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Hypoxia and Fetal Programming of Cardiovascular Dysfunction

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Hypoxia and Fetal Programming of Cardiovascular Dysfunction

by

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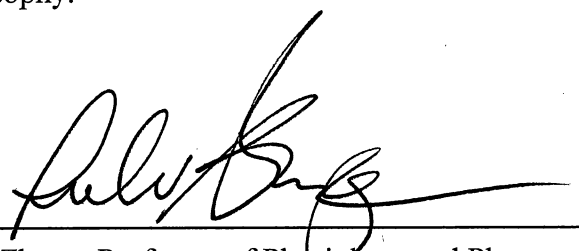
Each person whose signature appears below certifies that this dissertation in his/her opinion is adequate, in scope and quality, as a dissertation for the degree Doctor of Philosophy.



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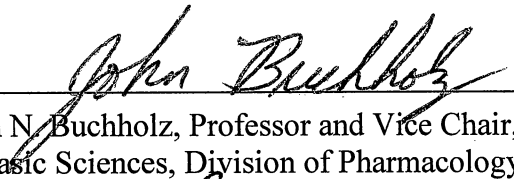

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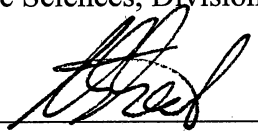

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ABBREVIATIONS

CVD	Cardiovascular disease
IUGR	Intrauterine growth restriction
IGF	Insulin like growth factors
RAS	Rennin-angiogenesis system
PPAR-α	Peroxisome proliferator-activated receptor
HPA	Hypothalamic-pituitary-adrenal
GR	Glucocorticoid receptors
MR	Mineralocorticoid receptors
GRE	Glucocorticoid-response elements
ROS	Reactive oxygen species
IPC	Ischemic preconditioning
11β-HSD2	11β-Hydroxysteroid dehydrogenase type 2
ATR	Agiotensin II receptor
GLUT1	Cardiac glucose transporter 1
PKC	Protein kinase C
SIDS	Sudden infant death syndrome
NO	Nitric oxide
I/R	Ischemia/reperfusion
HIF	Hypoxia-inducible factor
RACK	for activated C-kinase

ABSTRACT OF THE DISSERTATION

Hypoxia and Fetal Programming of Cardiovascular Dysfunction

by

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Dr. Zhang, Chairperson

Human epidemiological studies have shown a clear association of adverse intrauterine environment and an increased risk of ischemic heart disease in later adult life. Of all the stresses to which the fetus is subjected, perhaps the most important and clinically relevant is that of hypoxia. The goal of this project is to test the hypothesis that chronic hypoxia during gestation adversely affects fetal cardiovascular development and impairs cardiac function in offspring. In the first part of project, we tested the hypothesis that chronic hypoxia adversely regulates contractility of fetal pulmonary arteries and veins in sheep residing at high altitude (3,801 m) for 110 days. Our studies demonstrate the heterogeneity of fetal pulmonary arteries and veins in response to long-term high-altitude hypoxia and suggest a likely common mechanism downstream of nitric oxide in fetal pulmonary vascular response to chronic hypoxia *in utero*, which may be associated with an increased risk of pulmonary hypertension observed in newborns caused by fetal hypoxia. The second part of project focused on the effect of fetal hypoxia on heart development. This was accomplished by using a rat model. Time-dated pregnant rats were divided between normoxic and hypoxia (10.5% O2 on days 15-21 of gestation). We demonstrated that prenatal hypoxia caused an increase in heart susceptibility to ischemia and reperfusion injury in male offspring in a sex-dependent manner. This was caused by

enhanced type 2 angiotensin II receptors (AT_2R) and reduced PKCε expression in the heart. Further studies revealed an important role of glucocorticoid in programming of angiotensin II receptors, resulting in increased ischemic vulnerability in the heart of offspring. Multiple glucocorticoid response elements (GREs) were identified at the AT_2R promoter, deletion of which increased the promoter activity. Consistently, we demonstrated that dexamethasone decreased AT_2R expression in the fetal heart, which was blocked by RU 486. Prenatal hypoxia decreased glucocorticoid receptor (GR) expression in adult hearts, resulting in decreased GR binding to the GREs at the AT_2R promoter. These findings provide a mechanistic understanding worth of investigation in humans in fetal origins of cardiovascular disease caused by intrauterine adverse environment.

CHAPTER ONE

INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of death in the world. The World Health Report indicates that CVD was responsible for 16.7 million deaths globally in 2003 and predicts that by 2010, CVD will be the leading cause of death in the developing countries. Many risk factors have been established for CVD, including smoking, diabetes, dyslipidemia, and hypertension.

The 'developmental origins of adult disease' hypothesis, first put forward by David Barker, states that adverse intrauterine influences early in development would cause permanent changes in physiology and metabolism, resulting in increased disease risk in adulthood. The both animal and human studies support this conclusion; however, the molecular mechanisms underlying the association between low birth weight and later adult disease are not fully understood. Some studies have focused on the "Catch-up growth," which plays an important role in amplifying the incidence of CVD (Eriksson et al. 1999). Catch up growth is rapid growth in infants or young children who were born small for their gestational age has an increased growth rate that catch up to normal size. Low birth weight and postnatal catch-up growth are intimately linked and independent risk factors for adult disease. Recent studies form rats and mouse show that accelerated postnatal growth is a trigger for the development of CVD and adverse outcomes in adult

life (Osmond and Barker, 2000). Recent observations have shown that impaired growth in infancy and accelerated postnatal weight gain exacerbate the effects of impaired prenatal growth. The exact reason why catch-up growth is harmful is not fully understood, but there are two possibilities. Catch-up growth reflects persisting changes in hormonal secretions. In response to fetal undernutrition, growth restriction *in utero* results in a decrease in the number of cells, these limited cells become overgrown as a result of catch-up growth (Barker, 1999).

Besides fetal undernutrition, a number of additional stimuli and stress or insults, namely, fetal exposure to cocaine, smoking, corticorsteriod exposure and hypoxia have been studied as being capable of inducing fetal programming in both human and animal models (Godfrey and Barker 2000; Seckl 2004; Slotkin 1998; Wu et al. 2004; Zhang 2005).

Maternal Undernutrition and Cardiovascular Programming

Maternal nutrition during all the stages of gestation plays an important role in the development of the fetus. It has been known that the fetus receives the nutrients and oxygen from its mother's dietary intake. Insufficient maternal nutrition reduces the rate of cell division, impairing fetal growth (Barker, 2000). During periods of maternal undernutrition, the delivery of nutrients to the fetus becomes compromised and alters fetal development. The undernourished fetus makes adaptations including epigenetic modification, which causes the changes in organ structure and gene expesssion, resulting in chronic diseases in the later life (McMillen and Robinson 2005. Gilbert et al., 2006; Tappia and Gabriel 2006; Barker 2007; Poston 2007; Rinaudo and Lamb 2008; Yajnik and Deshmukh 2008). A number of conditions can cause intrauterine growth restriction

(IUGR), including nutritional inadequacies, CVD in mother, preeclampsia, hypoxia, drug abuse, high blood pressure and smoking (Harding, 2001). IUGR is usually classified as symmetric and asymmetric. Symmetric growth restriction implies a fetus whose entire body is proportionally small. A fetus with asymmetric IUGR has a normal head dimension but a small abdominal circumference (due to decreased liver size), scrawny limbs (because of decreased muscle mass) and thinned skin (because of decreased fat). Asymmetric growth restriction implies a fetus who is undernourished and keeps most of its energy maintaining growth of important organs, such as the brain and heart. If the insult causing asymmetric growth restriction is long or severe enough, the fetus may not compensate and becomes symmetrically growth-restricted. In addition, Asymmetric IUGR fetuses are more susceptible to developing the risk factors for CVD including hypertension (Barker, 1997a and 1997b).

Vascular endothelial dysfunction in children is associated with low body weight (Leeson et al, 1997). Abnormalities in nitric oxide-cGMP pathway in male offspring has been found in fetal protein restriction study, which may explain the lack of endothelium-dependent and –independent relaxation and the occurrence of vascular dysfunction and hypertension in the male offspring (Brawley et al., 2003 and 2004).

A variety of under nutrition models exist, including total calorie restriction, sodium restriction, and reduced protein intake. Both global maternal undernutrition and specific protein restriction result in reduced birth weight, increased blood pressure and impaired glucose tolerance in the offspring (Langley-Evans et al., 1995; Woodall et al., 1996). It has been found that maternal body compostition and diet during early or mid-pregnancy has effect on cardiovascular function and nephron number in sheep

(Gopalakrishnan et al., 2005). In a rat model, restricting maternal protein reduces the cardiomyocyte numbers, which may be due to increased apoptosis or reduced cellular proliferation (Corstius et al., 2005). In addition, maternal nutrient restriction results in the hypertrophy of the left ventricle, which is considered an important component in the development of later heart failure and heart disease (Dhalla et al., 2006, 2007). Furthermore, there is an increase in the amount of interstitial fibrosis in the left ventricle in the adult offspring when the mother has a low protein diet during gestation, which might contribute to reduce cardiac contractility and heart disease in adult offspring (Lim et al., 2006). Maternal undernutrition leads to pathologic cardiac remodeling, diastolic dysfunction, and increased sensitivity to ischemic injury during adult life (Xu et al., 2006)

It has been shown that a maternal low protein diet can induce changes in the gene and protein levels of myocardial Ca^{2+}-cycling proteins, which might contribute to the depressed contractile function of the neonatal heart (Tappia et al., 2009). It is well known that growth hormones and insulin like growth factors (IGFs) are involved in the development and physiology of the cardiovascular system. Maternal undernutrition decreases the expression of IGFs, which impairs cardiac growth and systolic function, resulting in the development of CVD later in life (Dong et al., 2005). In addition, Kawamura et al. (2007) also reported that fetal undernutrition activated the local rennin-angiogenesis system (RAS), partly contributing to the occurrence of cardiac remodeling in later life. Maternal nutrient restriction upregulated a number of genes related to hypertrophy in the fetal heart (Han et al., 2004). Another important aspect of maternal undernutrition is the reduction of the density of β_1-adrenergic receptors (Fernadez-Twinn

et al. 2006). It has been found that the pups have higher levels of circulating epinephrine (Petry et al. 2000). Excessive activation of the β-adrenergic system is harmful to the heart and plays an important role in the development of heart failure (Port and Bristow 2001). It is likely that the long term increased adrenergic stimulation will result in compromised cardiac function and possibly heart failure.

Maternal protein restriction also alters the compostion of the plasma membrane and the fatty acid content of the cardiomyocytes of the offspring (Tappia et al., 2005). It has been observed that there are the increased levels of sphingomyelin and lysophosphatidylcholine, which could induce increased myocardial apoptosis in response to maternal protein restriction. Additionally, levels of peroxisome proliferator-activated receptor (PPAR-α), an important transcription factor involved in regulation of genes encoding multiple enzymes for fatty acid oxidation and, was increased in response to reduced maternal protein intake (Tappia et al., 2005).

Maternal Corticosteroid Excess and Cardiovascular Programming

Secretion of glucocorticoids from the adrenal cortex is controlled by hypothalamic-pituitary-adrenal (HPA) axis through a negative-feedback loop. Glucocorticoids exert their effects by binding intracellular glucocorticoid receptors (GRs), members of the nuclear hormone superfamily of ligand-activated transcription factors. In addition, in some tissues, glucocorticoids bind to mineralocorticoid receptors (MRs). GRs and MRs are activated by ligand binding, then the receptor–ligand complex translocates to the nucleus, binding to glucocorticoid-response elements (GREs) in the promoter of target genes to influence gene transcription (Yamamoto, 1985).

Glucocorticoids regulate important cardiovascular, metabolic, immunological and other homoeostatic functions. Steroid hormones are increased in stress and involved in organ development, maturation. Given that glucocorticoids play an important role in lung development, glucocorticoids are given antepartum to women at increased risk for delivering prematurely in order to accelerate lung development and reduce the likelihood of the infant suffering respiratory distress syndrome (RDS) or bronchopulmonary dysplasia (BPD) (Ballard, 1987).

Although glucocorticoids are highly lipophilic molecules and can be cross biological barriers, such as the placenta, fetal glucocorticoids are generally lower than the maternal levels (Klemcke, 1995). This is due to higher level of 11β-Hydroxysteroid dehydrogenase type 2 (11β-HSD2), which converts steroid hormones into an inactive form (Lopez-Bernal and Craft 1981; Lopez-Bernal et al., 1980). This allows only a low level of maternal steroids to pass through the placenta. However, 11β-HSD2 does not deactivate all steroids. Administration of a steroid that 11β-HSD2 does not have a high affinity for, will lead to high fetal glucocorticoids. Additionally, a defect or inhibition of 11β-HSD2 or high levels of maternal steroids can overwhelm the protection of 11β-HSD2 and enhance levels of steroids in fetus. Given that fetal exposure to excessive amounts of glucocorticoids results in intrauterine growth restriction, it has been hypothesized that this placental 11β-HSD barrier plays a key role to protect the fetus from adverse effects of maternal glucocorticoids. Many studies have shown that 11β-HSD2 activity is influenced by maternal environmental factors such as maternal undernutrition, and IUGR (Shams et al., 1998). It has been demonstrated that corticosterone are associated with programming of hypertension in the absence of

changes in birth weight, which suggested that levels of glucocorticoids associated with physiological stress may overcome the placental 11β-HSD2 barrier, leading to influence the developing fetus.

The HPA axis is an important target in programming, and studies in various animal models have shown that prenatal glucocorticoid excess affects activity of this axis at several levels. For example, exposure to glucocorticoids *in utero* during the last third of pregnancy reduces MR and GR levels in the hippocampus. It is thought that the decrease in hippocampal GR expression reduces the sensitivity of feedback and, thus, permanently alters the 'set point' of the HPA axis (Plotsky and Meaney, 1993).

Glucocorticoid treatment during pregnancy reduces birth weight in both animals and humans (Nyirenda et al., 1998; Ikegami et al., 1997; Newnham et al., 1999; French et al., 1999; Bloom et al., 2001). Fetal excessive exposure to glucocorticoid is linked with a variety of pathologies such as hypertension and insulin resistance (Nyirenda et al., 1998). Fetal glucocorticoid overexposure is associated with an irreversible decrease in nephron number in rodents (Ortiz et al., 2001 and 2003) and sheep (Wintour et al., 2003). Glucocorticoids exposure prenatally altered activity of the renin–angiotensin–aldosterone system (RAAS) and vascular function in a region-specific manner (O'Regan et al., 2004 and Hodoke et al., 2006) and have increased expression of the AT_1 and AT_2 (Wintour et al., 2003). Excessive glucocorticoid exposure *in utero* can influence the heart both directly and indirectly. There were alterations of cardiac noradrenergic innervation and sympathetic activity of baroreceptor response in the offspring exposed to glucocorticoid prenatally (Wintour et al., 2003). It has been reported the changes in expression of several genes in the heart including cardiac glucose transporter 1 (GLUT1), Akt/PKB,

specific uncoupling proteins, peroxisome-proliferator-activated receptor γ (PPAR- γ) and calreticulin (Langdown et al., 2001 and 2003). The entry of glucose into the cardiac myocytes, the rate limiting step of glycolysis in these cells, is controlled by GLUT. Compared to the rats not exposed to dexamethasone *in utero*, the levels of the GLUT1 are higher in the adult organism exposed to dexamethasone prenatally (Langdown et al., 2001). However, this difference is not observed when the pups are less than 2 weeks old, suggesting that this alteration may be secondary to other changes that trigger this alteration. An increase in the Akt/PKB pathway was also observed, which may be responsible for the observed change in GLUT1 expression. However, this correlation has not been shown to be causative. Additionally, cardiac expression of protein kinase C (PKC) isoforms was increased in the pups of the mothers given dexamethasone. Both pro (β_1, β_2, and δ) and anti (α and ε) apoptotic isoforms were upregulated (Langdown et al., 2001). The effect of these changes on cardiac function and susceptibility to disease has not been fully understood but clearly significant long term changes take place in response to the glucocorticoids overexposure during development.

Maternal Drug Use and Cardiac Programming

Cigarette smoking is the leading cause of disease and death in the United States. It is estimated that approximately 25% of pregnant women smoke in the United States. Maternal smoking during pregnancy is associated with several adverse developmental outcomes in the offspring, including preterm delivery, spontaneous abortion, growth restriction, increased risk of sudden infant death syndrome (SIDS), elevated blood pressure, cardiovascular disease as well as long-term behavioral and psychiatric disorders. However, the underlying physiological mechanisms for these effects are not

fully understood. Nicotine is one of the major components in cigarette smoking, which is likely to contribute to the occurrence of cardiovascular disorders. Because nicotine is an agonist of nicotinic acetylcholine receptors, exposure to nicotine early in life may cause permanent changes in nicotinic receptors and consequent cell function (Slotkin, 1998). Nicotine decreases birth weight by increasing rate of early delivery and slowing fetal growth so even full term infants exposed to nicotine are small for gestational age (Lambers and Clark, 1996). It has been shown that maternal cigarette smoking acutely increases fetal heart rate, probably due to an increase in sympathetic activity. Fetal nicotine exposure does not change the heart function in rat pups, but produces intolerance to neonatal hypoxia, resulting in a sharp decrease in heart rate (Slotkin et al., 1997). It is currently being investigated that this effect may play a important role in sudden infant death syndrome, and it could also have effects in later life if the change is persistent. It has been demonstrated that fetal and neonatal nicotine exposure alters vascular function in adult offspring in a gender-dependent manner, possibly resutling in an increased risk of cardiovascular dysfunction in adult life (Xiao et al., 2007). In addition, fetal nicotine exposure increased heart susceptibility to ischemia-reperfusion injury and decreased expression of myocardial PKCε expression in the adult both male and female offspring (Lawrence et al. 2008). It has been shown that materal nicotine exposure can induce increased risk for behavioral and psychiatric disorders in later life (Wakschlag et al. 2002).

In the United States, more than 100,000 infants are exposed to cocaine *in utero* each year. Cocaine can cross the placenta and accumulate in the fetus (Schenker et al. 1993). Cocaine can cause irreversible structural damage to the heart, greatly accelerate

cardiovascular disease, and initiate sudden cardiac death. There is an established connection between cocaine use and myocardial infarction, arrhythmia, heart failure, and sudden cardiac death (Mone et al., 2004). Numerous mechanisms have been postulated to explain how cocaine contributes to these conditions. It has been shown that cocaine blocks K^+ channels, increases L-type Ca^{2+} channel current, and inhibit Na^+ influx during depolarization, possibling causing for arrhythmia. Fetal cocaine exposure has a clear effect on β-adrenergic signaling in animal models. Fetal cocaine exposure decreased K^+ induced norepinephrine release and increased in ionomyocin induced norepinephrine release in the cardiac adrenergic nerve ternimals (Snyder et al., 1995). Cocaine is directly toxic to fetal myocardiocytes inducing apoptosis, which is clearly deleterious to the heart (Bae and Zhang 2005b). Fetal cocaine exposure also increases the sensitivity to ischemia/reperfusion injury in the adult offspring in a sex-specific manner. It has been found that prenatal cocaine treatment decreased levels of PKCε in adult offspring (Zhang et al. 2007). PKCε has been demonstrated to be cardioprotective (Chen et al. 2001) so the downregulation of PKCε may explain the increased cardiac susceptibility to ischemic injury in the offspring. It has been demonstrated that a direct effect of cocaine in epigenetic modification of DNA methylation and programming of cardiac PKCε gene repression linking cocaine exposure *in utero* and pathophysiological consequences in the heart of adult offspring (Meyer et al., 2009).

Fetal Hypoxia and Cardiac Programming

Ischemic heart disease is a major cause of death among people in the western world. In addition to other risk factors, recent epidemiological studies have shown a clear association of adverse intrauterine environment and an increased risk of ischemic

heart disease in adult offspring (Barker et al., 1989, 1993). Of all the stresses to which the fetus is subjected, perhaps hypoxia is one of the most important and clinically relevant stresses that can adversely affect fetal development. The fetus may experience prolonged hypoxic stress under many different conditions, including pregnancy at high altitude; pregnancy with anemia; cigarette smoking and cocaine abuse during pregnancy; preeclampsia; and heart, lung and kidney disease. About 140 million people living in over 2,500 m above the sea level are exposed to chronic hypoxic conditions (Moore et al., 2004). Epidemiological studies have indicated that high altitude pregnancies increase the risk of IUGR and low birth weight (Jensen, 1997; Moore et al., 2001; Moore, 2003). Those factors are known to cause premature birth, infant mortality; and, an increased risk of developing cardiovascular diseases.

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; Moore, 2003). Fetal undergrowth appears to prompt programming, the process whereby a stimulus or insult acting at a critical period of development in early life alters gene expression pattern for life (Sayer et al., 1997). Additionally, chronic hypoxia suppresses fetal cardiac function, alters cardiac gene expression pattern, and increases heart to body weight ratio (Kamitomo et al., 1992; Xiao et al., 2000; Rouwet et al., 2002; Zhang, 2005). Studies in a pregnant rat model also demonstrated that fetal hypoxia caused a premature exit from the cell cycle of cardiomyocytes and myocyte hypertrophy (Bae et al., 2003). It is likely that the increase in size of cardiomyocytes is compensatory for reduced myocyte number. The reduction in cardiomyocyte number is likely influenced by either increased program cell death or

reduced cellular proliferation. Fetal hypoxia induced apoptosis via increased caspase 3 activity and Fas, and depressed Bcl-2 and heat shock protein 70 (Hsp 70) expression in fetal hearts (Bae et al., 2003). Hsp 70 plays an important role to protect from ischemia. Our lab has demonstrated that fetal hypoxia decreased endothelial nitric oxide (NO) synthase in the heart (Li, et al., 2003). Although whether NO protects the heart remains controversial, a number of studies show exogenous and endogenous NO to be cardioprotective in ischemia/reperfusion (I/R) injury (Bolli, 2001). In addition, studies in sheep show that long-term high altitude hypoxia decreases fetal cardiac output, and causes a redistribution of blood flow in favor of the heart and brain (Kamitomo et al., 1992). In a rat study, maternal hypoxia increases in hypoxia-inducible factor 1α (HIFα) expression (Bae et al., 2003). HIF is a heterodimeric transcription factor that plays a pivotal role in cellular sensing and response to low oxygen tension. After birth, cardiomyocytes are highly differentiated and rarely replicate, an inappropriate prenatal loss of cardiomyocytes through apoptosis is likely to result in a permanent reduction of the number of function units in myocardium and result in cardiac dysfunctions in infants and adults. During the early developmental period, either excessive and/or persistent cardiomyocyte loss through apoptosis has been suggested to lead to various cardiac diseases (James et al., 1998).

The rat model of fetal hypoxia shows catch-up growth postnatally, and no difference is found in body weight between control and hypoxic offspring, which is consistent with human epidemiologic findings that coronary heart disease reflects fetal undernutrition and consequent small body size at birth combined with improved postnatal nutrition and "catch up" growth in childhood. High altitude sheep study showed the

altered cardiac function in response to fetal hypoxia as demonstrated by decreased cardiac output and lower contractility (Kamitomo et al., 2002; Gilbert, 1998; Browne et al., 1997). In rat model, it has been demonstrated that fetal hypoxia results in significantly increases the susceptibility of the adult heart to I/R injury by decreasing postischemic recovery of left ventricular function and increasing myocardial infarct size (Xiao et al., 2000; Bae et al., 2003; Li et al., 2003, 2004; Xu et al., 2006). In addition, prenatal hypoxia abolishes the protective affects afforded by heat stress against I/R injury and decreases HSP70 and PKCε in the left ventricles of adult offpring (Li et al., 2003). Furthermore, fetal hypoxia increases oxidative stress and downregulates a list of genes involved in cell signaling, communication, defense, proliferation, and survival in fetal heart. It is not clear how many of those genes have lasting and effect on heart in the later adult life. Oxidative stress is as an underlying factor for the adverse uterine environment and the programming of the increased risk for developing chronic disease in adult life (Franco Mdo et al., 2002; Luo et al., 2006). Oxidative stress also has been reported to reduce lifespan by promoting apoptosis and organ damage in male offspring exposed to protein-restricted diet before birth (Langley-Evans and Sculley, 2005). Thus, it is reasonable to speculate that reduction of oxidative stress during pregnancy might prevent CVD and other chronic diseases in later life. In addition to fetal hypoxia, fetal exposure to glucocorticoids, nicotine (Lawrence et al., 2008) or cocaine (Bae et al., 2005; Bae and Zhang, 2005a) caused an epigenetic programming in the heart and resulted in increased heart susceptibility to I/R injury in adult offspring.

