ϵ -TIP (TIP) for 20 min before subjecting them to 20 min of ischemia and 30 min of reperfusion in a Langendorff preparation. PKC ϵ (A), phospho-PKC ϵ (p-PKC ϵ) (B), PKC δ (C), and phospho-PKC δ (p-PKC δ) (D) protein abundance in the left ventricle were determined with Western blot analyses and normalized to actin and an internal control (IC). Data are means \pm S.E.M. *, $P < 0.05$, +PKC ϵ -TIP versus -PKC ϵ -TIP. $n = 5$ rats.

Murriel and Mochly-Rosen, 2003; Przyklenk et al., 2003). In agreement with the present findings, a study in PKC ϵ knockout mouse model demonstrated that PKC ϵ expression was not required for normal cardiac function under physiological conditions, but PKC ϵ activation was necessary and sufficient for acute cardioprotection during cardiac ischemia and reperfusion (Gray et al., 2004). In the present study, we found that PKC ϵ -TIP significantly increased ischemic injury and decreased postischemic recovery of left ventricular function in control males, and in the presence of PKC ϵ -TIP there was no difference in heart susceptibility to ischemic and reperfusion injury between the control and hypoxic males. The selectivity of PKC ϵ -TIP was demonstrated by its inhibition of phospho-PKC ϵ but not phospho-PKC δ in the control heart. The lack of effect of PKC ϵ -TIP on ischemic injury of the heart in hypoxic males is consistent with its lack of effect on phospho-PKC ϵ that has already been inhibited in the heart of hypoxic group. In contrast to the males, PKC ϵ -TIP inhibited phospho-PKC ϵ and decreased postischemic recovery of left ventricular function to the same extent in both control and hypoxic groups in

females, with no difference in ischemic vulnerability of the heart in females between control and hypoxic groups. These findings provide the cause-and-effect evidence of the functional importance of PKC ϵ in the gender dichotomy of increased heart susceptibility to ischemic and reperfusion injury in offspring resulting from fetal hypoxia. This is in agreement with previous studies showing a key role of PKC ϵ in cardioprotection against ischemia and reperfusion injury (Dorn et al., 1999; Cross et al., 2002; Gray et al., 2004; Pierre et al., 2007).

The finding that prenatal hypoxia resulted in a decrease in PKC ϵ protein levels in the heart of male adult offspring suggests in utero epigenetic programming of PKC ϵ gene repression in the heart. The ratio of phospho-PKC ϵ /PKC ϵ was not significantly different between the control and hypoxic groups, suggesting that fetal hypoxia repressed PKC ϵ gene expression resulting in decreased phospho-PKC ϵ , rather than inhibited its activities per se. 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, 2003; Jaenisch and Bird, 2003). DNA methylation is a chief mechanism in epigenetic modification of gene expression pattern. Our recent study demonstrated an epigenetic mechanism of DNA methylation in programming of cardiac PKC ϵ gene repression, linking fetal cocaine exposure and pathophysiological consequences in the heart of adult male offspring in a gender-dependent manner (Zhang et al., 2009). In this study, eight transcription factor binding sites, Stra13 at -1723, PPARG at -1688, E2F at -1621, Egr-1 at -1008, MTF1 at -603, SP1 at -346, SP1 at -268, and MTF1 at -168, which contain CpG dinucleotides in their core binding sites, were identified at the promoter of PKC ϵ gene in the rat. Prenatal cocaine treatment caused an increase in CpG methylation at both SP1 binding sites of -346 and -268 resulting in the decreased SP1 binding to the PKC ϵ promoter and PKC ϵ gene repression in the heart of male offspring. In contrast in females, increased methylation was observed only at SP1 binding site of -268, which did not change PKC ϵ gene expression in the heart. Whether and to what extent fetal hypoxia induces a differential and sex-dependent pattern of DNA methylation in the PKC ϵ promoter remains an intriguing area for the future investigation.

Unlike PKC ϵ , the role of PKC δ in ischemia and reperfusion injury is less clear and is somewhat controversial. Inhibition of PKC δ during reperfusion has been shown to decrease reperfusion-induced injury (Murriel and Mochly-Rosen, 2003). Other studies demonstrated the cardioprotective effects of PKC δ (Kawamura et al., 1998; Zhao et al., 1998; Bouwman et al., 2006). It has been demonstrated that estrogen deficiency decreases ischemic tolerance in the aged rat heart through decreases in both PKC δ and PKC ϵ levels (Hunter et al., 2007). The present finding that PKC δ was significantly decreased in the heart of male but not female offspring that were exposed to hypoxia before birth suggests a possible mechanism of PKC δ in the sex dichotomy of increased heart susceptibility to ischemia and reperfusion injury in males. In agreement, a previous study demonstrated that prenatal nicotine exposure caused a significant decrease in PKC δ protein levels in the heart of female but not male offspring, which was associated with the increased heart vulnerability to ischemic injury in females compared with males (Lawrence et al., 2008).

Our investigation has demonstrated in a rat model that fetal hypoxia results in the increased heart susceptibility to ischemia and reperfusion injury in male offspring in a sex-dependent manner, which is caused by fetal programming of PKC ϵ gene repression resulting in a down-regulation of PKC ϵ expression in adult male hearts. Although a role of PKC δ is also suggested, its causal effect in sex-dependent programming of heart vulnerability to ischemic injury in offspring remains to be determined. Although it may be difficult to translate the present findings directly into the humans due to the paucity of epidemiological evidence in humans to link prenatal hypoxia per se and cardiovascular disease in later adult life, 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, given that hypoxia is one of the most important and clinically relevant stresses to the fetus and large epidemiological stud-

ies have indicated a link between in utero adverse stimuli during pregnancy and an increased risk of ischemic heart disease in the adulthood.

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