Hypoxia and Pulmonary Vessels and Nitric Oxide (NO)

Pulmonary vasoconstriction and high pulmonary vascular resistance are hallmarks of the fetal circulation. Following birth, pulmonary vascular resistance falls and pulmonary blood flow increases immediately as the lung expands with air and functions in oxygen exchange. Oxygen plays an important role in the transition of high pulmonary resistance in the newborn (Cornfield et al., 1992, 1996; North et al., 1996; Black et al., 1997). Fetal hypoxia is one of the major factors associated with persistent pulmonary hypertension in the newborn (Abman et al., 1990). It has been shown that newborns at high altitude have increased pulmonary vascular resistance (Niermeyer 2007; Herrera et al., 2007). Abnormal vasoconstriction and changes in vessel morphology are two prominent pathological features in lungs subjected to chronic hypoxia (Jeffery and Wanstall 2001; Runo and Loyd 2003). Vascular remodeling occurs not only in pulmonary arteries (PA) but also in pulmonary veins (PV) in a number of species including rat (Takahashi et al., 2001), sheep (Johnson et al., 1997), and human (Chazova et al., 1995).

Endothelium-derived nitric oxide or nitric oxide-like substance modulates the response of pulmonary vasculature. NO plays an important role in the regulation of the pulmonary circulation in the fetus. Although there are many studies of NO and pulmonary vascular reactivity in the newborn and adult, to our knowledge, studies of the effect of chronic hypoxia *in utero* on contractility and endothelium-dependent relaxation of fetal pulmonary vessels are limited. In the well-defined animal model of pregnant sheep maintained at high altitude of 3,801 m for 110 days during gestation (maternal PaO_2: 60 mmHg and fetal PaO_2: 19 mmHg), a recent study demonstrated that chronic hypoxia *in utero* attenuated PKG-mediated relaxation in PA in near-term fetal lambs,

which was due in part to inhibited cGMP-dependent protein kinase activity and enhanced Rho kinase activity (Gao et al., 2007). Nonetheless, it is unknown to what extent chronic hypoxia affects the up-stream mechanisms at eNOS levels and endothelium-dependent relaxation in pulmonary vessels in the fetus.

Protein Kinase C (PKC)

PKC comprises a multigene family of related serine/threonine kinases that sit at the crossroads of many signal transduction pathways and are implicated in G protein-coupled receptor and other growth factor-dependent cellular responses. PKC are highly homologous kinases and several different isoforms can be present in a cell. They are further classified into three subfamilies: the classical isozymes (α, βI, βII, γ), the novel isoforms (δ, ε, η, θ), and the atypical isoforms (ζ, λ). The classical isoforms are responsive to diacylglycerol (DAG) and Ca^{2+}. Novel isoforms are responsive only to DAG. Atypical isoforms activation is independent of both DAG and Ca^{2+}.

PKCε belongs to novel isoforms that are involved in wide cellular function. While the mechanisms whereby prenatal hypoxia causes an increase in vulnerability of ischemic injury in the heart of adult offspring has not been fully elucidated, the down regulation of PKCε gene expression in the heart appears to have an important role. PKC plays a pivotal role in cardioprotection from cardiac I/R injury (Gray et al. 1997; Murriel and Mochly-Rosen, 2003). Studies in a PKCε knock-out mouse model have demonstrated that PKCε expression is not required for cardiac function under normal physiological conditions, but PKCε activation is necessary and sufficient for acute cardioprotection during cardiac I/R (Gray et al., 2004). Expression of a PKCε-activating peptide or

cardiac-specific overexpression of PKCε confers cardioprotection against I/R-mediated cardiac damage (Dorn et al., 1999; Ping et al., 2002; Inagaki K, et al., 2003). 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, Figure 1). 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; Murriel and Mochly-Rosen, 2003; Przyklenk et al., 2003).

PKCε is highly expressed in heart tissue and plays an important role in ischemic preconditioning (IPC) (Gray et al., 1997).). IPC is the observation that many tissues, including the heart, suffer less damage from an ischemic insult if the tissue has been preconditioned by a prior short ischemic period (Eisen et al. 2004). IPC protects the heart from ischemia and reperfusion-induced damage by inducing myocardial adaptation to the ensuing prolonged ischemic event. IPC appears to be induced triggered by many factors including adenosine, opioid receptors, bradykinin, nitric oxide, free radicals, and calcium (Eisen et al. 2004). Despite a number of activators myocardial IPC appears to be induced via two possible pathways. One pathway is activation of K-ATP channels (Grover et al., 1992) and the other is activation of PKCε which has been shown is necessary for IPC cardioprotection (Hassouna et al., 2004; Kawamura et al. 1998; Korge et al., 2002; Ping et al. 1997). IPC and other activators of PKCε result in PKCε being translocated from the

cytosolic fraction to the particulate fraction. IPC is not only an important protective mechanism in the presence of PKCε but also a physiologic activator of PKCε that can modulate the activity of PKCε if it is still intact in a system.

Unlike PKCε, the role of PKCδ in I/R injury is less clear and somewhat controversial. Selective activation of PKCδ caused increased damage from ischemic insults in neonatal cardiac myocytes, in adult isolated rat cardiac myocytes and in isolated hearts infused with activator prior to ischemia (Chen et al., 2001). Furthermore, inhibition of PKCδ during reperfusion has been shown to decrease reperfusion-induced injury (Murriel and Mochly-Rosen, 2003). Using an ex vivo model of cardiac ischemia, it has been found that the administration of the PKCδ inhibitor only at reperfusion resulted in cardiac protection that was additive to that obtained by applying the PKCε activator before ischemia (Inagaki et al., 2003). There is also evidence to suggest that reactive oxygen species (ROS) formation during I/R mediated injury of the myocardium (Semenza, 2000). Free radicals during I/R may induce a proapoptotic signal that induces further damage to the muscle. ROS are induced early after reperfusion of the heart following ischemia, which may also be responsible for reperfusion injury (Bolli et al., 1989; Flaherty et al., 1994; Jordan et al., 1999). This may activate a variety of signaling pathways, including activation of PKC (Konishi et al., 1997). Several studies showed that H_2O_2 and ROS generate oxidative stress, which also results in activation and translocation of PKCδ (Majumder et al., 2001). Since reperfusion has also been shown to generate free radicals contributing to myocardial injury, it is possible that reperfusion activates PKCδ activation and translocation. A study in knockout mice lacking PKCδ demonstrates a loss of ROS formation by the endothelium when subjected to cell stress

agents such as UV and tumor necrosis factor α (TNF-α) (Leitges et al., 2001). Therefore, it is also possible that PKCδ is upstream of free radical formation and causes mitochondrial damage. However, other studies demonstrated the cardioprotective effects of PKCδ (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 PKCδ and PKCε levels (Hunter et al., 2007). Previous studies demonstrated that prenatal nicotine exposure caused a significant decrease in PKCδ protein levels in the heart of female but not male offspring, which was associated with the increased heart vulnerability to ischemic injury in the females as compare with the males (Lawrence et al., 2008).

Angiotensin II (Ang II) Receptors

Many studies have demonstrated that Ang II plays important role in the cardiovascular system under different physiological or pathological conditions, such as hypertension, cardiac hypertrophy, heart failure and ischemic heart disease (Daemen et al., 1991; Baker et al., 1993; Ruiz-Ortega et al., 2007; Billet et al., 2008; Rush and Aultman, 2008). Ang II plays a fundamental role in the regulation of cardiovascular homeostasis, and has been implicated in programming of cardiovascular disease induced by adverse *in utero* environment during the fetal development (Daemen et al., 1991; Chung et al., 1998; Diep et al., 1999; Chassagne et al., 2000). The renin-angiotensin system (RAS) is hyperactivated during myocardial ischemia, infarction and I/R induced injury (Dudley et al., 1990; Horiuchi et al., 1999). Ang II has two main specific G-protein-coupled receptor subtypes: AT_1R and AT_2R. There are two different types of

AT_1R in rat and mouse. $AT_{1a}R$ predominate in the cardiovascular system, lungs, ovaries and hypothalamus. However, the $AT_{1b}R$ subtype prevails in the anterior pituitary, several periventricular brain areas, adrenal cortex and uterus. The subtypes are equally distributed in the spleen, liver and kidneys. In the rat, the AT_{1a} gene has been mapped to chromosome 17, and the AT_{1b} gene to chromosome 2 (Inagami et al., 1995). In humans, only one type of AT_1 has been described and mapped to chromosome 3. The AT_2R is widely distributed in fetal tissues, however its expression sharply declines in most organs and practically vanishes from many tissues during ontogeny. Some studies have shown that the function and signaling mechanisms of AT_1R and AT_2R are different, and both receptors seem to exert opposite effects in terms of cardiovascular hemodynamics and cell growth and differentiation (Chung et al., 1998; Stoll and Unger, 2001). Recent studies have demonstrated a link between fetal insults to differential epigenetic modifications of *in utero* AT_1R and AT_2R genes expression in the adrenal, kidney and the resultant alteration of their expression pattern in adult life of offspring. This may lead to the development of hypertension (McMullen and Langley-Evans, 2005; Bogdarina, et al., 2007; Singh et al., 2007). The mechanism of fetal hypoxia induced ontogeny of AT_1R and AT_2R in the offspring heart has not been explored yet. Both AT_1R and AT_2R exist in cardiac myocytes and play significant pathophysiological roles in heart diseases (Sechi et al., 1992; Matsubara, 1998; Horiuchi et al., 1999; Schneider and Lorell, 2001).

Previous studies have shown that the AT receptor genes are developmentally regulated in a tissue-specific manner (Tufro-McReddie et al., 1993; Butkus et al., 1997; Wintour et al., 1999; Cox et al., 2005). These findings suggest that the diverse actions of Ang II during development may be mediated by tissue-specific temporal patterns of AT_1

and AT_2 mRNA expression. The ontogeny of cardiac AT_1 and AT_2 gene expression has been studied in the last third trimester of gestation in fetal sheep and newborn lambs, which demonstrated a rapid decrease in AT_2, but not AT_1, mRNA after birth (Samyn et al., 1998). Previous study also determined that AT_1 and AT_2 receptors gene expression is higher in fetal and newborn hearts than adults (Everett et al., 1996).

Additionally, evidence showed that chronic hypoxia increases the plasma levels of renin (Gould and Goodman, 1970) and Ang II (Zakheim et al., 1976), which has stimulated interest in the contribution of renin angiotensin system to hypoxia. Experimental evidence suggests an important role of the Ang II and its receptors in hypoxic/ischemic brain injury (Li et al., 2008). It has been found that AT_1R and AT_2R expression was upregulated in PA in hypoxic rats (Chassagne et al., 2000), suggesting a participation of these receptors in the remodeling process. However, whether exposure to chronic hypoxia induces cardiac changes in AT_1R and AT_2R expression is not clear.

The tissue specific and ontogeny-dependent expression of the AT_2R gene suggest possible developmental, neurological and reproductive of roles of Ang II via the AT_2R and that the biological roles of this receptor is closely related to its unique expression pattern. Previous study showed that in maternal low protein diet rat models, expression of the $AT_{1b}R$ gene in the adrenal gland is upregulated, which is due to significantly undermethylated of promoter of $AT_{1b}R$ gene (Bogdarina et al., 2007). A DNA segment between -44bp and +58bp in promoter region of the rat AT_2 gene is important for the basal promoter activity of the AT_2R gene. In this DNA segment, there is a TATA box consensus sequence, which contains CpG dinucleotides. It is possible prenatal hypoxia can regulate the methylation of the TATA box. Whether and to what extent fetal hypoxia

would induce a differential and sex-dependent pattern of DNA methylation in the AT_2 promoter remains an intriguing area for the future investigation. In addition, the expression of AT_1R and AT_2R are regulated by glucocorticoids (Matsubara, 1998). It has been suggested in rats that glucocorticoids play an important role in fetal programming of AT_1R and AT_2R expression pattern in offspring (McCullen and Langley-Evans, 2005a, 2005b). GREs in rat $AT_{1a}R$ and $AT_{1b}R$ gene promoters have been identified previously (Guo et al., 1995; Bogdarina et al., 2009). $AT_{1a}R$ promoter harbors positive GREs and $AT_{1b}R$ contains negative GREs.

Sex Dichotomy

A number of studies have reported sex differences in the incidence and progression of cardiovascular diseases such as coronary artery disease, heart failure, cardiac hypertrophy, and sudden cardiac death (Gilbert et al., 2006; Grigore et al., 2008; Ojeda et al., 2008). Many recent studies have provided evidence that indicates a sex dichotomy also exists in the physiological responses to developmental challenges relating to the programming of subsequent cardiorenal function. Sex differences can be quite early in embryonic development and are independent of sex hormones. Additionally, sex steroids have a profound effect on the development and progression of programmed disease states.

It has been reported that men often have higher risk for cardiovascular disease than premenopausal women of similar age (Reckelhoff, 2001; Wiinberg et al., 1995), which may partly be associated to intrinsic sex differences in cardiovascular and renal function (Miller et al., 1999; Schwertz et al., 1999). For example, sex chromosomes

facilitate the development of sexual dimorphism and X-linked genes have a key role in coding for sexually dimorphic traits. Compared to male embryos, several genes located on the X chromosome are more expressed in bovine and human female (Gutierrez-Adan et al., 2000, Peippo et al., 2002). It has been demonstrated that a number of genes expressed in placenta are significantly related with the sex of the fetus, which is that genes are expressed at higher levels in female placentas, including those with roles in immune regulation like *JAK1*, *IL2RB*, *Clusterin*, *LTBP*, *CXCL1*, and *IL1RL1* (Sood et al., 2006).

In most cases, the male offspring exhibits a more severely impacted phenotype while the female offspring are either less affected or completely spared. Sex steroids also play an important role in the difference. Androgens have been linked with the progression of renal injury (Reckelhoff et al., 1998; Sandberg and Ji, 2003), while estrogens seem to be protective in renal function (Sakemi et al., 1995; Sandberg and Ji, 2003). Additionally, it seems that sex may exert different effects during fetal and adult life. In animal models of intrauterine malnutrition, the 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 I/R 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.

Epigenetic Mechanisms in Fetal Programming

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; Drake and Walker, 2004). Epigenetic changes are defined as reversible changes that occur as a result of heritable modifications without involving the alteration of primary DNA sequence (Ho and Tang 2007). DNA methylation is a chief mechanism in epigenetic modification of gene expression pattern and occurs at cytosines of the dinucleotide sequence of CpG. CpG dinucleotides are primarily clustered together in regions referred to as CG islands while being relatively uncommon in other parts of the genome. CG islands are most commonly found in the promoter region of genes and the methylation status of these islands plays an important role to regulate the transcription of the associated gene. Indeed, in many animal models, it has been known that environmental manipulations alter methylation at specific genes. Changes in the methylation status of gene promoter region resulting in inappropriate activation or suppression of gene expression have been linked to disease states. Notably, a number of cancers are linked with global hypomethylation with discrete regions of hypermethylation in the promoter of tumor suppressor genes (Jones and Baylin 2002; Jones and Laird 1999). It has been demonstrated that altered maternal diet during pregnancy can increase methylation of the agouti gene and the mouse AxinFu gene, which alters the phenotype of offspring (Waterland et al., 2006). Fetal undernutrition can cause changes in DNA methylation, which affects gene transcription, notably the expression of GRs in the liver (Maclennan et al., 2004 and Lillycrop et al., 2005). The GR itself may play a direct role to mediate epigenetic changes, possibly underlying

glucocorticoid programming. Thus, glucocorticoid treatment induces differential demethylation of target gene promoters, and this demethylation persists after steroid withdrawal in cultured fetal hepatocytes (Thomassin et al., 2001).

The recent study also demonstrated an epigenetic mechanism of DNA methylation in programming of cardiac PKCε 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). PKCε promoter was cloned and sequenced in our lab. The promoter was found to be rich in CpG sites and contained 8 putative promoter 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) that contain at least one potential methylation site (CpG). It has been demonstrated that prenatal cocaine exposure causes a significant increase in methylation status of CpG dinucleotides at the SP1 bindings sties which resulted in decreased SP1 binding in the promoter (Zhang, et al., 2009). The importance of SP1 in PKCε expression was confirmed using a luciferase reporter assay (Zhang et al.., 2009). Fetal environment impacting methylation status of specific genes has been also reported in other models. In a rat maternal protein deprivation model, there was an increase in DNA methylation at specific sites of the PPARα promoter in the adult offspring (Lillycrop et al. 2008).

Central Hypothesis

The central hypothesis of my project is that fetal hypoxia cause dysfunction of pulmonary vascular contractility and fetal programming of PKCε, AT_1R and AT_2R genes

in the heart, resulting in an increase in heart susceptibility to ischemia and reperfusion injury in offspring in a sex-dependent manner.

Significance

Epidemiologic evidence has indicated a correlation between adverse intrauterine environment and increased risk of hypertension and ischemic heart disease in the adult. Animal studies suggest that fetal exposure to chronic hypoxia can cause *in utero* programming leading to an increased risk of adult disease. It has been known that gender differences exist in susceptibility to and mortality from various cardiovascular diseases. However, 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. Additionally, the mechanisms whereby fetal hypoxia causes an increase in the vulnerability of ischemic injury in the heart of adult offspring are not clear. Our purposed studies will give insight into how chronic hypoxic exposure during pregnancy on programming of some specific genes, such as PKCε, AT receptor, and GR. We also can gain a better understanding of the correlation of PKCε, AT receptor, and GR. The possibility that fetal hypoxia may result in programming of some specific genes in the offspring with a consequence of increased cardiac vulnerability provides a mechanistic understanding worthy of investigation in human, given that hypoxia is one of the most important and clinically relevant stresses to the fetus. Our studies will delineate how prenatal hypoxia can adversely impact development of the fetus and induce disease phenotype in the adult offspring.

CHAPTER TWO

EFFECT OF LONG-TERM HIGH-ALTITUDE HYPOXIA ON FETAL PULMONARY VASCULAR CONTRACTILITY

By

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Abstract

Hypoxia in the fetus and/or newborn is associated with an increased risk of pulmonary hypertension. The present study tested the hypothesis that long-term high-altitude hypoxemia differentially regulates contractility of fetal pulmonary arteries (PA) and veins (PV) mediated by differences in endothelial NO synthase (eNOS). PA and PV were isolated from near-term fetuses of pregnant ewes maintained at sea level (300 m) or high altitude of 3,801 m for 110 days (arterial Po(2) of 60 Torr). Hypoxia had no effect on the medial wall thickness of pulmonary vessels and did not alter KCl-induced contractions. In PA, hypoxia significantly increased norepinephrine (NE)-induced contractions, which were not affected by eNOS inhibitor N(G)-nitro-l-arginine (l-NNA). In PV, hypoxia had no effect on NE-induced contractions in the absence of l-NNA. l-NNA significantly increased NE-induced contractions in both control and hypoxic PV. In the presence of l-NNA, NE-induced contractions of PV were significantly decreased in hypoxic lambs compared with normoxic animals. Acetylcholine caused relaxations of PV but not PA, and hypoxia significantly decreased both pD(2) and the maximal response of acetylcholine-induced relaxation in PV. Additionally, hypoxia significantly decreased the maximal response of sodium nitroprusside-induced relaxations of both PA and PV. eNOS was detected in the endothelium of both PA and PV, and eNOS protein levels were significantly higher in PV than in PA in normoxic lambs. Hypoxia had no significant effect on eNOS levels in either PA or PV. The results demonstrate heterogeneity of fetal pulmonary arteries and veins in response to long-term high-altitude hypoxia and suggest a likely common mechanism downstream of NO in fetal pulmonary vessel response to chronic hypoxia in utero.

Introduction

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. Nearly 140 million people residing at over 2,500 m above the sea level are permanently exposed to chronic hypoxic conditions (Moore et al., 2004). Pulmonary vasoconstriction and high pulmonary vascular resistance are hallmarks of the fetal circulation. Following birth, pulmonary vascular resistance falls and pulmonary blood flow increases immediately as the lung expands with air and functions in oxygen exchange. Oxygen plays a vital role in the transition of high pulmonary resistance in the fetus to low pulmonary resistance in the newborn (Black et al., 1997; Cornfield et al., 1992, 1996; Fineman et al., 1995; North et al., 1996). Chronic hypoxia in utero is one of the major factors associated with persistent pulmonary hypertension in the newborn (Abman, 1999; Niermeyer, 2007). It has been shown that newborns at high altitude have elevated pulmonary vascular resistance (Herrera et al., 2007; Niermeyer 2003, 2007).

Among other mechanisms, oxygen-induced changes in endothelial NO production play a key role and contribute significantly to pulmonary vasodilation after birth (Black et al., 1997; Cornfield et al., 1992, Fineman et al., 1995; Shaul et al., 1993). Although there are many studies of NO and pulmonary vascular reactivity in the newborn and adult, to our knowledge, studies of the effect of chronic hypoxia in utero on contractility and endothelium-dependent relaxation of fetal pulmonary vessels are limited. This deficit is partly due to a limit of animal models of chronic in utero hypoxia, in which small fetal pulmonary vessels can be isolated and studied in an organ bath. In the well-defined

animal model of pregnant sheep maintained at a high altitude of 3,801 m for 110 days during gestation [maternal arterial PO_2 (Pa_{O2}) of 60 Torr and fetal Pa_{O2} of 19 Torr], a recent study demonstrated that chronic hypoxia in utero attenuated PKG-mediated relaxation in pulmonary arteries in near-term fetal lambs, which was due in part to inhibited cGMP-dependent protein kinase activity and enhanced Rho kinase activity (Gao et al., 2007). Nonetheless, it is unknown to what extent chronic hypoxia affects the upstream mechanisms at endothelial NO synthase (eNOS) levels and endothelium-dependent relaxation in pulmonary vessels in the fetus.

The present study was designed to test the hypothesis that chronic hypoxia during gestation differentially regulates pulmonary vascular contractility and relaxation in near-term fetal lambs. We determined the effect of chronic hypoxia on KCl- and norepinephrine-induced contractions, endothelium-dependent and -independent relaxations, and eNOS protein levels in pulmonary vessels of fetal lambs. Because both pulmonary arteries and veins contribute significantly to pulmonary vascular resistance in the fetus and newborn (Gao and Raj, 2005), we studied the effect of hypoxia on both pulmonary arteries and veins in fetal lambs.

Materials and Methods

Experimental Animals

Pregnant sheep of the same age and breed were obtained from the Nebeker Ranch in Lancaster, CA. Normoxic controls were maintained at the Nebeker Ranch (altitude: ~300 m; Pa_{O2}: 102 ± 2 Torr). For hypoxic exposure, pregnant sheep were transported to the Barcroft Laboratory, White Mountain Research Station, Bishop, CA (3,801-m

altitude; Pa_{O2}: 60 ± 2 Torr) at 30 days of gestation and maintained at high altitude for ~110 days, as previously described (Xiao et al., 2001, 2004). Near-term pregnant sheep (~140 days of gestation; term being 147 days) were transported to the Animal Care Facility at Loma Linda University. Ewes were anesthetized with thiamylal (10 mg/kg) administered via an external jugular vein, and anesthesia was maintained on 1.5–2.0% halothane in oxygen throughout surgery. Pulmonary arteries and veins were obtained from near-term fetuses of both normoxic control and chronically hypoxic pregnant sheep. All procedures and protocols used in the present study were approved by the Institutional Animal Care and Use Committee of Loma Linda University and followed the guidelines by the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Tissue Preparation and Contraction Studies

Preparation of pulmonary vessels was conducted in ice-cold Krebs solution (pH 7.4) of the following composition (in mM): 115.21 NaCl, 4.7 KCl, 1.80 $CaCl_2$, 1.16 $MgSO_4$, 1.18 KH_2PO_4, 22.14 $NaHCO_3$, 0.03 EDTA, and 7.88 dextrose. The Krebs solution was oxygenated with a mixture of 95% O_2 and 5% CO_2. Fourth-generation pulmonary arteries and veins were dissected and cut into rings of 4 mm in length (diameter of 1.5–2.0 mm for artery and 0.8–1.3 mm for vein). Isometric tensions of vessel rings were measured in Krebs solution in tissue baths at 37°C, as described previously (Xiao et al., 2001, 2004). After 60 min of equilibration in the tissue bath, each ring was stretched to the optimal resting tension, as determined by the tension developed in response to 120 mM KCl added at each stretch level. Norepinephrine-induced concentration-dependent contraction curves were determined by cumulative addition of the agonist in approximate one-half log increments in the absence or presence of eNOS

inhibitor N^G-nitro-L-arginine (L-NNA; 100 μM, pretreatment for 20 min). For relaxation studies, tissues were precontracted with submaximal concentration (3 μM) of norepinephrine, followed by acetylcholine and sodium nitroprusside added in a cumulative manner, respectively. The relaxation responses to acetylcholine and sodium nitroprusside were expressed as the percentage of norepinephrine precontractions.

Immunoblotting

Protein levels of eNOS were determined by Western blot analysis, as described previously (Xiao et al., 2001). Briefly, pooled segments of fourth-generation pulmonary arteries and veins, respectively, were homogenized in a lysis buffer containing 20 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, pH 7.4. Homogenates were then centrifuged at 4°C for 10 min at 12,000 *g*, and the supernatants were collected. Protein was quantified in the supernatant using a protein assay kit from Bio-Rad. Samples with equal protein were loaded on 7.5% polyacrylamide gel with 0.1% sodium dodecyl sulfate and separated by electrophoresis at 100 V for 90 min. Proteins then were transferred onto nitrocellulose membranes. Nonspecific binding sites on the membranes were blocked in a Tris-buffered saline solution containing 5% dry milk for 1 h at room temperature. The membranes were incubated with mouse eNOS monoclonal antibody (1:500) overnight at 4°C. The membranes then were incubated with secondary horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody (1:2,000). Proteins were visualized with enhanced chemiluminescence reagents, and the blots were exposed to Hyperfilm. For comparison of eNOS between arteries and veins, the bands were measured as absolute density values.

For comparison of eNOS between normoxia and hypoxia for a given vessel type, eNOS bands were normalized to those of actin used as a loading control. Results were quantified by the Kodak electrophoresis documentation and analysis system and Kodak ID image analysis software.

Histological Analysis and Immunohistochemistry

Pulmonary vessel rings were fixed in 10% neutral buffered formalin and embedded in paraffin. Immunohistochemical detection of eNOS and Von Willebrand Factor (vWF) was performed using Pharmingen Anti-Ig HRP Detection Kit, as described previously (Xiao et al., 2007). Briefly, tissue slices (4 μm thick) of vessel rings were incubated with primary antibodies against eNOS (1:100) or vWF (1:200) for 60 min at room temperature. After rinsing the slices three times in phosphate-buffered saline for 15 min, the slices were incubated with biotinylated goat anti-mouse IgG or anti-rabbit Igs (1:50) for 60 min at room temperature. The samples were then exposed to streptravidin-HRP and reacted with diaminobenzidine substrate solution according to the manufacture's recommendations and counterstained with hematoxylin. The negative control of eNOS staining was performed in the absence of eNOS antibody. For histological analysis of medial wall thickness, tissue slides were stained with hematoxylin and eosin. The slices were viewed with an Olympus BH-2 microscope, and images were captured with an attached SPOT digital camera imaging system.

Materials

Norepinephrine, L-NNA, vWF antibody, and sodium nitroprusside were obtained From Sigma (St. Louis, MO). Mouse anti-eNOS monoclonal antibody was from Transduction Laboratory (Lexington, KY). Anti-Ig HRP Detection Kits were from BD Biosciences (San Diego, CA). Electrophoresis and Western blotting reagents were from Bio-Rad (Hercules, CA).

Data Analysis

Concentration-response curves were analyzed by computer-assisted nonlinear regression to fit the data using GraphPad Prism (GraphPad Software, San Diego, CA) to obtain the values of pD_2 ($-\log EC_{50}$) and the maximum response. Results were expressed as means ± SE, and the differences were evaluated for statistical significance ($P < 0.05$) by Student's *t*-test or two-way ANOVA followed by Bonferroni posttests.

Results

Effect of Chronic Hypoxia on Medial Wall Thickness of Pulmonary Vessels

Fig. 1 shows the effect of hypoxia on medial wall thickness of 4th generation pulmonary arteries and veins in near-term fetal lambs. The medial wall was significantly thicker in pulmonary arteries than that in pulmonary veins. Chronic hypoxia showed no significant effect on medial wall thickness in either pulmonary arteries or veins in fetal lambs (Fig. 1).

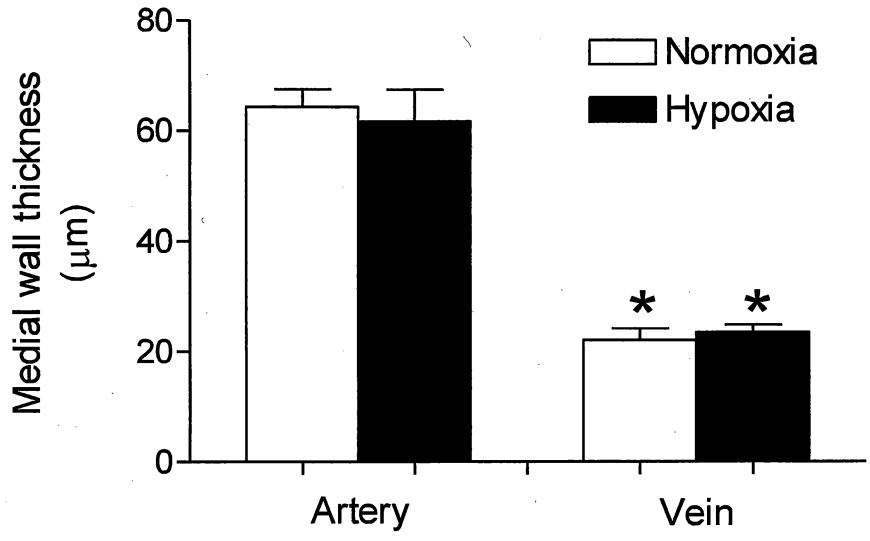

Figure 1. Effect of chronic hypoxia on medial wall thickness of fetal pulmonary arteries and veins. Bar graphs show medial wall thickness of pulmonary arteries and veins obtained from near-term fetal lambs of normoxic control and hypoxic ewes. Data were analyzed by two-way ANOVA with vessel type as one factor and hypoxia as the other. The asterisk (*) indicates a significant difference ($P < 0.05$) from artery ($n = 4$).

Effect of Chronic Hypoxia on KCl- and Norepinephrine-Induced Contractions

The effect of hypoxia on KCl-induced contractions is illustrated in Fig. 2. In both normoxic and hypoxic lambs, KCl-induced contractions were significantly greater in pulmonary veins than arteries. Chronic hypoxia had no significant effect on KCl-induced contractions in either pulmonary arteries or veins (Fig. 2).

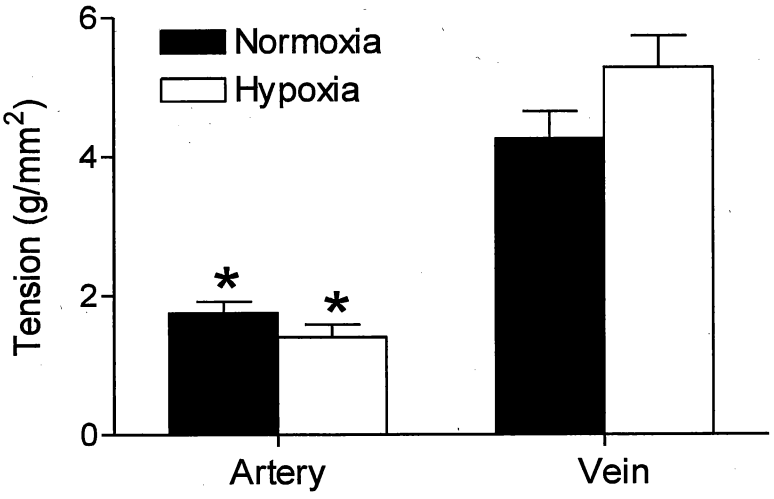

Figure 2. Effect of chronic hypoxia on KCl-induced contractions of fetal pulmonary arteries and veins. Bar graphs show KCl-induced contractions of pulmonary arteries and veins obtained from near-term fetal lambs of normoxic control and hypoxic ewes. Data were analyzed by two-way ANOVA with vessel type as one factor and hypoxia as the other. The asterisk (*) indicates a significant difference ($P < 0.05$) from vein ($n = 5$).

Fig. 3 shows the effect of hypoxia on norepinephrine-induced concentration-dependent contractions of fetal pulmonary vessels in the absence or presence of the eNOS inhibitor L-NNA. In pulmonary arteries, L-NNA had no significant effect on norepinephrine-induced contractions in either control or hypoxic vessels (Fig. 3, *top*). Chronic hypoxia significantly increased the maximal response of norepinephrine-induced contractions in pulmonary arteries regardless of L-NNA treatment (Fig. 3, *bottom* and Table 1). In contrast to arteries, L-NNA significantly increased norepinephrine-induced maximal contractions of pulmonary veins from both control and hypoxic fetal lambs, but the effect of L-NNA was significantly reduced in hypoxic fetal lambs (Fig. 3, *bottom* and Table 1).

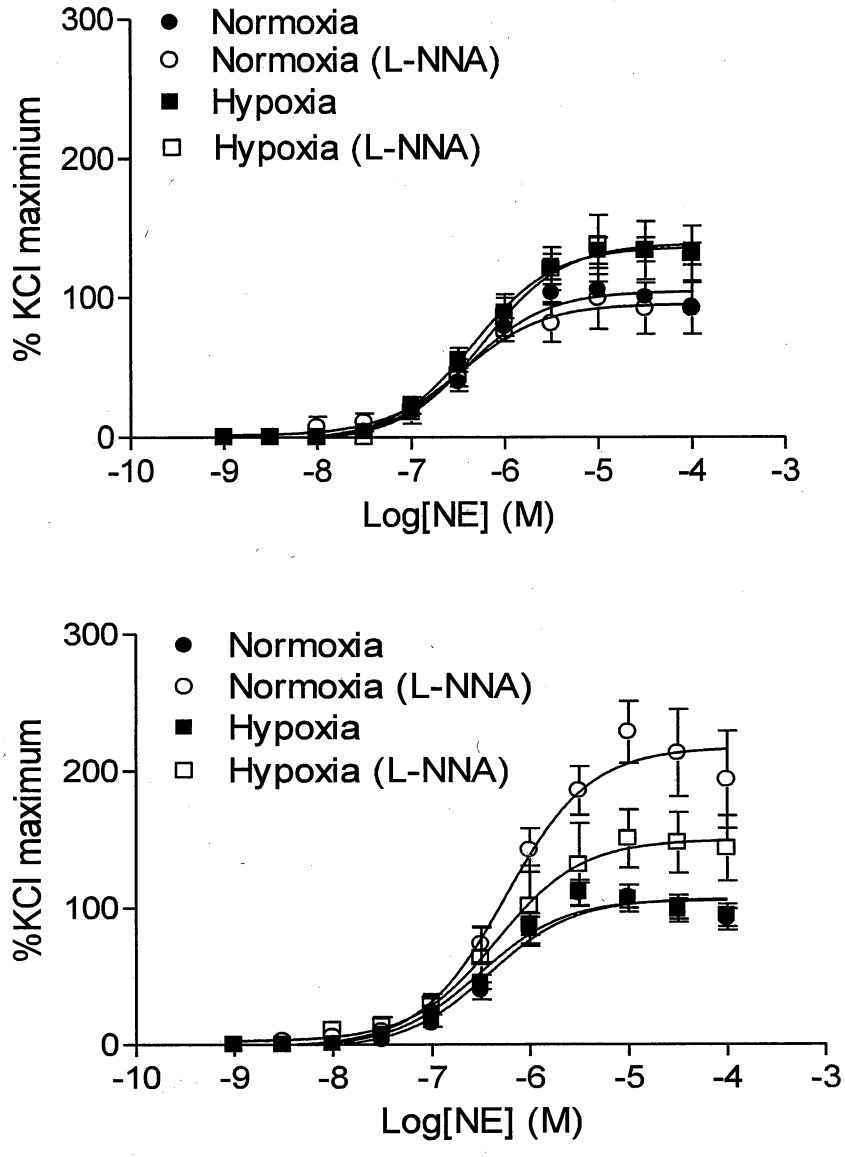

Figure 3. Effect of chronic hypoxia on norepinephrine-induced contractions of fetal pulmonary arteries (upper panel) and veins (lower panel). Norepinephrine (NE)-induced contractions were determined in the absence or presence of L-NNA (100 μM, 20 min) in pulmonary arteries and veins obtained obtained from near-term fetal lambs of normoxic control and hypoxic ewes. Data are expressed as percent of KCl (120 mM)-induced contractions and are means ± SEM of tissues from 5 to 6 animals. The pD_2 values and the maximal responses are presented in Table 1.

Table 1. Effect of chronic hypoxia on norepinephrine-induced contractions of pulmonary arteries and veins from fetal lambs in the absence or presence of L-NNA.

	Normoxia		Hypoxia	
	pD_2	E_{max}	pD_2	E_{max}
Artery				
-L-NNA	6.41 ± 0.07	104.6 ± 2.6	6.34 ± 0.08	136.4 ± 4.0 [a]
+L-NNA	6.45 ± 0.17	95.3 ± 5.7	6.23 ± 0.14	138.9 ± 7.7 [a]
Vein				
-L-NNA	6.43 ± 0.09	106.6 ± 3.6	6.53 ± 0.09	106.0 ± 3.5
+L-NNA	6.25 ± 0.12	217.1 ± 10.1	6.35 ± 0.19	149.7 ± 10.1[a,b]

pD_2: -log EC_{50}, E_{max}: maximum response. [a] $P < 0.05$, normoxia *vs.* hypoxia; [b] $P < 0.05$, +L-NNA *vs.* –L-NNA. N = 5 to 6 in each group.

Effect of Chronic Hypoxia on Endothelium-Dependent and Independent Relaxations

The endothelium-dependent relaxations induced by acetylcholine were determined in pulmonary vessels pre-contracted with 3 μM norepinephrine. Acetylcholine had no effect in pulmonary arteries, but produced concentration-dependent relaxations of pulmonary veins in both control and hypoxic fetal lambs (Fig. 4). Hypoxia significantly decreased pD_2 (6.10 ± 0.08 *vs.* 6.43 ± 0.10, $P < 0.05$) and the maximal relaxation (41.6 ± 2.1% *vs.* 49.7 ± 2.4%, $P < 0.05$) induced by acetylcholine in pulmonary veins (Fig. 4). Unlike acetylcholine, sodium nitroprusside produced concentration-dependent relaxations in both pulmonary arteries and veins. Hypoxia significantly decreased sodium nitroprusside-induced relaxations in both pulmonary arteries and veins (Fig. 5 and Table 2).

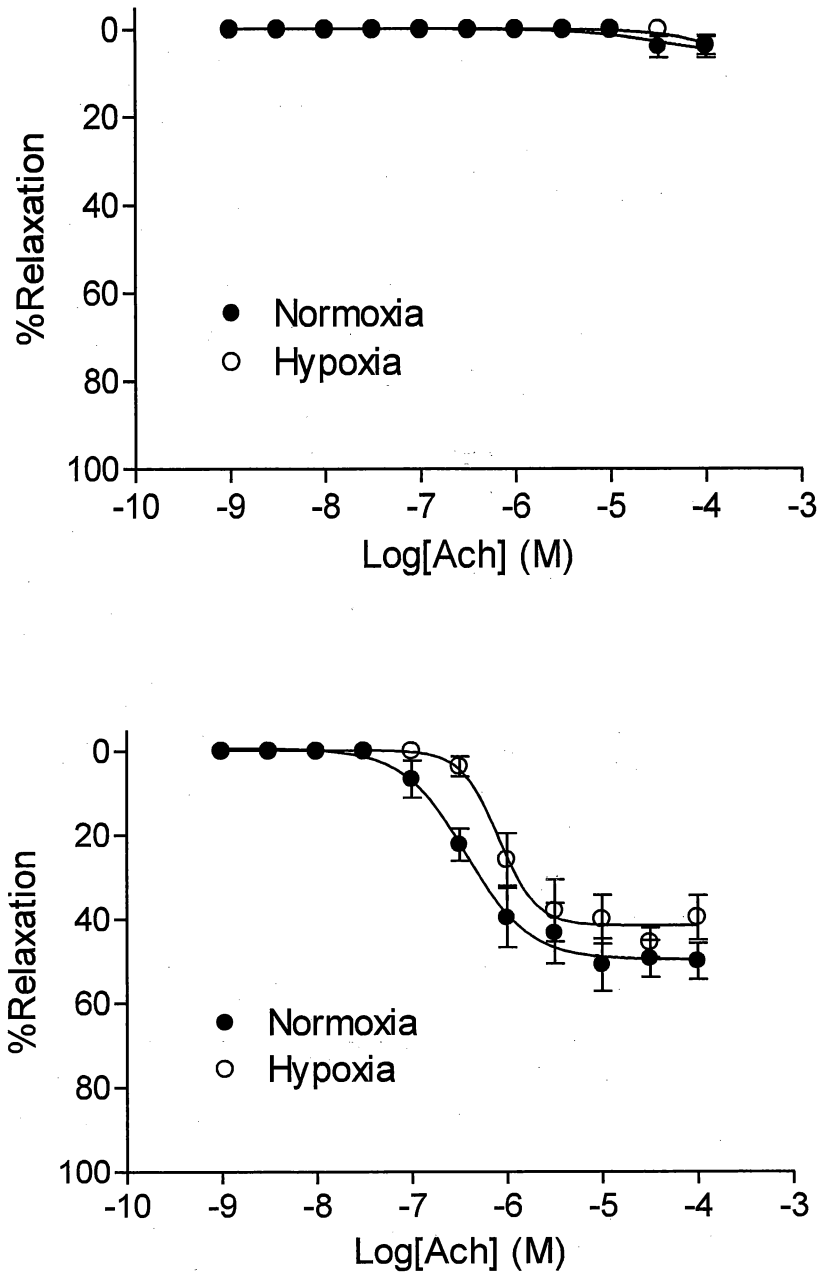

Figure 4. Effect of chronic hypoxia on acetylcholine-induced relaxations in fetal pulmonary arteries (upper panel) and veins (lower panel). Acetylcholine (Ach)-induced relaxations were determined in pulmonary arteries and veins (pre-contracted with 3 μM norepinephrine) obtained from near-term fetal lambs of normoxic control and hypoxic ewes. Data are means ± SEM of tissues from 5 to 6 animals. The pD_2 value and the maximal response are presented in the text.

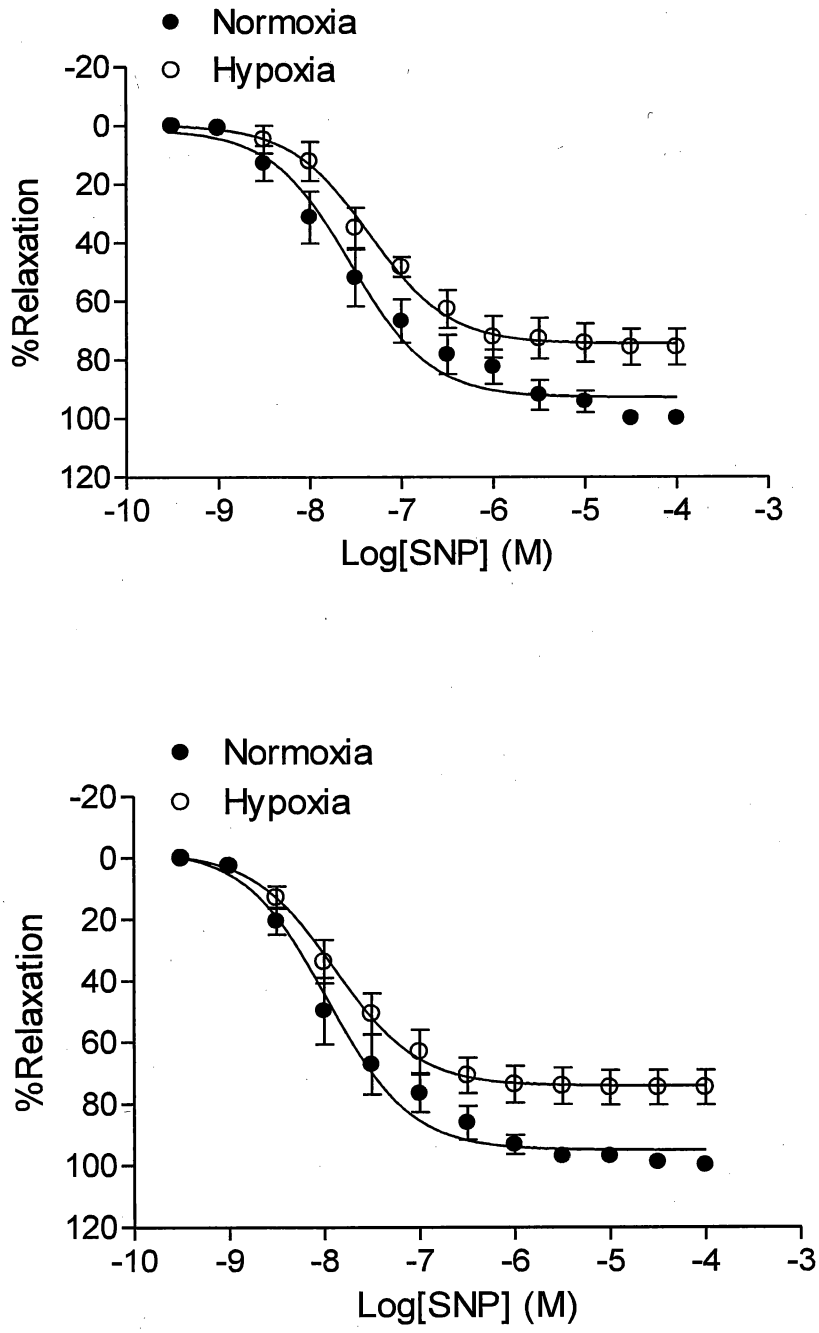

Figure 5. Effect of chronic hypoxia on sodium nitroprusside-induced relaxations in fetal pulmonary arteries (upper panel) and veins (lower panel). Sodium nitroprusside (SNP)-induced relaxations were determined in pulmonary arteries and veins (pre-contracted with 3 μM norepinephrine) obtained from near-term fetal lambs of normoxic control and hypoxic ewes. Data are means ± SEM of tissues from 5 to 6 animals. The pD_2 value and the maximal response are presented in Table 2.

Table 2. Effect of chronic hypoxia on sodium nitroprusside-induced relaxations in pulmonary arteries and veins from fetal lambs.

	Normoxia		Hypoxia	
	pD_2	E_{max}	pD_2	E_{max}
Artery	7.5 ± 0.1	92.9 ± 2.5	7.3 ± 0.1	74.5 ± 2.4[a]
Vein	8.0 ± 0.1	95.1 ± 2.1	7.9 ± 0.1	74.3 ± 2.1[a]

pD_2: -logEC_{50}, E_{max}: maximum response. [a] $P < 0.05$, normoxia *vs.* hypoxia. N = 5 to 6 in each group.

Effect of Chronic Hypoxia on eNOS Expression

eNOS protein levels and distribution in fetal pulmonary vessels were determined with immunohistochemistry and immunoblotting. As shown in Fig. 6, eNOS immunoreactivity was primarily detected in the endothelium of the pulmonary arteries and veins. Additionally, the endothelium was labeled with vWF (Fig. 6). Both immunoblotting and immunohistochemistry analyses showed significantly higher levels of eNOS expression in pulmonary veins than that in arteries (Fig. 6). In either pulmonary arteries or veins, eNOS protein levels were not significantly different between the control and hypoxic fetal lambs (Fig. 7).

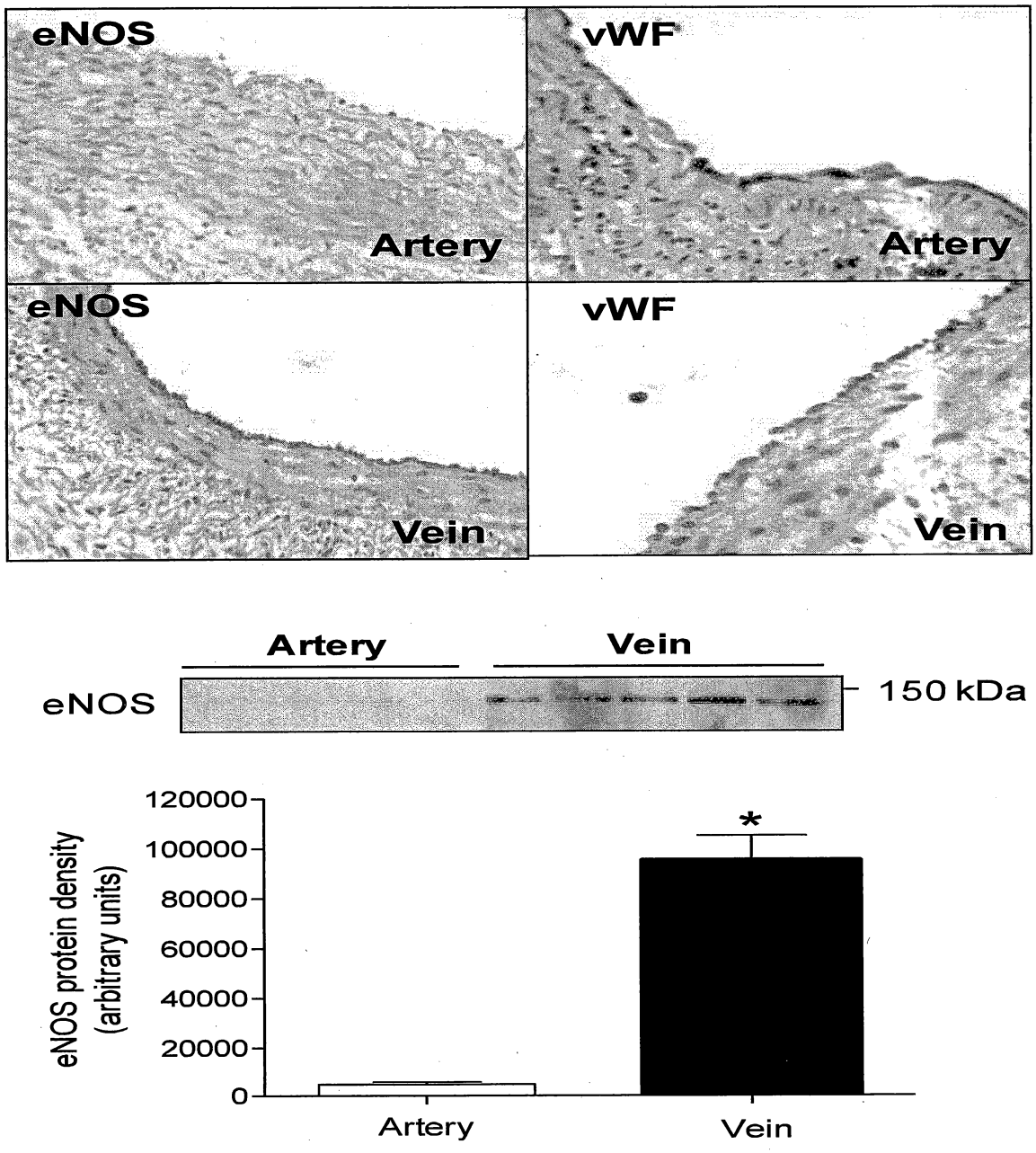

Figure 6. Localization and density of eNOS in fetal pulmonary arteries and veins. eNOS localization and density were determined in fetal pulmonary arteries and veins by immunohistochemical staining (upper panel) and immunoblotting (lower panel), respectively. eNOS expression was detected in the endothelium of fetal pulmonary arteries and veins. Additionally, the endothelium was labeled with vWF. Western blot illustrates eNOS bands detected by the monoclonal antibody at the expected size of ~140 kDa. Data are means ± SEM of tissues from 4 animals in each group. The asterisk (*) indicates a significant difference ($P < 0.05$) from artery.

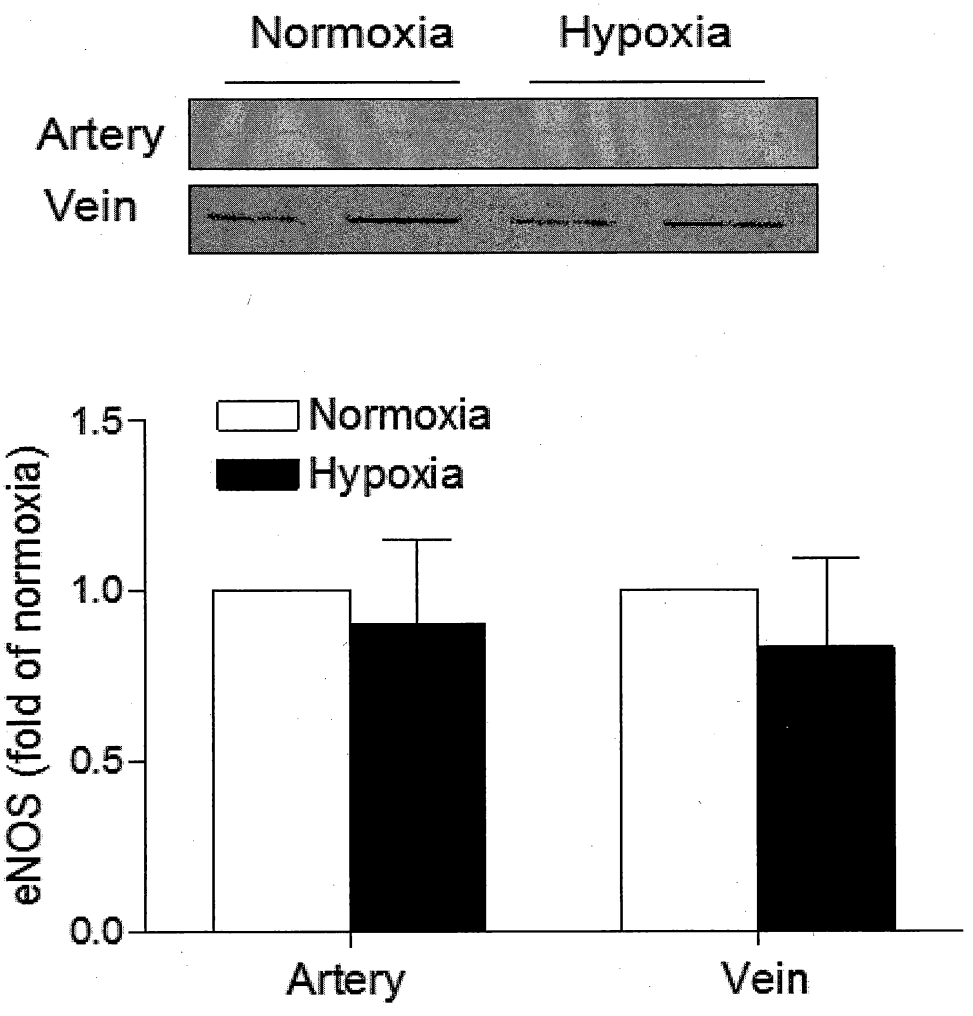

Figure 7. Effect of chronic hypoxia on eNOS protein levels in fetal pulmonary arteries and veins. eNOS protein levels were determined by Western blot in pulmonary arteries and veins obtained from near-term fetal lambs of normoxic control and hypoxic ewes. Data are means ± SEM of tissues from 4 animals in each group.

Discussion

The major findings of the present study are that chronic hypoxia in utero differentially regulates contractions and relaxations of small pulmonary arteries and veins in near-term fetal lambs by *1*) increasing norepinephrine-induced contractions of pulmonary arteries; *2*) decreasing norepinephrine-induced contractions of pulmonary veins in a NO-dependent manner; *3*) reducing endothelium-dependent relaxations selectively in pulmonary veins; *4*) decreasing endothelium-independent relaxations of both pulmonary arteries and veins; and *5*) having no significant effect on eNOS protein levels in either pulmonary arteries or veins. Additionally, chronic hypoxia has no significant effect on KCl-induced contractions and medial wall thickness in either pulmonary arteries or veins in fetal lambs.

In the present study, we found no significant difference in medial wall thickness of small pulmonary vessels between normoxic and hypoxic fetal lambs. Although it is unlikely that samples of hypoxic and normoxic vessels shrink differently during tissue fixation and thus prevent the observation of treatment differences in medial wall thickness, this possibility cannot be excluded. Nonetheless, the present finding is consistent with previous studies in rats and guinea pigs, in which chronic in utero hypoxemia did not change pulmonary arterial structure (Geggel et al., 1986; Murphy et al., 1986). Additionally, hypoxia did not cause hyperplasia or hypertrophy of the media of pulmonary arteries in near-term bovine fetuses (Bentitz et al., 1986). This is further supported with the finding that KCl-induced contractions were not significantly different in pulmonary vessels obtained from control and hypoxic fetal lambs, suggesting the same vascular smooth muscle mass in control and hypoxic pulmonary vessels. Additionally, the

finding suggests that hypoxia had no significant effect on voltage-gated calcium channel density in fetal pulmonary vessels. A recent study showed increased KCl-induced contractions of fourth-generation pulmonary arteries from highland (3,600 m above sea level) newborn (average 10 days of age) lambs compared with lowland (580 m above sea level) newborn lambs (Herrera et al., 2007). Although medial wall thickness was not measured, the authors suggested a greater vascular smooth muscle mass in highland newborn lambs. Taken together, these studies suggest that pulmonary vascular remodeling in hypoxic infants may not occur during fetal life but rather in the transition from prenatal to postnatal life and during the early neonatal period, when changes in pulmonary structure and function are particularly sensitive to hypoxia (Niermeyer, 2003, 2007). Whether hypoxic fetuses are more vulnerable to pulmonary vascular remodeling during the early neonatal period is not clear and remains an intriguing area for future investigation.

Despite the thinner media, fetal pulmonary veins showed greater contractions to KCl compared with arteries. This is consistent with previous findings in the pulmonary circulation that, during the perinatal period, veins exhibit greater contractions than arteries in response to a variety of stimuli (Arrigoni et al., 1999; Gao and Raj, 2005). The present study has demonstrated a complex relationship between the effects of chronic hypoxia on fetal pulmonary arteries vs. veins and the role of NO in utero vs. in vitro. In the absence of L-NNA, hypoxia significantly increased the maximal response of norepinephrine-induced contractions in pulmonary arteries, but not veins, in fetal lambs. This suggests that in utero, when NO is present, the site of resistance under conditions of hypoxia in the intact pulmonary circulation is greater in pulmonary arteries than in veins. It has been

demonstrated that chronic hypoxia produces an increase in α_1-adrenoreceptor gene expression and increases pulmonary vascular smooth muscle contractile sensitivity, which is thought to play an important role in the development of pulmonary hypertension (Lal et al., 1999; Salvi 1999). Fetal pulmonary vascular beds are under α1-adrenergic control, and fetal pulmonary arteries contract to norepinephrine (Nuwayhid et al., 1975; Thompson and Weiner, 1993; Irish et al., 1998). The finding that norepinephrine-induced contractions in the presence of L-NNA were significantly decreased in pulmonary veins of hypoxic fetal lambs is intriguing and suggests a compensatory adaptation mechanism in vascular contractility of pulmonary veins to chronic in utero hypoxia. Given that both arteries and veins contribute almost equally to pulmonary vascular resistance during perinatal development (Gao and Raj, 2005), the opposite changes in vascular contractility of pulmonary arteries and veins, demonstrated in the present study, may result in minimal changes in pulmonary vascular resistance in near-term fetal lambs in response to chronic in utero hypoxia.

The finding that L-NNA had no effect on norepinephrine-mediated contractions of fetal pulmonary arteries suggests a lack of basal inhibitory effect of eNOS in the regulation of pulmonary arterial contractility in near-term fetal lambs. This is supported by the findings of the minimal eNOS levels and the lack of acetylcholine-induced relaxations in pulmonary arteries. This is consistent with the previous study in fetal lambs showing a lack of acetylcholine-induced relaxations in pulmonary arteries ((Steinhorn et al., 1993; Gao et al., 1995). Additionally, it was demonstrated that calcium ionophore A23187 failed to relax pulmonary arteries in fetal lambs (Irish et al., 199). Taken together, these studies have demonstrated that the endothelium is not functional in NO-

mediated relaxation in pulmonary arteries in near-term fetal lambs resulting from minimal eNOS protein levels. Nevertheless, sodium nitroprusside produced concentration-dependent relaxations in pulmonary arteries. Because sodium nitroprusside is an NO donor and relaxes vascular smooth muscle via activation of guanylate cyclase and increasing cGMP, the finding suggests that the downstream pathway of cGMP-dependent protein kinase is fully functional in fetal pulmonary arteries, as demonstrated in the present as well as in previous studies (Irish et al., 1998; Gao et al., 2003, 2007; Steinhorn et al., 1993). The present study demonstrated that chronic hypoxia had no effect on eNOS protein levels and endothelium-dependent relaxation in fetal pulmonary arteries, albeit it decreased the downstream pathway of cGMP-dependent relaxations. The similar finding of decreased cGMP-dependent relaxations in fetal pulmonary arteries was obtained in a recent study (Gao et al., 2007). Because of the lack of NO-dependent relaxation and the lack of effect of chronic hypoxia on eNOS, the decreased downstream cGMP-dependent relaxations may minimally affect pulmonary arterial tone in the fetus but may be detrimental in the transition of pulmonary arterial contractility and structure from prenatal to postnatal life, in which eNOS/NO becomes a key mechanism in the regulation of pulmonary arterial reactivity.

In contrast to pulmonary arteries, pulmonary veins in near-term fetal lambs have much higher levels of eNOS in the endothelium and relax significantly to acetylcholine. Additionally, inhibition of eNOS by L-NNA significantly increased norepinephrine-induced contractions, suggesting a significant component of basal eNOS activity in the inhibition of pulmonary vein contractility. This is consistent with previous studies (Gao et al., 1995; Steinhorn et al., 1993; Tzao et al., 2001). It has been demonstrated in intact

fetal sheep that acetylcholine produces a decrease in pulmonary vascular resistance and an increase in pulmonary blood flow, which are blocked by eNOS inhibitors L-NNA and L-NMMA (Abman et al., 1990; Tiktinsky et al., 1992). Given the lack of eNOS-mediated relaxation in pulmonary arteries, eNOS/NO-mediated regulation of fetal pulmonary vascular resistance and pulmonary blood flow resides primarily in pulmonary veins. In the present study, we have shown that chronic in utero hypoxia results in a significant decrease in NO-mediated relaxations in fetal pulmonary veins. This is supported by the finding that acetylcholine-induced relaxation of pulmonary vein was significantly decreased in hypoxic fetal lambs. Additionally, the effect of eNOS inhibitor L-NNA in increasing norepinephrine-induced contractions was significantly decreased in pulmonary vein of hypoxic compared with normoxic fetal lambs. The findings that hypoxia had no significant effect on eNOS protein levels in pulmonary veins suggests that the inhibition may occur at downstream pathways. Consistent with this notion, the present study demonstrated that sodium nitroprusside-induced relaxations were significantly decreased by chronic hypoxia in both pulmonary veins and arteries. In the same animal model, a recent study demonstrated that 8-Br-cGMP (stimulator of PKG) caused a similar relaxation of pulmonary veins obtained from control and hypoxic fetal lambs (Longo LD, personal communication). Taken together, these findings suggest a likely mechanism of decreased soluble guanylate cyclase (sGC) and reduced cGMP production in fetal pulmonary vessels in response to in utero chronic hypoxia. Indeed, long-term high-altitude hypoxemia significantly decreased sGC abundance and catalytic activity in carotid arteries of fetal lambs (Williams et al., 2006). Additionally, chronic hypoxia

decreased expression of sGC in rat pulmonary artery smooth muscle cells (Hassoun et al., 2004).

In conclusion, we have demonstrated heterogeneity in responses of pulmonary arteries and veins in near-term fetal lambs to long-term high-altitude hypoxemia. Although chronic hypoxia in utero has no significant effect on the medial wall thickness of pulmonary vessels in fetal lambs, it significantly increases vasoconstriction of pulmonary arteries and decreases vasorelaxation of pulmonary veins. Furthermore, our studies demonstrate that chronic hypoxia has no significant effect on eNOS protein levels in either pulmonary arteries or veins in near-term fetal lambs and point to a likely common mechanism of decreased sGC in fetal pulmonary arteries and veins in response to chronic hypoxia in utero. This is consistent with the growing literature demonstrating the importance of sGC in addition to eNOS in the regulation of perinatal as well as adult pulmonary circulation (Tzao et al., 2001, Dumitrascu et al., 2006; Vermeersch et al., 2007). Future studies are needed to determine the effect of chronic hypoxia on sGC protein levels and catalytic activity in fetal pulmonary vessels.

Acknowledgments

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CHAPTER THREE

PRENATAL HYPOXIA CAUSES A SEX-DEPENDENT INCREASE IN HEART SUSCEPTIBILITY TO ISCHEMIA AND REPERFUSION INJURY IN ADULT MALE OFFSPRING: ROLE OF PKCε

By

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Abstract

The present study tested the hypothesis that PKCε plays a key role in the sex dichotomy of heart susceptibility to ischemia and reperfusion injury in adult offspring resulted from prenatal hypoxic exposure. Time-dated pregnant rats were divided between normoxic and hypoxic (10.5% O_2 from day 15 to 21 of gestation) groups. Hearts of 3-month-old progeny were subjected to ischemia and reperfusion (I/R) injury in a Langendorff preparation. Pre-ischemic values of left ventricle (LV) function were the same between control and hypoxic animals. Prenatal hypoxia significantly decreased post-ischemic 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 PKCε and phospho-PKCε levels in the LV of the male, but not female, rats. The PKCε translocation inhibitor peptide (PKCε-TIP) significantly decreased phospho-PKCε 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, PKCε-TIP inhibited PKCε phosphorylation and decreased post-ischemic recovery of LV function equally well in both control and hypoxic animals. PKCε-TIP had no effect on PKCδ 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 PKCε gene repression resulting in a down-regulation of PKCε function in the heart of adult male offspring.

Introduction

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, heart, lung and kidney disease, or with hemoglobinopathy. There is clear evidence of a link between hypoxia and fetal intrauterine growth restriction (Unger et al., 1988; Moore, 2003). 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; Moore, 2003). Additionally, chronic hypoxia suppresses fetal cardiac function, alters cardiac gene expression pattern, and increases heart to body weight ratio (Kamitomo et al., 1992; Murotsuki et al., 1997; Martin et al., 1998; Xiao et al., 2000; Rouwet et al., 2002; Zhang, 2005).

Animal studies have suggested a possible link between prenatal hypoxia and increased risk of cardiovascular disease in offspring (Heydeck et al., 1994; Roigas et al., 1996; Butler et al., 2002; Peyronnet et al., 2002; Davis et al., 2003; Li et al., 2003; Jones et al., 2004; Mone et al., 2004; Zhang, 2005). Studies in a pregnant rat model demonstrated that maternal hypoxia caused an increase in HIF-1α 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). Additionally, 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, the 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 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.

Additionally, 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 Cε (PKCε) plays a pivotal role of cardioprotection during cardiac ischemia and reperfusion injury (Chen et al., 2001; Ping et al., 2001; Murriel and Mochly, 2003). Studies in a PKCε knock-out mouse model have demonstrated that PKCε expression is not required for cardiac function under normal physiological conditions, but PKCε activation is necessary and sufficient for acute cardioprotection during cardiac ischemia and reperfusion (Gray et al., 2004). Herein, 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 PKCε gene

repression resulting in a down-regulation of PKCε 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. 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 mmHg (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, the 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 mmHg) with gassed (95% O_2-5% CO_2) Krebs-Heinseleit 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 (mmHg) and its first derivative (dP/dt). LV end diastolic pressure (LVEDP) was set at about 5 mmHg. Control hearts were perfused continuously, subjected to 20 min of global ischemia by stopping the perfusion, and then followed by 30min reperfusion. Some hearts were perfused with 5 μM PKCε translocation inhibitor peptide (EAVSLKPT; PKCε-TIP; Calbiochem) or 5 μM scrambled PKCε translocation inhibitor peptide (LSETKPAV; Scrambled PKCε-TIP; Calbiochem) for 20 min prior ischemia and reperfusion with no wash-out period respectively. Both in vivo study (Przyklenk et al., 2003) and isolated buffer-perfused rat heart study (Bae and Zhang, 2005; Pierre et al., 2007) confirmed that PKCε-TIP, an octapeptide confirmed to selectively inhibit translocation of the PKCε. LV functional parameters, LV developed pressure (LVDP), heart rate (HR), dP/dt_{max}, dP/dt_{min}, and LVEDP were continuously recorded with an on-line computer. Pulmonary artery effluent was collected as an index of coronary flow.

Myocardial Infarct Size

Myocardial infarct size was measured as previously described (Bae and Zhang, 2005). Briefly, at the end of reperfusion, left ventricles were collected, cut into four slices, incubated with 1% triphenyltetrazolium chloride (TTC) solution for 15 min at 37°C, and immersed in formalin for 30 min. Each slice was then photographed (Kodak digital camera) separately, and the areas of myocardial infarction (MI) in each slice were

analyzed by computerized planimetry (Image-Pro Plus), corrected for the tissue weight, summed for each heart, and expressed as a percentage of the total left ventricle weight.

Lactate Dehydrogenase (LDH) Activity Measurement

LDH activity was measured as previously described (Pierre et al., 2007). Briefly, 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, Saint Louis, MO), following the manufacture's directions.

Western Blot Analysis

At the end of reperfusion, left ventricles were isolated, and protein levels of PKCε, phospho-PKCε, PKCδ, and phospho-PKCδ 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 μg/ml leupeptin, and 5μg/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 (Hercules, CA). Samples with equal proteins were loaded on to 7.5% polyacrylamide gel with 0.1% sodium dodecyl sulfate and were separated by electrophoresis at 100 V for 2 h. Proteins were then transferred to nitrocellulose membrane and incubated with primary antibodies for PKCε, PKCδ (Santa Cruz Biotechnology, Santa Cruz, CA), phospho-PKCε, and phospho-PKCδ (Upstate Biotechnolgy; Lake Placid, NY), respectively. After washing,

membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Amersham, Arlington Heights, IL). Proteins were visualized with enhanced chemiluminescence reagents, and blots were exposed to Hyperfilm. Results were quantified with the Kodak electrophoresis documentation and analysis system and Kodak ID image analysis software. To minimize any confounding influence of variability among gels, internal control were loaded in each gel and band intensities were normalized to actin and internal control.

Statistical Analysis

Data were expressed as means ± SEM. Experimental number (n) represents offspring from different dams. Statistical significance ($P < 0.05$) was determined by two-way ANOVA followed by Neuman-Keuls post hoc testing.

Results

Body Weight and Baseline Cardiac Function

As shown in table 1, neither male nor female 3-month-old offspring showed significant difference in body mass and heart weight between control and prenatally hypoxic groups. LVDP, HR, dP/dt_{max}, dP/dt_{min}, and coronary flow rate at baseline were not significantly different among all groups in either male or female offspring (Table 1).

Table 3. Pre-ischemic left ventricle functional parameters

	BW	HW	HR	LVEDP	LVDP	dP/dt_{max}	dP/dt_{min}	CF
	g		bpm	mmHg		mmHg/s		ml/min
Male								
C	492±11	1.3±0.1	253±6.9	5.2±0.2	100.5±2.8	3789±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±142	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±155	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±71	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±156	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±92	1863±135	8.9±0.3

BW, body weight; C, control; H, hypoxia; S-TIP, scrambled PKC-TIP; TIP, PKC-TIP; HW, heart weight; CF, coronary flow. n = 5-11

Post-Ischemic Recovery of LV Function in Male Hearts

Global ischemia for 20 min caused a persistent impairment in LV function in all five groups. As shown in Fig. 8, compared with the control group, there were significant decreases in post-ischemic 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). Recovery of LV function was not significantly different between the control and Scrambled PKCε-TIP group (Fig. 8). Inhibition of PKCε with PKCε-TIP, but not Scrambled PKCε-TIP, resulted in significant decreases in postischemic recovery of LVDP, dP/dt_{max} and dP/dt_{min} in the heart of control animals (Fig. 8). In contrast, it had no effect on post-ischemic recovery of LV function in the heart of hypoxic animals (Fig. 8). In the presence of PKCε-TIP, there was no

difference in post-ischemic 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 PKCε-TIP (Fig. 8). Post-ischemic recovery of LV function in female hearts. In contrast to the finding in the male offspring, prenatal hypoxia showed no effect on post-ischemic recovery of LVDP, dP/dt_{max} and dP/dt_{min} in the female offspring (Fig. 9). PKCε-TIP significantly decreased post-ischemic recovery of LV function in the hearts of both control and hypoxic groups (Fig. 9). There was no difference in post-ischemic recovery of LV function between the control and hypoxic animals either in the absence or presence of PKCε-TIP.

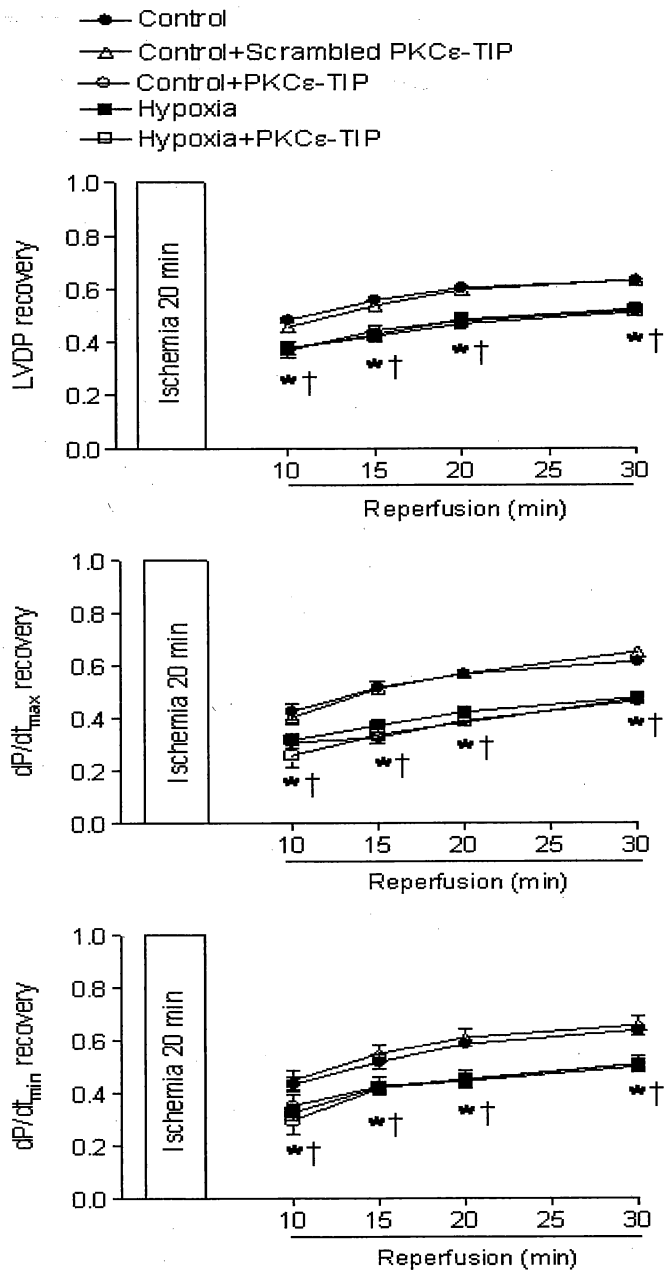

Figure 8. Effect of prenatal hypoxia on post-ischemia recovery of left ventricle function in male offspring. Hearts were isolated from 3-month-old male offspring that exposed to normoxia (control) or hypoxia before birth, and were pretreated in the absence or presence of 5 μM PKCε-TIP for 20 min before subjecting to 20 min of ischemia and 30 min of reperfusion in a Langendorff preparation. Post-ischemic recovery of left ventricle function during reperfusion was measured relative to the pre-ischemic values. LVDP, left ventricular developed pressure; dP/dt_{max}, maximal rate of contraction; dP/dt_{min}, maximal rate of relaxation. Data are means ± SEM. * $P < 0.05$, *vs.* control, n = 5-11

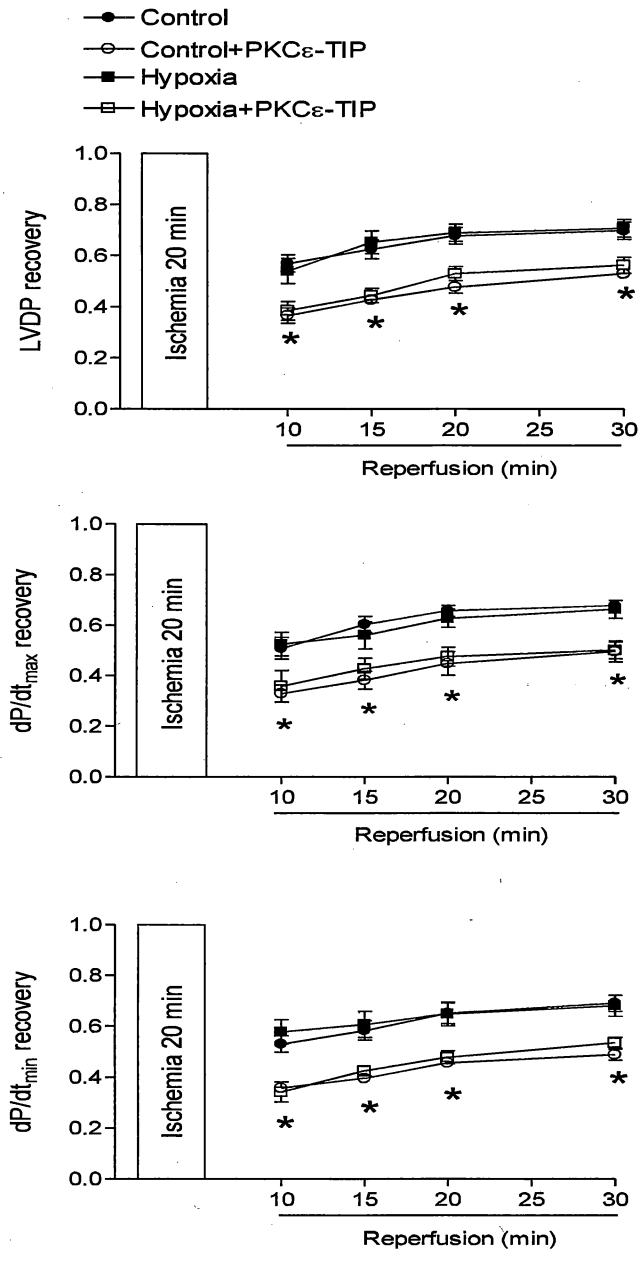

Figure 9. Effect of prenatal hypoxia on post-ischemia recovery of left ventricle function in female offspring. Hearts were isolated from 3-month-old female offspring that exposed to normoxia (control) or hypoxia before birth, and were pretreated in the absence or presence of 5 μM PKCε-TIP for 20 min before subjecting to 20 min of ischemia and 30 min of reperfusion in a Langendorff preparation. Post-ischemic recovery of left ventricle function during reperfusion was measured relative to the pre-ischemic values. LVDP, left ventricular developed pressure; dP/dt_{max}, maximal rate of contraction; dP/dt_{min}, maximal rate of relaxation. Data are means ± SEM. * $P < 0.05$, +PKCε-TIP *vs.* -PKCε-TIP, n = 5-6

Myocardial Infarction and Lactate Dehydrogenase (LDH) Release

In male animals, ischemia and reperfusion-induced increase in LVEDP was significantly higher in the hearts of hypoxic group as compared with that in the control group (Fig. 10A). This was consistent with the significant increases in myocardial infarct size and LDH release in the hearts of hypoxic animals (Fig. 10B and 10C). There were no differences in LVEDP, myocardial infarct size, and LDH release between control and Scrambled PKCε-TIP groups (Fig. 10). PKCε-TIP, but not Scrambled PKCε-TIP, significantly increased LVEDP (Fig. 10A), myocardial infarct size (Fig. 10B), and LDH release (Fig. 10C) in the control group. In contrast, it had no effects on LVEDP, myocardial infarct size, and LDH release in the hypoxic group (Fig. 10). In the presence of PKCε-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 heart of hypoxic group in the absence of PKCε-TIP (Fig. 10). 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. 11). PKCε-TIP increased LVEDP (Fig. 11A), myocardial infarct size (Fig. 11B), and LDH release (Fig. 11C) to the same extent in both the control and hypoxic groups.

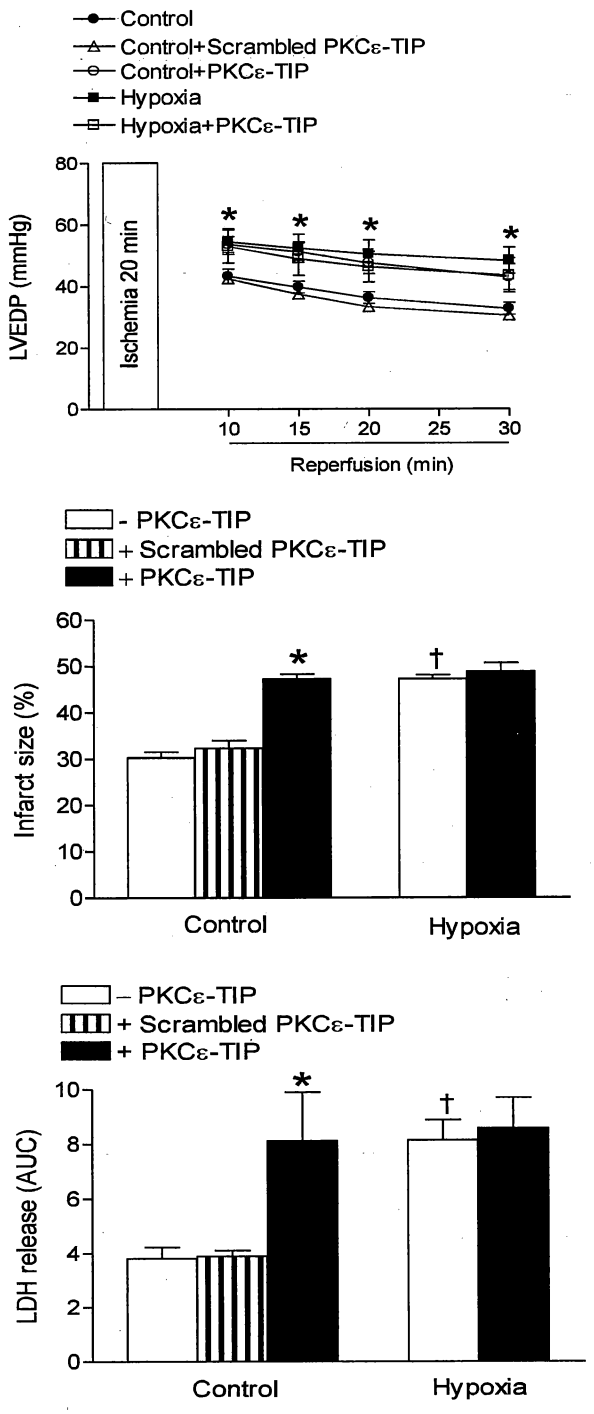

Figure 10. Effect of prenatal hypoxia on ischemia and reperfusion injury of left ventricle in male offspring. Hearts were isolated from 3-month-old male offspring that exposed to normoxia (control) or hypoxia before birth, and were pretreated in the absence or presence of 5 μM PKCε-TIP for 20 min before subjecting to 20 min of ischemia and 30 min of reperfusion in a Langendorff preparation. A: Left ventricle end diastolic pressure (LVEDP) was measured during reperfusion. B: Infarct size of the left ventricle was measured at the end of reperfusion. C: Lactate dehydrogenase (LDH) release over 30 min of reperfusion was measured. Data are means ± SEM. * $P < 0.05$, *vs.* control, n = 5-11

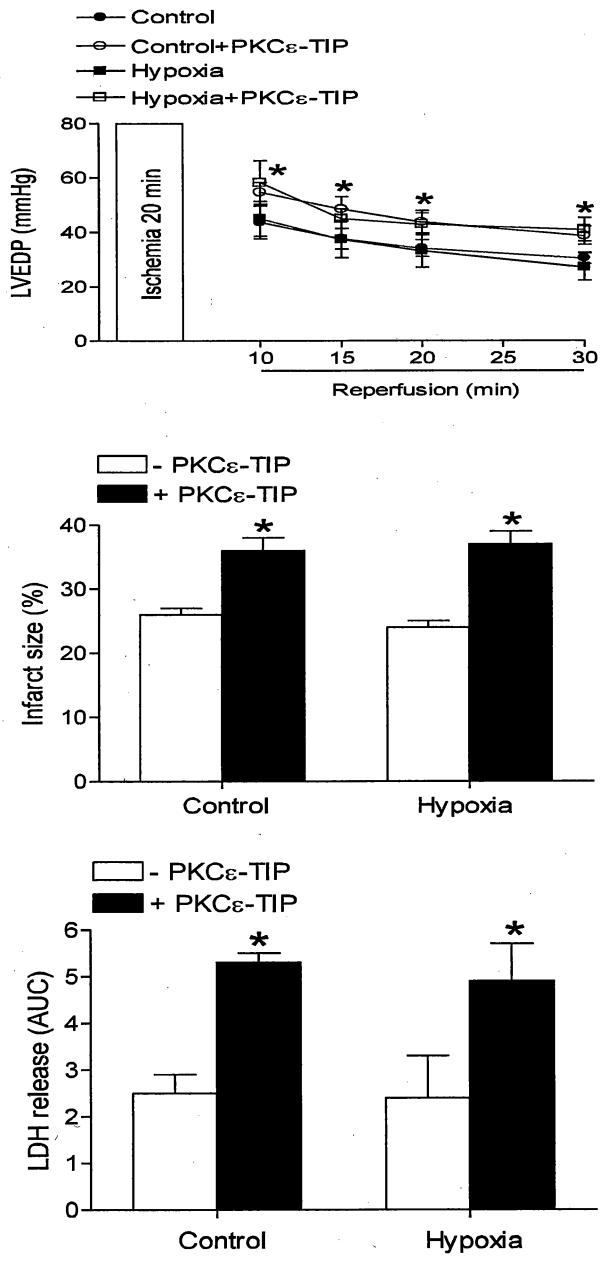

Figure 11. Effect of prenatal hypoxia on ischemia and reperfusion injury of left ventricle in female offspring. Hearts were isolated from 3-month-old female offspring that exposed to normoxia (control) or hypoxia before birth, and were pretreated in the absence or presence of 5 μM PKCε-TIP for 20 min before subjecting to 20 min of ischemia and 30 min of reperfusion in a Langendorff preparation. A: Left ventricle end diastolic pressure (LVEDP) was measured during reperfusion. B: Infarct size of the left ventricle was measured at the end of reperfusion. C: Lactate dehydrogenase (LDH) release over 30 min of reperfusion was measured. Data are means ± SEM. * $P < 0.05$, +PKCε-TIP *vs.* -PKCε-TIP, n = 5

Western Blot

In male animals, there was a significant decrease in PKCε protein levels in the LV of hypoxic group as compared with that in the control group (Fig. 12A). This was accompanied by a significant decrease in phospho-PKCε in the hypoxic group (Fig. 12B). PKCε-TIP had no effect on PKCε levels in the LV in either control or hypoxic groups (Fig. 12A). It significantly decreased phospho-PKCε in the LV in the control group (Fig. 12B). In contrast, it had no further effect on decreased phospho-PKCε in the hypoxic group (Fig. 12B). In the presence of PKCε-TIP, there was no significant difference in phospho-PKCε levels between the control and hypoxic groups, which were the same as that found in the hypoxic group in the absence of PKCε-TIP (Fig. 12B). Similar to PKCε, prenatal hypoxia also decreased PKCδ protein levels in the LV of hypoxic group as compared with that in the control group (Fig. 12C). However, phospho-PKCδ was not significantly different between the control and hypoxic groups (Fig. 12D). Unlike its inhibitory effect on phospho-PKCε in the control hearts, PKCε-TIP did not affect phospho-PKCδ levels in either control or hypoxic groups (Fig. 12D). In females, there were no significant differences in PKCε, phospho-PKCε, PKCδ, and phospho-PKCδ levels in the LV between the control and hypoxic groups (Fig. 13). PKCε-TIP decreased phospho-PKCε in the LV to the same extent in both the control and hypoxic groups (Fig. 13B).

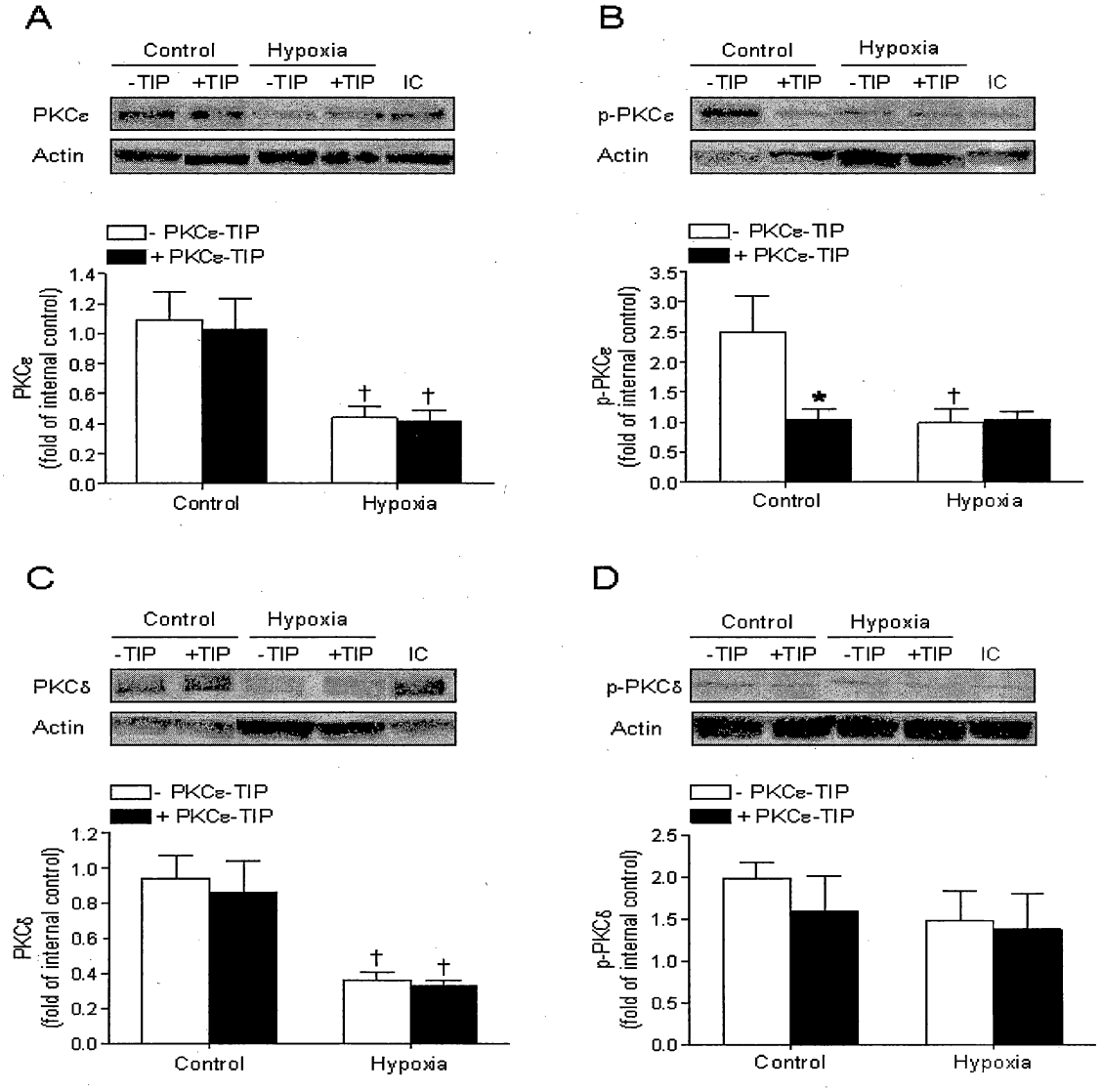

Figure 12. Effect of prenatal hypoxia on PKCε and PKCδ expression in left ventricle of male offspring. Hearts were isolated from 3-month-old male offspring that exposed to normoxia (control) or hypoxia before birth, and were pretreated in the absence or presence of 5 μM PKCε-TIP (TIP) for 20 min before subjecting to 20 min of ischemia and 30 min of reperfusion in a Langendorff preparation. PKCε, phospho-PKCε (p-PKCε), PKCδ and phospho-PKCδ (p-PKCδ) protein abundance in left ventricle were determined with Western blot analyses and normalized to actin and internal control (IC). Data are mean ± SEM. [a] $P < 0.05$, hypoxia *vs.* control, [b] $P < 0.05$, +TIP *vs.* -TIP, n = 5

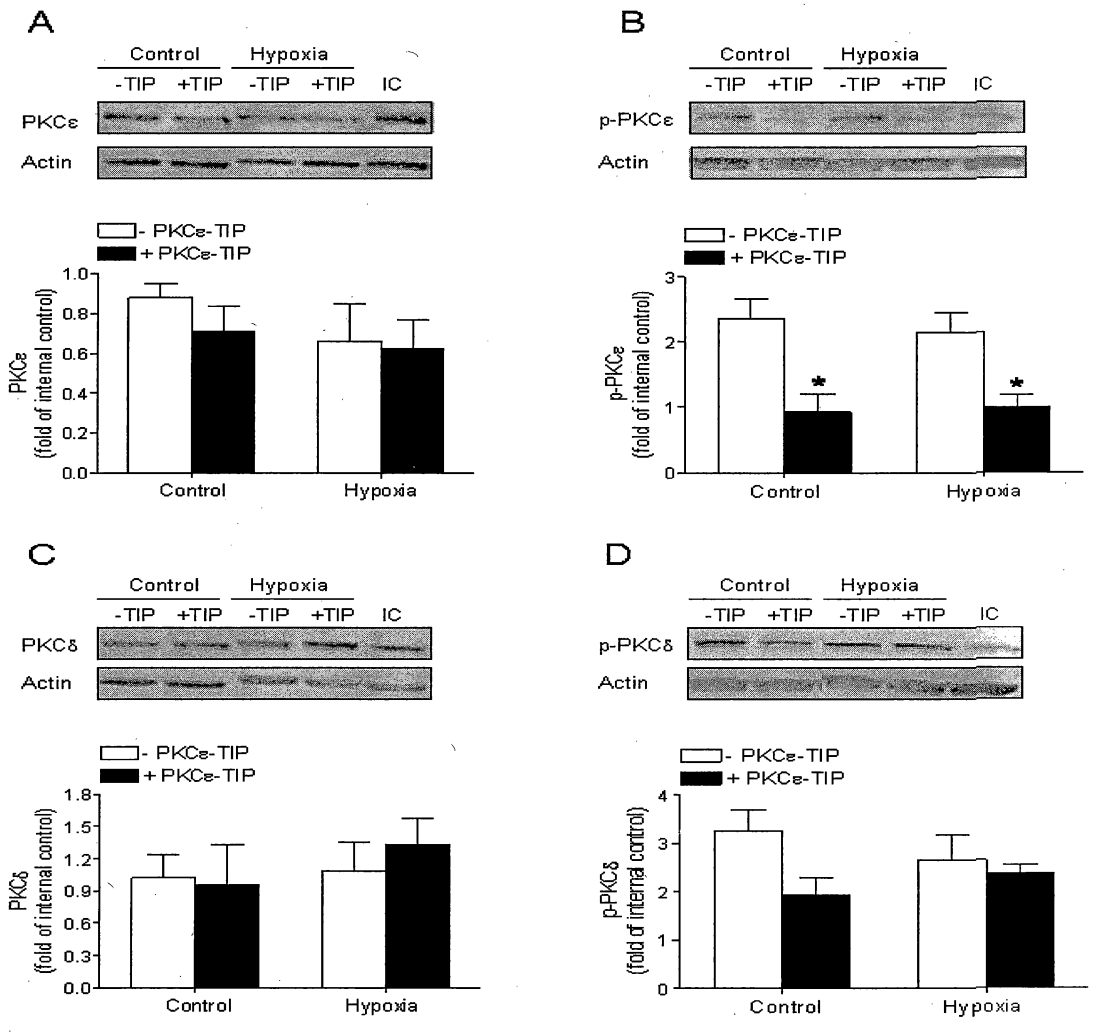

Figure 13. Effect of prenatal hypoxia on PKCε and PKCδ expression in left ventricle of female offspring. Hearts were isolated from 3-month-old female offspring that exposed to normoxia (control) or hypoxia before birth, and were pretreated in the absence or presence of 5 μM PKCε-TIP (TIP) for 20 min before subjecting to 20 min of ischemia and 30 min of reperfusion in a Langendorff preparation. PKCε, phospho-PKCε (p-PKCε), PKCδ and phospho-PKCδ (p-PKCδ) protein abundance in left ventricle were determined with Western blot analyses and normalized to actin and internal control (IC). Data are mean ± SEM. * $P < 0.05$, +TIP *vs.* -TIP, $n = 5$.

Discussion

The present study clearly demonstrated sex dichotomy in manifestation of increased cardiac vulnerability to ischemia and reperfusion injury in adult offspring resulting from fetal hypoxia. The lack of effect of prenatal hypoxia on the baseline left ventricular function in adult offspring is in agreement with the previous results obtained in other models (Bae et al., 2005; Lawrence et al., 2008), supporting the notion that it is possible and perhaps common for an organ to be programmed and then vulnerable for life without evidence until a late-life stressor challenges its adaptive capabilities. Contrast with the finding in the male offspring that showed a significant increase in ischemia and reperfusion injury in the left ventricle, hearts from female animals showed a resistance to hypoxic-mediated programming of heart vulnerability to ischemic injury. 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 caused by adverse intrauterine environments. Additionally, 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 an organ and/or tissue specificity 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. Although the results are conflicting, it has been shown 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 Langendorff 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, compared with male cardiomyocytes (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). Additionally, 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.

The finding that fetal hypoxia had no significant effect on coronary flow rate either at the baseline or during postischemic recovery in both male and female offspring suggests that the target mechanisms of sex-dependent cardiac programming reside in cardiomyocytes rather than coronary vasculature. The present study demonstrated that fetal hypoxia caused a sex-dependent PKCε gene repression in the heart of male offspring. 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 adult offspring, which corresponded to the decreased post-ischemic 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 a PKCε translocation inhibitory peptide (PKCε-TIP) in the present study. The activation of PKC isozymes is initiated by their translocation to the unique sub-cellular sites and binding to isozyme-specific anchoring proteins, receptors for activated C-kinase (RACKs), a family of membrane-associated PKC anchoring proteins that act as molecular scaffolds to localize individual PKCs to distinct membrane microdomains. 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 3-10 nM, and has been widely used to study the role of PKCε in cardiac function (Zhou et al., 2002; Murriel and Mochly-Rosen, 2003; Przyklenk et al., 2003). A previous study has shown that 5 μM of PKCε-TIP inhibits PKCε translocation in the heart of adult rat in the Langendorff preparation (Pierre et al., 2007). Consistent with the previous studies (Przyklenk et al., 2003; Bae and Zhang, 2005; Pierre et al., 2007), the present study showed that PKCε-TIP

had no significant effects on left ventricular function at the baseline levels. This is in agreement with the findings obtained in a PKCε knock-out mouse model, which demonstrated that PKCε expression was not required for normal cardiac function under physiological conditions, but PKCε activation was necessary and sufficient for acute cardioprotection during cardiac ischemia and reperfusion (Gray et al., 2004). In the present study, we found that PKCε-TIP significantly increased ischemic injury and decreased postischemic recovery of left ventricular function in control males, and in the presence of PKCε-TIP there was no difference in heart susceptibility to ischemic and reperfusion injury between the control and hypoxic males. The selectivity of PKCε-TIP was demonstrated by its inhibition of phospho-PKCε but not phospho-PKCδ in the control heart. The lack of effect of PKCε-TIP on ischemic injury of the heart in hypoxic males is consistent with its lack of effect on phospho-PKCε that has already been inhibited in the heart of hypoxic group. In contrast to the males, PKCε-TIP inhibited phospho-PKCε and decreased postischemic recovery of left ventricular function to the same extent in both control and hypoxic groups in females, consisting with the 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 PKCε 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 PKCε in cardioprotection against ischemia and reperfusion injury (Gray et al., 1997; Dorn et al., 1999; Liu et al., 1999; Chen et al., 2001; Cross et al., 2002; Pierre et al., 2007).

The finding that prenatal hypoxia resulted in a decrease in PKCε protein levels in the heart of male adult offspring suggests *in utero* epigenetic programming of PKCε gene repression in the heart. The ratio of phospho-PKCε/PKCε was not significantly different between the control and hypoxic groups, suggesting that fetal hypoxia repressed PKCε gene expression resulting in decreased phospho-PKCε, 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 PKCε gene repression, linking fetal cocaine exposure and pathophysiological consequences in the heart of adult male offspring in a gender-dependent manner (Zhang et al., 2008). 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 PKCε 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 PKCε promoter and PKCε 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 PKCε gene expression in the heart. Whether and to what extent fetal hypoxia induces differential and sex-dependent pattern of DNA methylation in the PKCε promoter remains an intriguing area for the future investigation.

Unlike PKCε, the role of PKCδ in ischemia and reperfusion injury is less clear and is somewhat controversial. Inhibition of PKCδ during reperfusion has been shown to decrease reperfusion-induced injury (Murriel and Mochly-Rosen, 2003). Other studies demonstrated the cardioprotective effects of PKCδ (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 PKCδ and PKCε levels (Hunter et al., 2007). The present finding that PKCδ was significantly decreased in the heart of male but not female offspring that exposed to hypoxia before birth suggests a possible mechanism of PKCδ in the sex dichotomy of increased heart susceptibility to ischemia and reperfusion injury in males. In agreement, previous studies demonstrated that prenatal nicotine exposure caused a significant decrease in PKCδ protein levels in the heart of female but not male offspring, which was associated with the increased heart vulnerability to ischemic injury in the females as compare with the 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 PKCε gene repression resulting in a down-regulation of PKCε expression in adult male hearts. Although a role of PKCδ is also suggested, its causal effect in sex-dependent programming of heart vulnerability to ischemic injury in offspring remains to be determined. Whereas 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 human, given that hypoxia is one of the most important and clinically relevant stresses to the fetus and large epidemiological studies have indicated a link between *in utero* adverse stimuli during pregnancy and an increased risk of ischemic heart disease in the adulthood.

CHAPTER FOUR

FETAL HYPOXIA CAUSES PROGRAMMING OF AT_2R EXPRESSION AND CARDIAC VULNERABILITY TO ISCHEMIC INJURY IN RAT OFFSPRING

by

Qin Xue, Chiranjib Dasgupta, Man Chen and Lubo Zhang

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Abstract

Angiotensin II plays an important role in cardiac function and has been implicated in programming of cardiovascular disease caused by adverse *in utero* environment during fetal development. The present study tested the hypothesis that fetal hypoxia alters angiotensin II receptor gene expression pattern and increases heart ischemic susceptibility in offspring. Cardiac AT_1 (AT_1R) and AT_2 (AT_2R) receptors decreased from the fetus to adult. Hypoxia downregulated AT_1R in the fetal heart, which was recovered in adult offspring. In contrast, AT_2R was significantly increased in both male and female offspring. Multiple glucocorticoid response elements (GREs) were identified at the AT_2R promoter, deletion of which increased the promoter activity. Consistently, dexamethasone decreased AT_2R expression in the heart, which was blocked by RU 486. Prenatal hypoxia decreased glucocorticoid receptor (GR) in adult hearts, resulting in decreased GR binding to the GREs at the AT_2R promoter. The inhibition of AT_2R improved postischemic recovery of left ventricular function and rescued the hypoxia-induced cardiac ischemic vulnerability in male offspring. In contrast, the inhibition of AT_1R decreased the postischemic recovery. The results demonstrate that fetal hypoxia causes programming of increased cardiac AT_2R gene expression by downregulating GR, which contributes to increased ischemic vulnerability of the heart in offspring.

Introduction

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 (Barker et al., 1986; Bateson et al., 2004; Gluckman et al., 2008;

McMillen and Robinson 2005). Hypoxia is one of the most important and clinically relevant stresses that can adversely affect fetal development. There is evidence of a link between hypoxia and fetal intrauterine growth restriction and an increased risk of cardiovascular disease in offspring (Heydeck et al., 1994; Roigas et al., 1996; Butler et al., 2002; Peyronnet et al., 2002; Davis et al., 2003; Li et al., 2003; Jones et al., 2004; Mone et al., 2004; Zhang 2005). Animal studies have demonstrated that fetal hypoxia causes a premature exit from the cell cycle of cardiomyocytes and myocyte hypertrophy (Bae et al., 2003), and results in an increased heart susceptibility to acute ischemia and reperfusion injury in adult male offspring in a sex-dependent manner (Li et al., 2003, 2004; Xu et al., 2006, Xue and Zhang, 2009).

Angiontensin II (Ang II) plays a fundamental role in the regulation of cardiovascular homeostasis, and has been implicated in programming of cardiovascular disease induced by adverse *in utero* environment during the fetal development (Bogdarina et al., 2007; Hadoke et al., 2006; Langley-Evans et al., 1999; Langley-Evans and Jackson, 1995; Sherman RC, Langley-Evans, 1998). Recent studies have demonstrated a link between fetal insults to differential epigenetic modifications of type 1 (AT_1R) and type 2 (AT_2R) Ang II receptor genes in the adrenal and kidney and the resultant alteration of their expression pattern in adult life, which may lead ultimately to the development of hypertension (Bogdarina et al., 2007; McMullen and Langley-Evans, 2005; Singh et al., 2007). Nevertheless, the effect of fetal hypoxia on the ontogeny of Ang II receptors in the heart has not been determined. Both AT_1R and AT_2R are expressed in cardiac myocytes and have significant pathophysiological roles in heart diseases (Matsubara, 1998; Sechi et al., 1992; Horiuchi et al., 1999; Schneider and Lorell,

2001, Xu et al., 2009). Yet the role of AT_1R and AT_2R in ischemia and reperfusion injury of the heart remains controversial, depending on systemic *vs.* local blockade as well as chronic *vs.* acute blockade of AT_1R and AT_2R. Although long-term systemic administration of AT_1R antagonists reduced ischemic injury, studies of the acute effects of AT_1R or AT_2R antagonists on the recovery of left ventricular function during reperfusion of the ischemic left atrium–perfused isolated working rat heart demonstrated the cardioprotection of AT_2R blockade, but not AT_1R blockade (Ford et al., 1996, 1998). Herein, we present evidence that fetal hypoxia causes programming of increased AT_2R gene expression in the heart of offspring by the down-regulation of glucocorticoid receptors (GRs), which contributes to the increased ischemic vulnerability of the heart in offspring resulted from fetal hypoxia.

Material 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% O_2 from day 15 to 21 of gestation, as described previously (Xue and Zhang, 2009). Hearts were isolated from near-term (21 d) fetuses, 3 weeks and 3 months old offspring. To isolated hearts, rats were anesthetized with 75 mg/kg ketamine and 5-mg/kg xylazine injected intramuscularly. For *ex vivo* studies, 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% CO_2, as reported previously (Meyer et al., 2009). Hearts were given 24 h of

recovery time before being treated with dexamethasone and RU 486 for 48 h. All procedures and protocols were approved by the Institutional Animal Care and Use Committee guidelines.

Western Blot Analysis

Protein was isolated from hearts of fetuses and offspring. Protein abundance of AT_1R, AT_2R, GRs were measured with Western blot analysis. In brief, hearts were homogenized in a lysis buffer. Homogenates were then centrifuged at 4°C for 10 minutes at 10,000 g, and supernatants collected. Nuclear extracts were prepared from hearts 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 7.5% polyacrylamide gel with 0.1% SDS and separated by electrophoresis at 100 V for 90 minutes. Proteins were then transferred onto nitrocellulose membranes. Nonspecific binding sites 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 AT_1R, AT_2R, and GR (Santa Cruze Biotechnology; Santa Cruz, CA). 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 of fetuses and offspring. RNA was extracted from hearts using TRIzol protocol (Invitrogen, Carlsbad, USA). $AT_{1a}R$, $AT_{1b}R$ and AT_2R mRNA abundance was determined by real-time RT-PCR using Icycler Thermal cycler (Bio-Rad, Hercules, CA), as described previously (Meyer et al., 2009). The primers used were: $AT_{1a}R$, 5'-GGAGAGGATTCGTGGCTTGAG-3' (forward) and 5'-CTTTCTGGGAGGGTTGTGTGAT-3' (reverse); $AT_{1b}R$, 5'-ATGTCTCCAGTCCCCTCTCA-3' (forward) and 5'-TGACCTCCCATCTCCTTTTG-3' (reverse); and AT_2R, 5'-CAATCTGGCTGTGGCTGACTT-3' (forward) and 5'-TGCACATCACAGGTCCAAAGA-3' (reverse). Real-time RT-PCR was performed in a final volume of 25 µl. We used the following RT-PCR protocol: 50°C for 10 min, 95°C for 5 min, followed by 40 cycles of 95°C for 10 s, 56°C for 30 s, 72°C for 10s. GAPDH was used as an internal reference and serial dilutions of the positive control was performed on each plate to create a standard curve. PCR was performed in triplicate, and threshold cycle numbers were averaged.

Site-Directed Mutagenesis and Reporter Gene Assay

Rat AT_2R promoter sequence was obtained from rat genome data base (http://www.ncbi.nlm.nih.gov/mapview). Primers flanking a fragment of 2220 base pairs (bp) AT_2R promoter region were designed and synthesized by IDT (Coralville, IA). Genomic DNA isolated from rat hearts was used as PCR template for DNA amplification. After second round of nested PCR, a 2130 bp amplified fragment spanning -2080 bp to +49 bp relative to the transcriptional start site was cloned into pCR4-TOPO

vector (Invitrogen) and sequenced. The KpnI/XhoI fragment flanking the AT_2R promoter region was then inserted into the luciferase reporter gene plasmid, pGL3 (Promega) to yield the full-length promoter-reporter plasmid. Promoter analyses identified the presence of multiple GREs. Site-specific deletions of GREs were constructed, respectively. All promoter constructs sequences were confirmed with DNA sequencing analyses. Reporter gene assay was performed using a rat embryonic heart-derived myogenic cell line H9c2, as described previously (Meyer et al., 2009). H9c2 cells were obtained from American Type Culture Collection (Rockville, MD) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. H9c2 cells were seeded in six-well plates (2×10^6 cells/plate) and transiently co-transfected with 1 μg of promoter/reporter vector along with 0.05 μg of internal control pRL-SV40 vector using Tfx-20 transfection reagents for eukaryotic cells (Promega) following manufacturer's instructions. After 48 hours, firefly and *Renilla reniformis* luciferase activities in cell extracts were measured in a luminometer using a dual-luciferase reporter assay system (Promega). The truncated promoter activities were then calculated by normalizing the firefly luciferase activities to *R. reniformis* luciferase activity.

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts were collected from hearts using NXTRACT CelLytic Nuclear Extraction Kit (Sigma). The oligonucleotide probes of GREs at rat AT_2R 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 (Meyer et al., 2009). 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 oligonucleotieds probes, 1× binding buffer, 1 µg of poly(dI-dC), and 5 µg of nuclear extracts. For competitions studies, increasing concentrations of non-labeled homologous and heterologous oligonucleotides were added to binding reactions. For super-shift assays, 2 µg of GR antibody (Santa Cruz Biotechnology) were added and further incubated for 1 hour at 4 °C. 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/cm^2). Membranes were blocked and then visualized using the reagents provided in the LightShift kit.

Hearts Subjected to Ischemia and Reperfusion

Hearts of 3 months old offspring were isolated and retrogradely perfused *via* the aorta in a modified Langendorff apparatus, as previously described (Xue and Zhang, 2009). 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 losartan (1

μM), or PD 123,319 (0.3 μM), or losartan plus PD 123,319 for 5 minutes before ischemia and throughout the period of ischemia and reperfusion. Left ventricular developed pressure (LVDP), heart rate (HR), dP/dt_{max}, dP/dt_{min}, and LV end diastolic pressure (LVEDP) were continuously recorded. Myocardial infarct size was measured at the end of reperfusion with 1% triphenyltetrazolium chloride, and was expressed as a percentage of the total left ventricular weight. LDH activity was measured in coronary effluent collected at 30 second 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 assay (TOX 7 kit, Sigma, Saint Louis, MO), following the manufacture's directions, 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.

Results

Effect of Fetal Hypoxia on AT_1R and AT_2R Protein and mRNA in the Heart

Protein and mRNA abundance of both AT_1R and AT_2R showed a development-dependent reduction in the heart, and no sex difference was observed (Figure 14). AT_2R mRNA decreased to less abundance that that of $AT_{1a}R$ and $AT_{1b}R$, resulting in a significant decrease in the $AT_2R/AT_{1a,b}R$ receptor ratio in the adult heart. Hypoxia caused

a significant decrease in protein abundance of AT_1R but not AT_2R, resulting in an increased AT_2/AT_1 ratio in the fetal heart (Figure 15A). This was associated with a decrease in $AT_{1b}R$ mRNA (Figure 15B). The same expression pattern persisted in the heart of 3 weeks old male offspring, whereas no significant differences in AT_1R and AT_2R were observed in females. In 3 months old offspring, prenatal hypoxia increased protein (Figure 15A) and mRNA (Figure 15B) abundance of AT_2R, but not AT_1R, in the male heart. In females, both AT_1R and AT_2R were increased and the AT_2R/AT_1R ratio was not significantly changed (Figure 15A). Consistently, $AT_{1b}R$ and AT_2R mRNA was significantly increased in the female heart (Figure 15B).

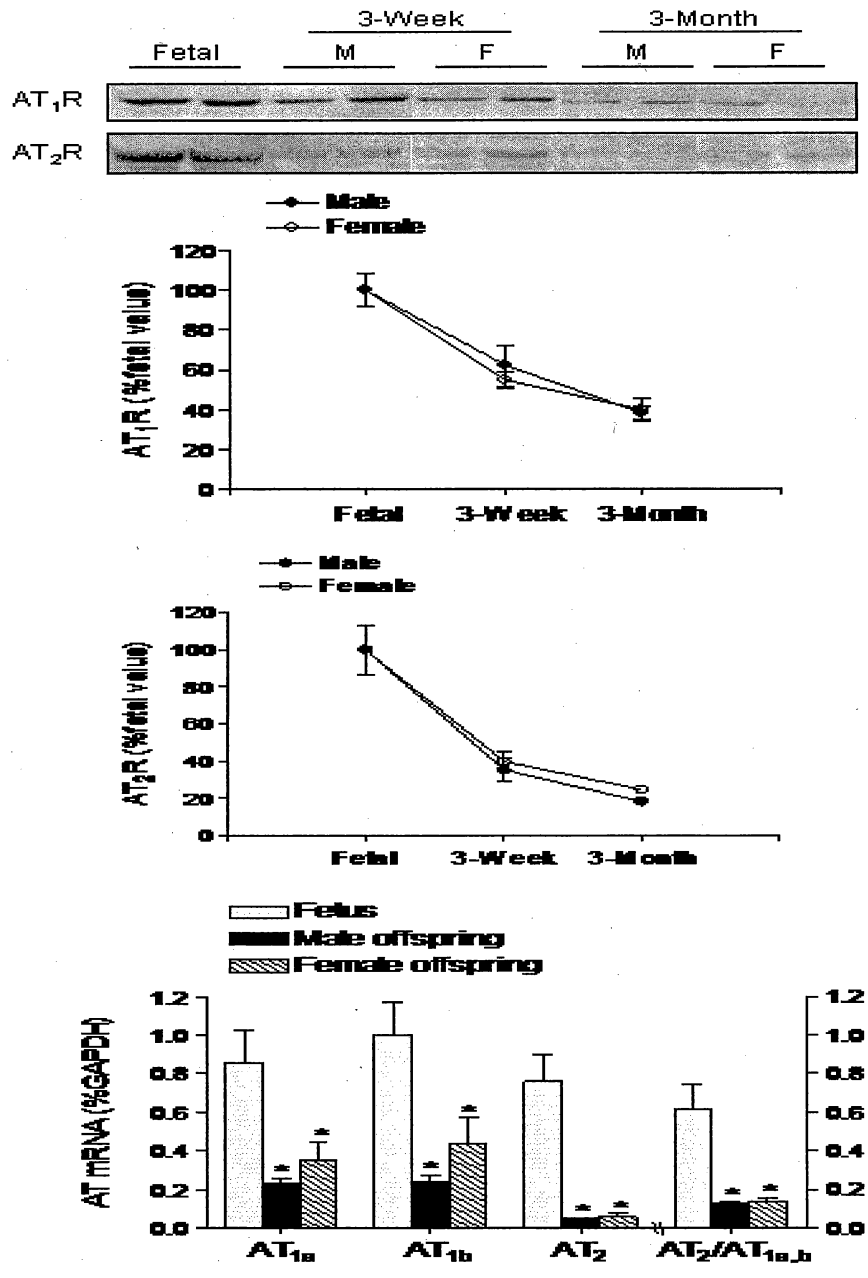

Figure 14. Effect of development on AT_1R and AT_2R protein and mRNA abundance. AT_1R and AT_2R protein and mRNA abundance was determined in hearts isolated from fetus, 3 weeks and 3 months old male (M) and female (F) rats. Data are mean ± SEM. * $P < 0.05$, *vs.* fetus. n = 5.

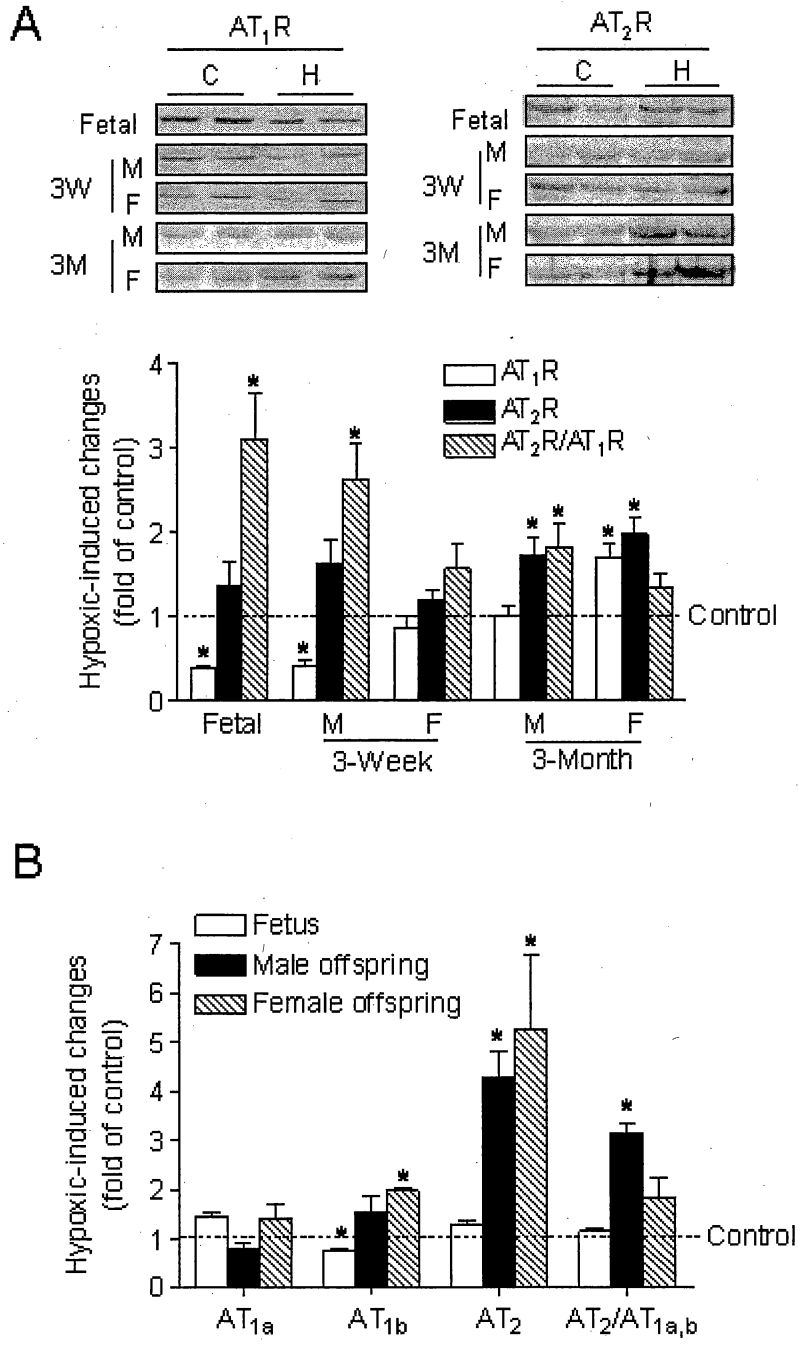

Figure 15. Effect of fetal hypoxia on AT_1R and AT_2R protein and mRNA abundance. Hearts were isolated from fetus, 3 weeks (3W) and 3 months (3M) old male (M) and female (F) offspring in control (C) and hypoxic (H) groups. A) Protein. B) mRNA. Data are means ± SEM. * $P < 0.05$, hypoxia *vs.* control. $n = 5$.

Inhibitory Effect of GREs on the AT_2R Promoter Activity

Rat AT_2R promoter has a TATAA element at -28 from the transcription start site (Figure 16). Deletion of the TATAA element significantly decreased the promoter activity (Figure 17). Multiple GREs were identified at rat AT_2R promoter. These include GRE1 (-1853), GRE2 (-1674), GRE3 (-1526), GRE4 (-1159), GRE5 (-945), GRE6 (-676), GRE7 (-107), GRE8 (+13) (Figure 3). Site-specific deletion of each GREs independently caused a significant up-regulation of the promoter activity (Figure 17). While the GRE4 deletion stimulated the luciferse activity by 2.43-fold, deletion of other GREs increased the promoter activity by 1.5- to 1.9-fold.

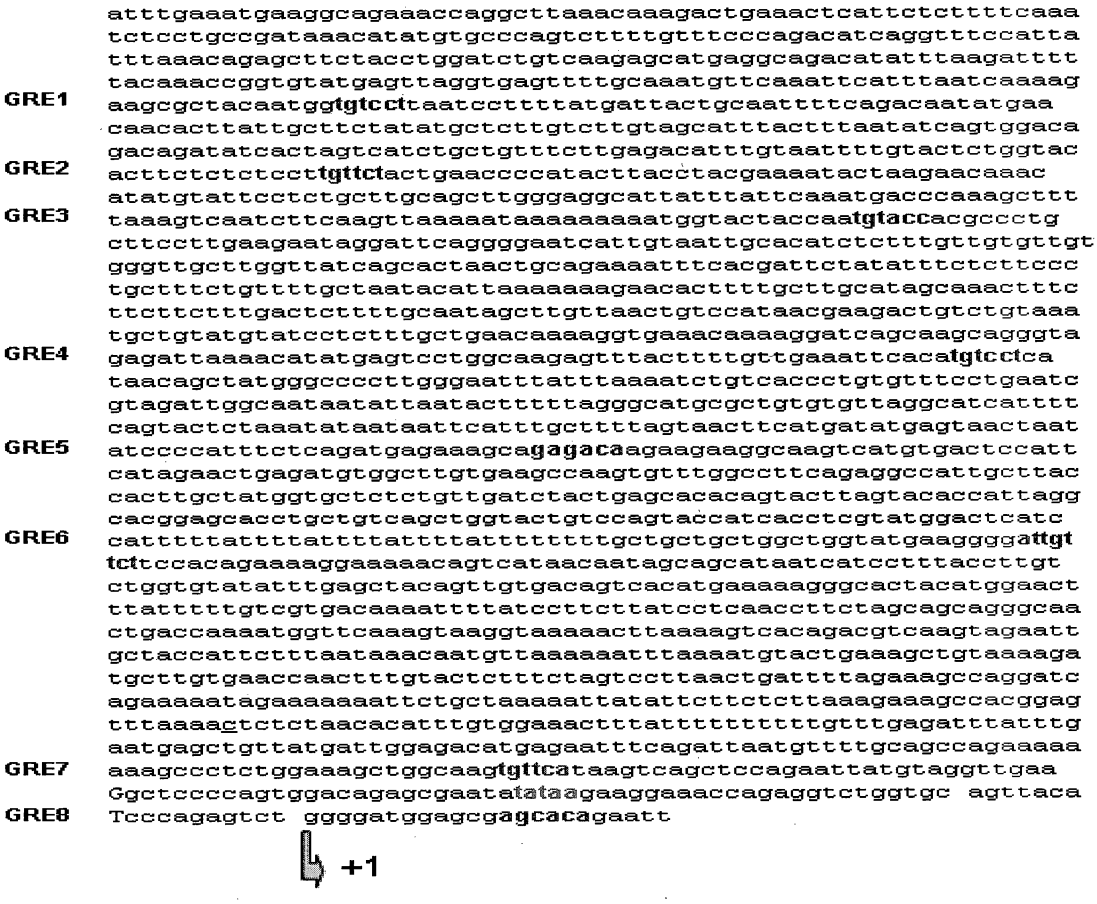

Figure 16. Rat AT_2R gene promoter sequence.

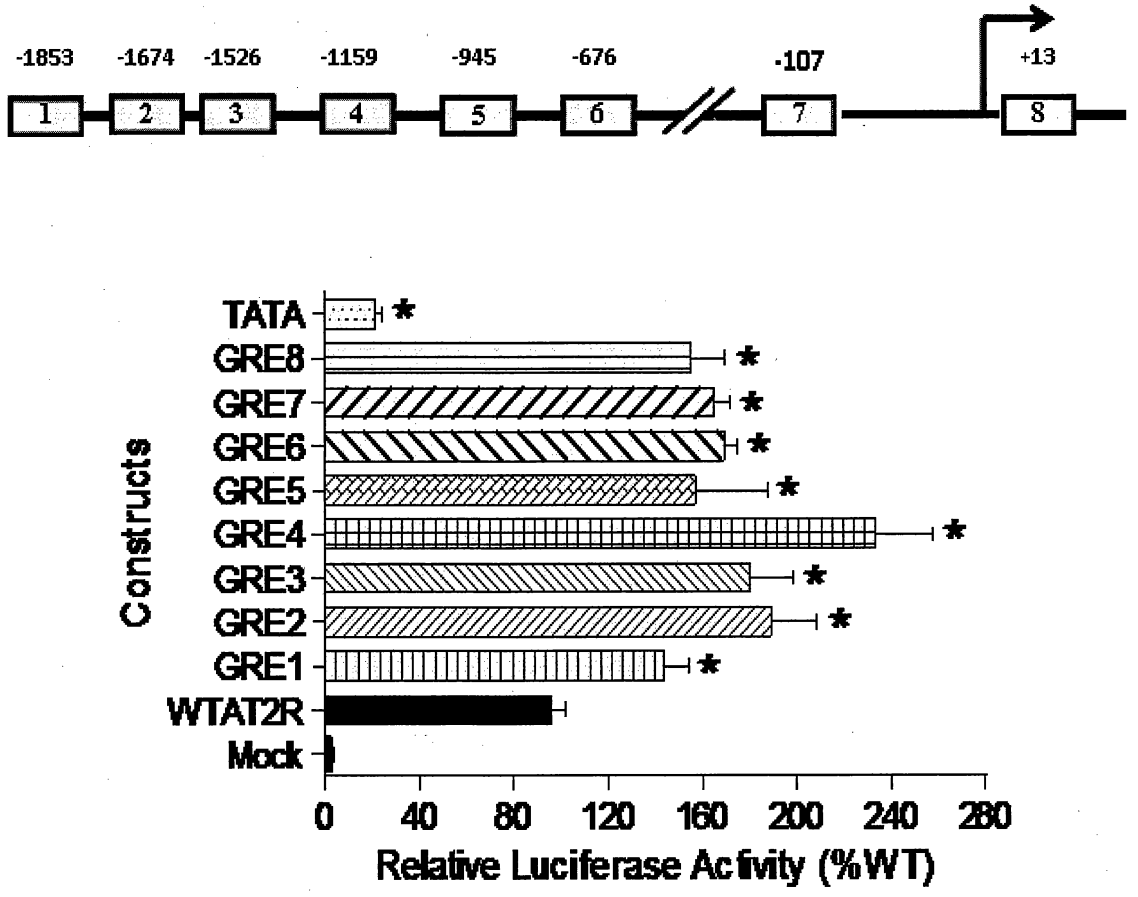

Figure 17. Effect of GREs on the AT_2R promoter activity. AT_2R promoter reporter gene constructs of wild type (WTAT2R) and site-specific deletion of GREs and TATA were transiently co-transfected with pRL-SV40 driven *R. reniformis* luciferase in a rat embryonic heart-derived myogenic cell line H9c2. After 48 h, firefly and *R. reniformis* luciferase activities in cell extracts were measured using a dual-luciferase reporter assay system. The promoter activities were then calculated by normalizing the firefly luciferase activities to *R. reniformis* luciferase activity. Data are mean ± SEM. * $P < 0.05$, *vs.* wild type. $n = 6$.

Binding of GRs to the GREs at the AT_2R Promoter

Binding of nuclear proteins to the putative GREs (Figure 18) at the AT_2R promoter was evaluated by electrophoretic mobility shift assay (EMSA). Immunoblot analysis confirmed the presence of GRs in the nuclear extracts used in EMSA (Figure 19). We first defined our criteria by analyzing GRE6, as it was originally suggested at the AT_2R promoter (Ichiki *et al*). GRE4 was included along with GRE6 in our initial analysis. As shown in figure 19 (top panels), incubation of nuclear extracts from rat hearts with double-stranded oligonucleotide probes of GRE4 and GRE6 resulted in a DNA-protein complex of similar electrophoretic mobility in EMSA, which was blocked by cold homologous, heterologous, a consensus GRE, or $AT_{1a}R$-GRE oligos. Identity of GRs in the nuclear extracts was confirmed by super-shift data shown in figure 19, in which the electrophoretic mobility of the gel-retarded complex formed between GRE6 and nuclear extracts was further retarded with an anti-GR antibody. We extended EMSA and competition experiments for GREs 1, 2, 3, 5, 7, and 8, and showed that each of the biotinylated EMSA oligos encompassing these sites reacted with nuclear extracts to form a gel-retarded band that has identical electrophoretic mobility as with that of GRE6 (Figure 19, lower panels).

GRE oligos used in EMSA

GRE 1 (-1853)	5'- CTACAATGGTGTCCTTAATCCTTTTA
GRE 2 (-1674)	5'-TCTCTCTCCTTGTTCTACTGAACCCC
GRE 3 (-1526)	5'-GTACTACCAATGTACCACGCCCTGCT
GRE 4 (-1159)	5'-GAAATTCACATGTCCTCATAACAGCT
GRE 5 (-947)	5'-TGAGAAAGCAGAGACAAGAAGAAGGC
GRE 6 (-676)	5'-ATGAAGGGGATTGTTCTTCCACAGAA
GRE 7 (-107)	5'-AGCTGGCAAGTGTTCATAAGTCAGCT
GRE 8 (+13)	5'-GGATGGAGCGAGCACAGAATTGAAAG

Consensus GRE oligos used for heterologous competition in EMSA

CGRE-S	5'-TATGGTTACAAACTGTTCTAAAAC
CGRE-AS	5'-GTTTTAGAACAGTTTGTAACCATA
AT1a-GRE-S	5'-AAGCTTGTACACTATTGTCTGAGTT
AT1a-GRE-AS	5'-AACTCAGACAATAGTGTACAAGCTT

Figure 18. Sequences of GRE oligos used in EMSA.

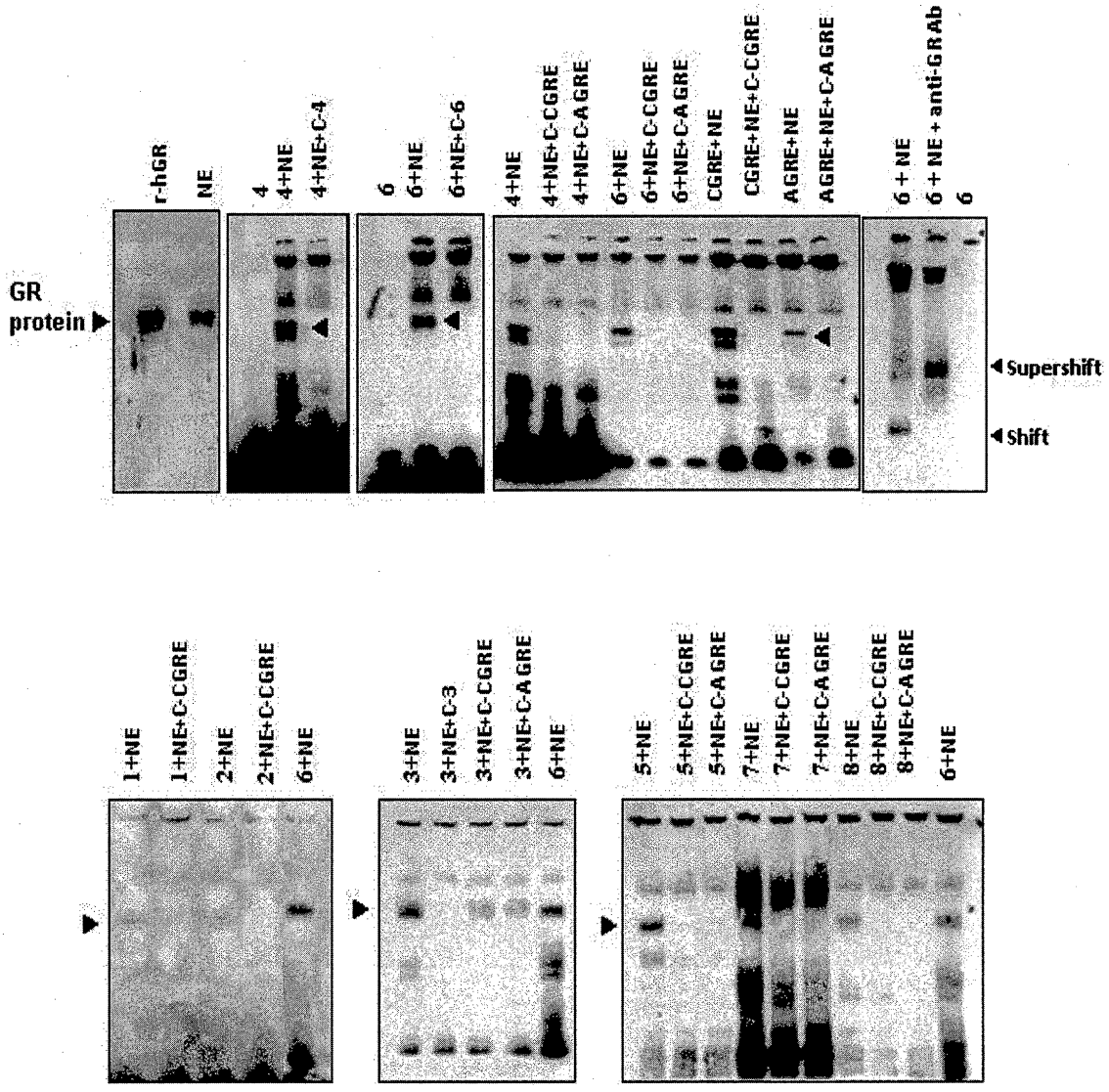

Figure 19. Characterization of GREs in the AT2R promoter. EMSA was performed with nuclear extracts (NE) and biotin labeled ds-oligo probes containing GREs (1 through 8), and consensus GRE (CGRE), AT1aR-GRE (AGRE) (oligo sequences are in *on-line* data supplement). GR, glucocorticoid receptor; r-hGR, human recombinant GR; C-3, C-4, C-6, C-CGRE, C-AGRE etc are cold ds-oligos used for competition experiments. Each EMSA generated band of same electrophoretic mobility as those of authentic GREs CGRE and AGRE, and competed out by homologous, heterlogous, and consensus oligos. The GRE6 probe forms a complex that was supershifted by glucocorticoid receptor antibody (anti-GR Ab).

Dexamethasone Inhibits AT_2R Expression in the Heart

Dexamethasone treatment for 48 hours produced a dose-dependent decrease in mRNA and protein abundance of AT_2R in the intact fetal rat hearts (Figure 20). RU 486 had no effect on AT_2R mRNA but blocked dexamethasone-induced reduction of AT_2R mRNA abundance (Figure 20).

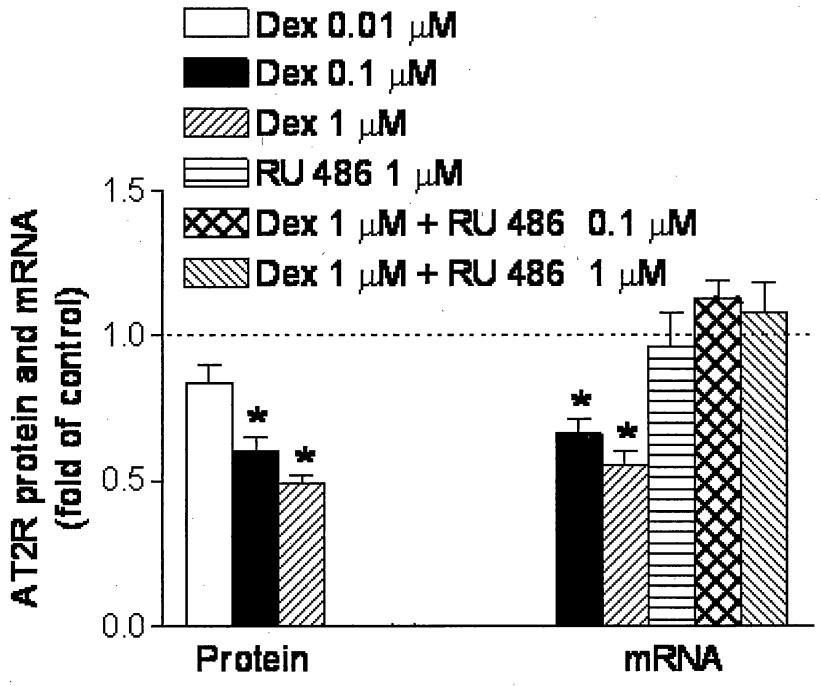

Figure 20. Effect of dexamethasone on AT_2R protein and mRNA abundance. Intact fetal hearts were treated with dexamethasone (Dex) for 48 hours in the absence or presence of RU 486. Data are mean ± SEM. * $P < 0.05$, *vs.* control. n = 6.

Prenatal Hypoxia Decreases GR Binding to GREs at the AT_2R Promoter in Offspring Hearts

Figure 21 shows the effect of prenatal hypoxia on GR expression in the hearts of adult offspring. In both male and female offspring, fetal hypoxia caused about 50% decreases in total cellular as well as nuclear GR protein abundance in the hearts. In accordance, there were similar extent decreases in GR bindings to GREs 4, 6, 7, 8 at the AT_2R promoter in offspring hearts (Figure 22A). The binding affinity of GR to GREs was determined in competition studies performed in pooled nuclear extracts from the hearts of adult offspring with the increasing ratio of unlabeled/labeled oligonucleotides encompassing the GRE4 at –1159 in the AT_2R promoter. Fetal hypoxia had no significant effect on the binding affinity of nuclear extracts to the GRE in the hearts of both male and female offspring (Figure 22B).

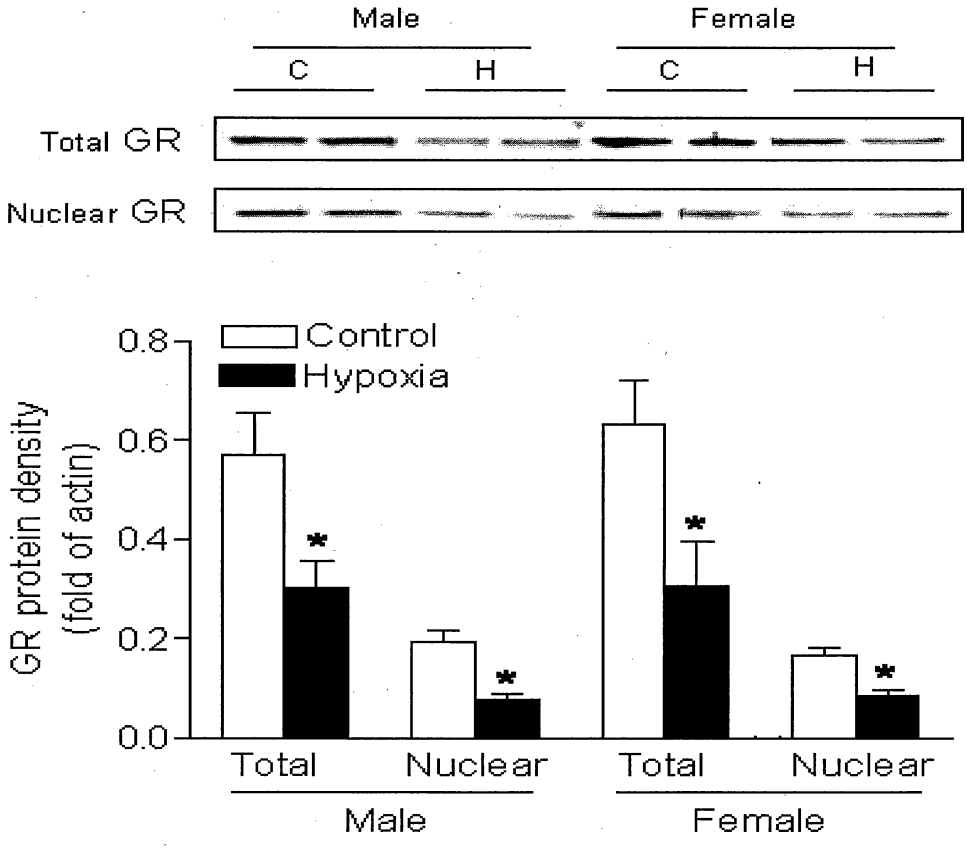

Figure 21. Effect of fetal hypoxia on glucocorticoid receptor protein abundance. Hearts were isolated from 3 months old male and female offspring in control (C) and hypoxic (H) groups. Total cellular and nuclear glucocorticoid receptor (GR) abundance was determined. Data are mean ± SEM. * $P < 0.05$, hypoxia *vs.* control. $n = 5$.

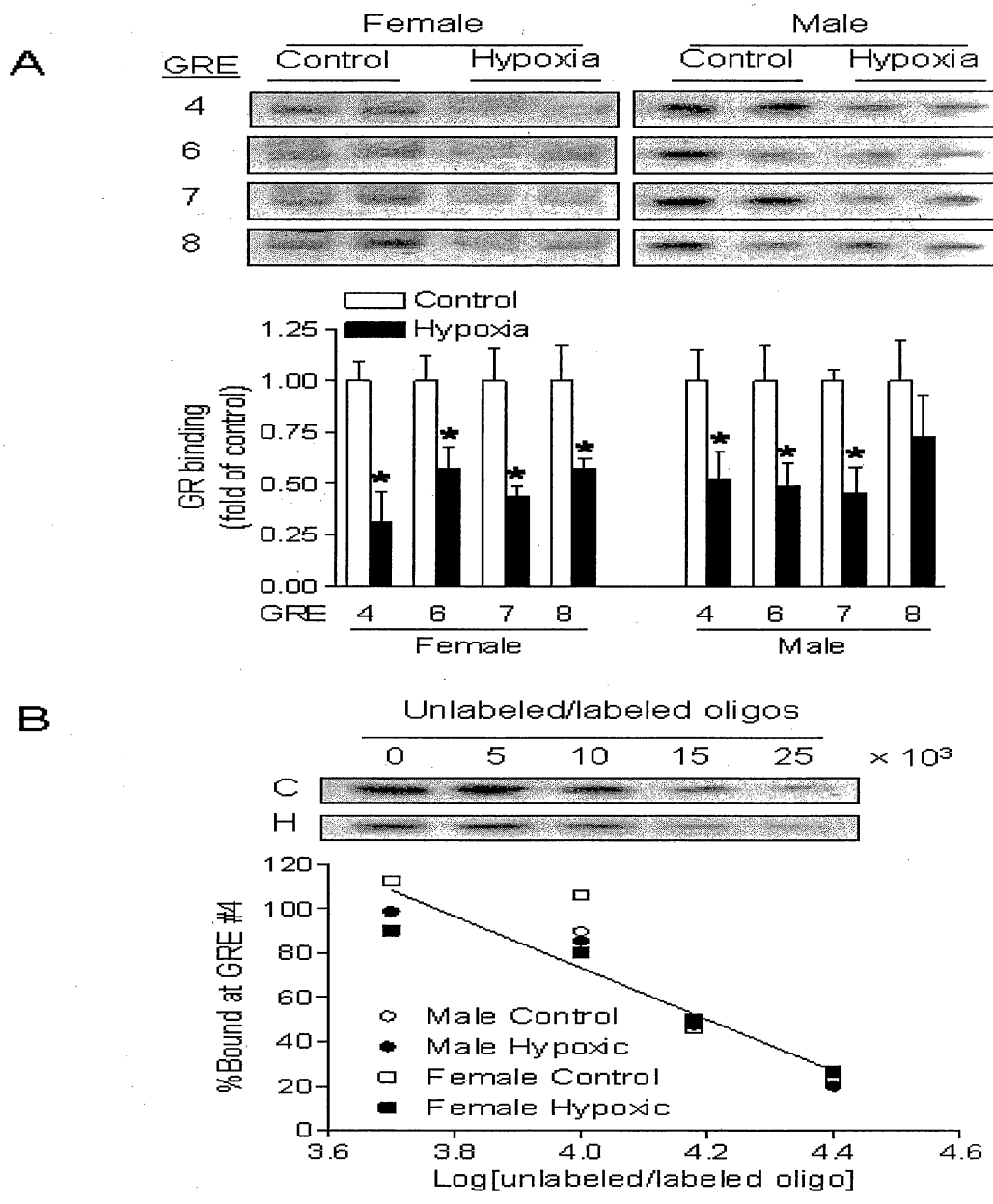

Figure 22. Effect of fetal hypoxia on GRE binding at the AT_2R promoter. Binding of the GREs was determined by EMSA in nuclear extracts from the hearts of male and female offspring in control (C) and hypoxic (H) groups. Data are mean ± SEM. * P <0.05, hypoxia *vs.* control. n = 5.

Functional Role of AT_2R in Acute Ischemia and Reperfusion Injury

The functional significance of AT_2R in modulating the post-ischemic recovery of left ventricle (LV) function after acute ischemia was determined in a Langendorff preparation using a selective AT_2R inhibitor, PD 123,319. As shown in Table 4, there were no significant differences in LV developed pressure (LVDP), heart rate (HR), dP/dt_{max}, dP/dt_{min} and coronary flow rate at the baseline among all groups. PD 123,319 significantly improved the post-ischemic recovery of LVDP (Figure 23), as well as dP/dt_{max} and dP/dt_{min} (Figure 24) in both male and female hearts. Consistently, PD 123,319 decreased LV end diastolic pressure (LVEDP) (Figure 24), myocardial infarct size and LDH release (Figure 23) after myocardial ischemia in both male and female animals. In contrast, an AT_1R selective inhibitor losartan impaired the post-ischemic recovery of LVDP (Figure 23) and dP/dt_{max} and dP/dt_{min} (Figure 24), and significantly increased LVEDP (Figure 24), myocardial infarct size and LDH release (Figure 23). In the presence of both PD 123,319 and losartan, there were no significant differences in the post-ischemic recovery of LV function and myocardial infarction (Figure 23 and Figure 24). The recovery of HR and coronary flow rate was not significantly different among all groups (data not shown). Further studies demonstrated that blockade of AT_2R with PD 123,319 rescued the myocardial phenotype of increased susceptibility to acute ischemia and reperfusion injury in male offspring that exposed to hypoxia before birth (Figure 25 and Figure 26).

Table 4. Pre-ischemic left ventricle functional parameters

	HR (bpm)	LVEDP (mmHg)	LVDP (mmHg)	dP/dt_{max} (mmHg/s)	dP/dt_{min} (mmHg/s)	CF (ml/min)
Male						
Control	261±5.8	5.3±0.2	106.1±3.7	3464±64.5	2235±93.7	11.7±0.6
PD	258±2.2	5.4±0.3	102.2±1.6	3310±80.7	2209±79.0	11.8±0.4
Losartan	254±6.6	5.0±0.1	113.1±2.1	3280±107.5	2248±94.5	11.0±0.6
PD+Losartan	252±6.0	4.9±0.1	108.6±3.3	3358±59.2	2251±64.1	12.4±0.3
Female						
Control	258±7.0	5.3±0.1	98.0±3.3	3303±69.6	1915±36.5	10.8±0.4
PD	261±7.1	5.1±0.1	96.0±2.5	3191±136.0	1947±53.3	10.8±0.3
Losartan	253±2.0	5.4±0.1	97.6±2.3	3424±173.6	1992±52.8	10.6±0.4
PD+Losartan	258±4.1	5.0±0.1	102.4±3.0	3262±131.3	2078±42.2	10.8±0.4

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; PD, PD123319. n = 5.

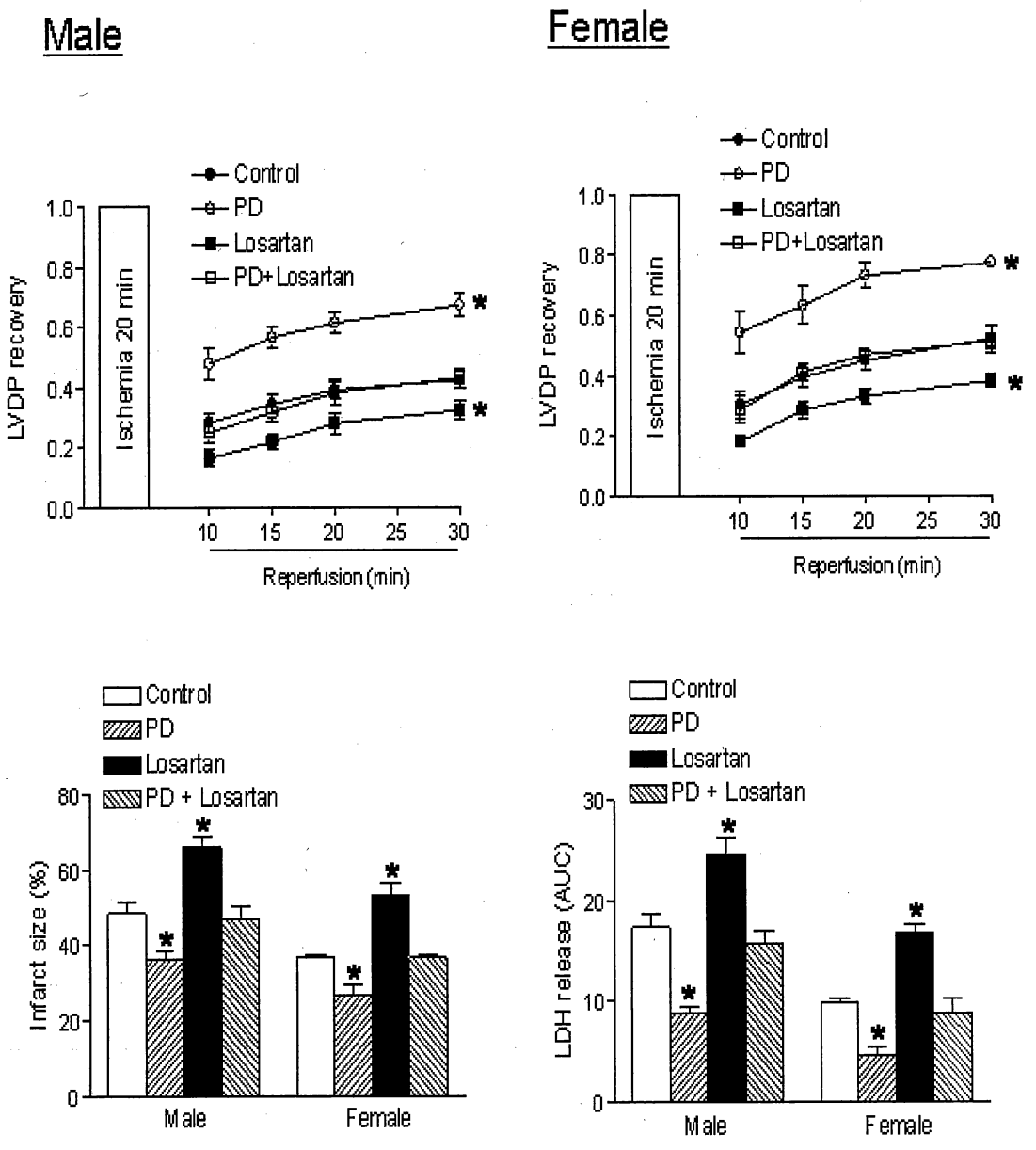

Figure 23. Effect of AT_1R and AT_2R inhibitors on cardiac ischemia and reperfusion injury. Hearts were isolated from 3 months old male and female rats and were pretreated in the absence or presence of losartan (1 μM), or PD123,319 (PD, 0.3 μM), or losartan + PD for 5 minutes before subjecting to 20 minutes of ischemia and 30 minutes of reperfusion in a Langendorff preparation. Post-ischemic recovery of left ventricular developed pressure (LVDP), infarct size, and lactate dehydrogenase (LDH) release were determined. Data are mean ± SEM. * $P < 0.05$, vs. control. $n = 5$.

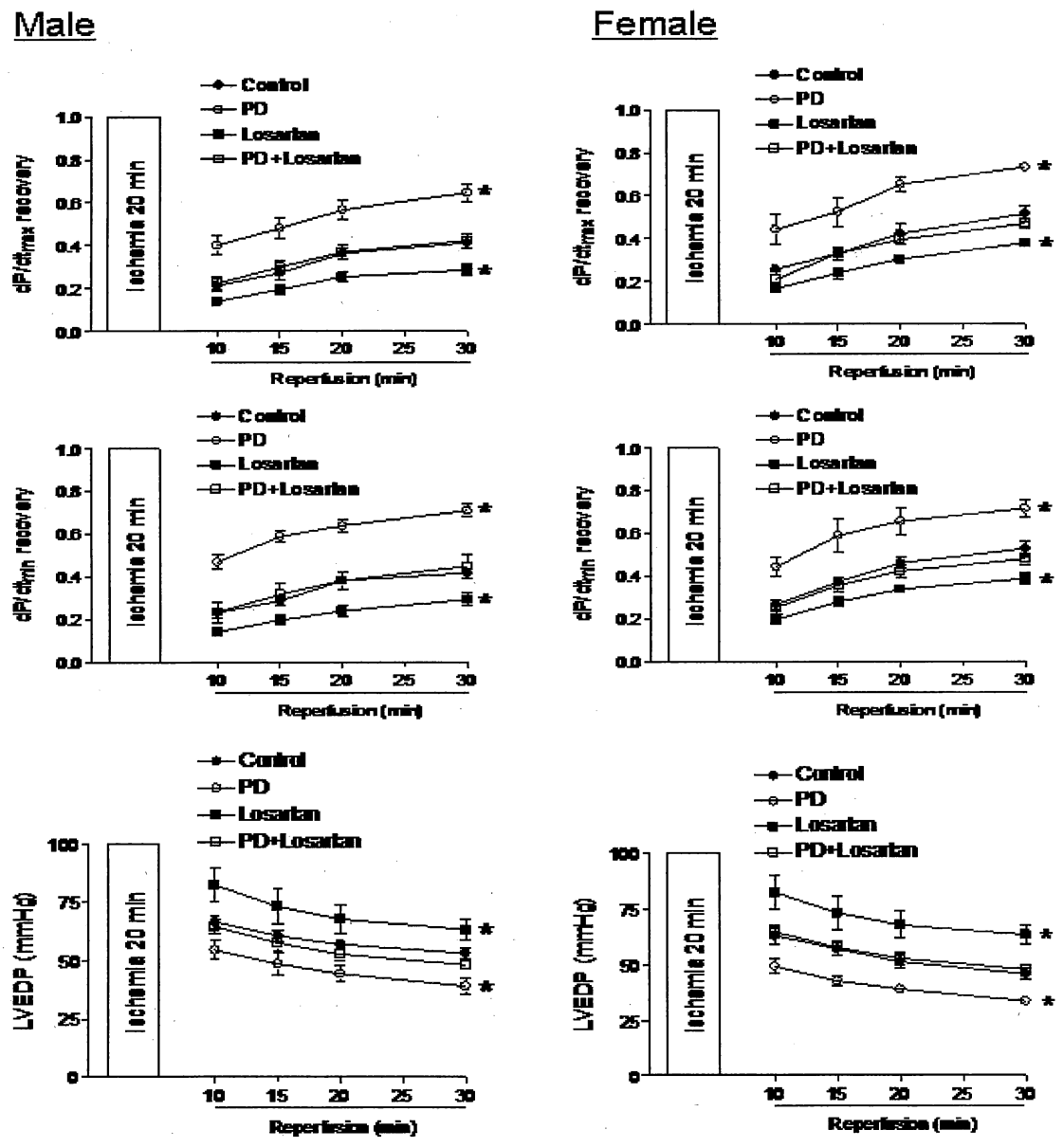

Figure 24. Effect of AT_1R and AT_2R inhibitors on cardiac ischemia and reperfusion injury. Hearts were isolated from 3 months old male and female rats and were pretreated in the absence or presence of losartan (1 μM), or PD123,319 (PD, 0.3 μM), or losartan + PD for 5 minutes before subjecting to 20 minutes of ischemia and 30 minutes of reperfusion in a Langendorff preparation. Post-ischemic recoveries of left ventricle dP/dt_{max}, dP/dt_{min}, and end diastolic pressure (LVEDP) were determined. Data are mean ± SEM. * $P < 0.05$, vs. control. $n = 5$.

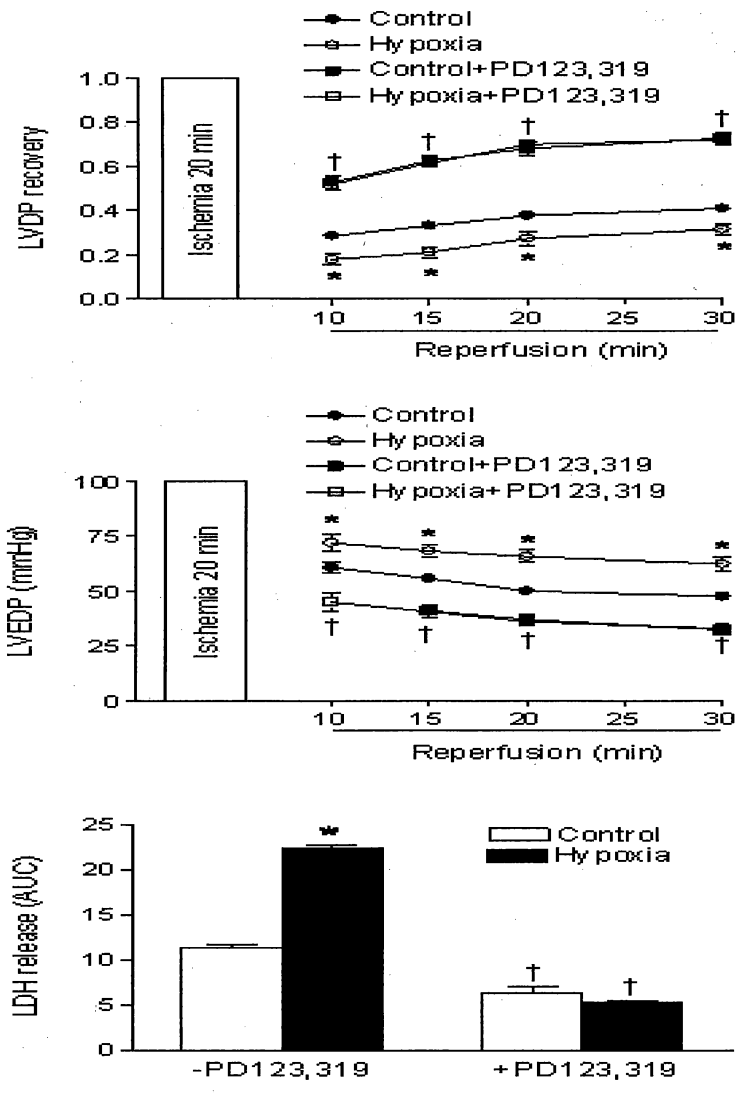

Figure 25. Rescue effect of PD123,319 on hypoxia-mediated ischemic vulnerability. Hearts were isolated from 3-month-old male offspring that had been exposed to normoxia (control) or hypoxia before birth, and were treated in the absence or presence of PD123,319 (0.3 μM) for 5 min before subjecting to 20 min of ischemia and 30 min 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 min of reperfusion was measured as area under curve (AUC). Data are mean ± SEM. * $P < 0.05$, hypoxia vs. control; † $P < 0.05$, +PD123,319 vs. -PD123,319. n = 5.

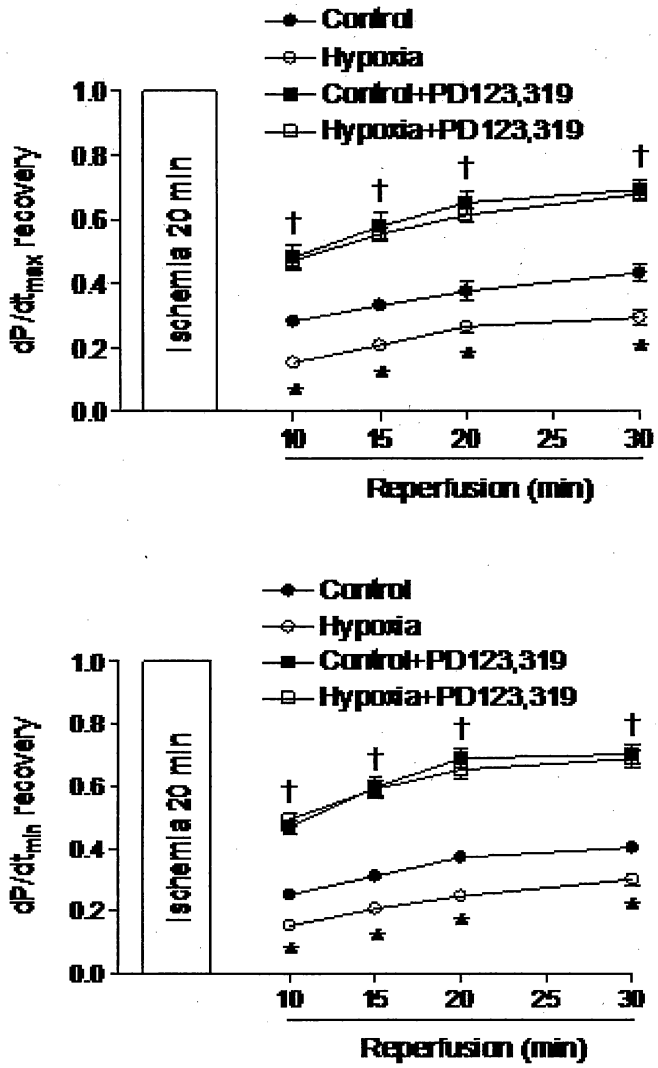

Figure 26. Rescue effect of PD123,319 on hypoxia-mediated ischemic vulnerability. Hearts were isolated from 3-month-old male offspring that had been exposed to normoxia (control) or hypoxia before birth, and were treated in the absence or presence of PD123,319 (0.3 μM) for 5 min before subjecting to 20 min of ischemia and 30 min of reperfusion in a Langendorff preparation. Post-ischemic recovery of dP/dt_{max} and dP/dt_{min} were determined. Data are mean ± SEM. * $P < 0.05$, hypoxia vs. control; † $P < 0.05$, +PD123,319 vs. -PD123,319. n = 5.

Discussion

The present study demonstrates a development-dependent down-regulation of both AT_1R and AT_2R in the heart from the fetus to adult. Previous studies have shown that the Ang II receptor genes are developmentally regulated in a tissue-specific manner, suggesting that the diverse actions of Ang II during development may be mediated by tissue-specific temporal patterns of AT_1R and AT_2R expression (Butkus et al., 1997; Tufro-McReddie et al., 1993; Wintour et al., 1999; Robillard et al., 1995; Shanmugam et al., 1996; Cox et al., 2005). The ontogeny of cardiac AT_1R and AT_2R gene expression has been studied in the third trimester of gestation in fetal sheep and newborn lambs, which demonstrated a rapid decrease in AT_2R, but not AT_1R, mRNA after birth (Samyn et al., 1998). Additionally, it has been shown that the expression of AT_1R and AT_2R is higher in fetal and newborn hearts than that in adults (Everett et al., 1997). The present study demonstrated further that the development-dependent reduction of AT_1R and AT_2R in the heart was the same in both males and females.

Fetal hypoxia down-regulated $AT_{1b}R$ mRNA and AT_1R protein expression, but had no effect on AT_2R expression in the fetal heart, which was sustained in 3 weeks old male offspring. This is consistent with previous findings showing a remarkable decrease in AT_1R mRNA and protein in neonatal rat brain and murine BV-2 cells exposed to hypoxia (Li et al., 2008). Studies in ovine also reported that maternal nutrient restriction decreased AT_1R, but had no effect on AT_2R in the fetus (Gilbert et al., 2007). Furthermore, fetal corticosterone exposure reduced mRNA expression of $AT_{1b}R$, but not $AT_{1a}R$ and AT_2R, in the kidney of near-term fetal rats (Singh et al., 2007). Nevertheless, the finding that fetal hypoxia resulted in increased AT_2R expression in the heart of adult

offspring is intriguing and suggests epigenetic reprogramming of up-regulating a fetal gene of pathophysiologic significance in the heart in a developmental-dependent manner (Papait & Condorelli, 2010; Swynghedauw et al., 2010; Matsubara, 1998). Although AT_2R usually are expressed at low levels in adult life, they are up-regulated in adult hearts under pathological conditions (Matsubara, 1998; levy, 2005). Consistent with the present finding, previous studies demonstrated that prenatal low-protein and/or corticosterone exposure caused an up-regulation of AT_2R in the kidney of offspring (McMullen and Langley-Evans, 2005; Singh et al., 2007).

The expression of AT_1R and AT_2R are regulated by glucocorticoids (Matsubara, 1998). It has been suggested in rats that glucocorticoids play an important role in fetal programming of AT_1R and AT_2R expression pattern in offspring (McCullen S and Langley-Evans, 2005a, 2005b). GREs in both $AT_{1a}R$ and $AT_{1b}R$ gene promoters in rodents have been identified previously (Guo et al., 1995, Bogdarina et al., 2009). $AT_{1a}R$ promoter harbors positive GREs and $AT_{1b}R$ contains negative GREs. Although it has been suggested that glucocorticoids inhibit AT_2R gene expression, GREs at AT_2R gene promoter have not been fully studied. The sequence of the rat AT_2R promoter region that we cloned is in agreement with Ichiki et al (1996). Similar sequences were also reported earlier by Koike et al (1995) and Mukoyama et al (1993). Each sequence indicated the presence of a putative TATAA box. The finding that deletion of the TATAA box region reduced the promoter activity nearly by 79% in the present study indicates an important role of the TATAA element in the transcription of AT_2R gene. However, there is an apparent disagreement in the transcription initiation site of the AT_2R gene between reports of Ichki et al (1996) and Koike et al (1995). We adopted the initiation start site as

indicated by Mukoyama et al (1993), because their data were based on direct cloning and expression of the isolated AT_2R mRNA, whereas results of Koike and Ichiki were derived from primer extension analysis (Koike and Ichiki). We have identified and characterized eight GREs at rat AT_2R gene promoter. These GRE sequences are imperfect representation (half sites) of the positive regulatory consensus GRE sequence, 5'GGTACAnnnTGTTCT-3' (Beato M, Cell, 1989), and their site-specific deletions increased the AT_2R promoter activity, indicating an inhibitory role of these GREs. GREs 1, 2, 3, 4, 6 and 7 satisfy 6 out of 8 probable criteria of the negatively regulated GRE that were defined earlier (Beato M, Cell, 1989, J Steroid Biochem, v 32, 737-748). GREs 5 and 8 meet lesser number of criteria.

Consistent to the presence of negatively regulated GREs at the AT_2R promoter, the dexamethasone treatment of isolated E17 fetal rat hearts caused a dose-dependent down-regulation of AT_2R protein and mRNA expression. This effect was reversed by RU486, indicating a GR-mediated response. Similar findings of glucocorticoid-mediated inhibition of AT_2R expression were obtained previously in PC12 cells (Kijima et al., 1995). The finding of multiple negatively regulated GREs at the AT_2R promoter suggests an important physiological function of glucocorticoids in the down-regulation of AT_2R gene expression in the postnatal development. Furthermore, the finding that fetal hypoxia caused significant decreases in both total cellular and nuclear GR protein abundance in the heart of adult offspring provides a mechanism that may contribute to the reverse of glucocorticoid-dependent developmental down-regulation of AT_2R in the heart. Indeed, fetal hypoxia caused about 50% decreases in GR bindings to the GREs at the AT_2R promoter in offspring hearts, which were similar to the extent of decrease in nuclear GR

protein abundance. The finding that the binding affinity of nuclear extracts to the GREs in the hearts was not significantly different between control and fetal hypoxic offspring suggests that the decreased GR binding to the GREs at rat AT_2R promoter is mainly mediated by the reduced GR density. To a less extent, $AT_{1b}R$ gene is also negatively regulated by glucocorticoids (Bogdarina et al., 2009). In the present study, the increased AT_1R protein abundance in the heart of female adult offspring by fetal hypoxia is mainly mediated by an up-regulation of $AT_{1b}R$ gene expression. Although the cause of the gender difference in programming of AT_1R and AT_2R expression pattern in the heart remains unclear, it has been shown in rats that fetal corticosterone exposure results in increased AT_2R in the kidney of both male and female offspring, but increased AT_1R only in females (Singh et al., 2007).

The functional significance of AT_1R and AT_2R in acute ischemia and reperfusion injury was investigated in isolated rat hearts in a Langendorff preparation. It has been well accepted that long-term, systemic administration of AT_1R blockers prevents the deleterious consequences of ischemia and reperfusion injury and reduces cardiac remodeling. The function that AT_2R plays in normal or diseased hearts is much less clear and appears controversial (Akishita et al., 2000; Brede et al., 2003; Senbonmastsu et al., 2000). The effects of long-term and systemic blockade of AT_1R or AT_2R (as well as receptor knockout studies) on the heart involve multiple mechanisms, whereas the acute and direct effects of AT1R and AT2R on modulating ischemia and reperfusion injury may be quite different as those seen in the long-term and systemic effects. Indeed, in an isolated working heart preparation, it has been demonstrated that AT_1R antagonist losartan decreased, whereas AT_2R antagonist PD 123,319 enhanced, the post-ischemic

recovery of left ventricle function after acute ischemia in rats (Ford et al., 1996, 1998). The present study confirmed this finding and demonstrated further that acute blockade of AT_2R improved myocardial infarction and inhibition of AT_1R exacerbated the myocardial injury. Additionally, the acute effects of AT_1R and AT_2R on ischemia and reperfusion injury in the heart were similar in both male and females animals. This finding suggests a gender-independent, direct cardioprotective effect of AT_1R and the opposite effect of AT_2R in acute ischemia reperfusion injury. Furthermore, the present finding of the lack of the effect with the combination of both AT_1R and the AT_2R blockade indicates an interaction between AT_1R and AT_2R in the heart, and suggests that the ratio of AT_2R to AT_1R is an especially important consideration in the cardiac susceptibility to acute ischemia and reperfusion injury. It has been proposed that the pathophysiological function of AT_2R is context-specific, *i.e.* the ratio of AT_2R to AT_1R (Booz, 2004).

The present finding of the increased ratio of AT_2R to AT_1R in the heart of male offspring exposed to hypoxia before birth suggests enhanced heart susceptibility to ischemia and reperfusion injury. Indeed, our previous studies in the same animal model demonstrated that fetal hypoxia caused an increase in heart susceptibility to ischemia and reperfusion injury in male offspring in a sex-dependent manner (Xue and Zhang, 2009). In the present study, the finding that the blockade of AT_2R with PD 123,319 rescued the myocardial phenotype of the increased ischemic susceptibility in male offspring of prenatal hypoxia provides the course-and-effect evidence for the role of increased AT_2R in fetal programming of enhanced cardiac vulnerability of acute ischemic injury. Whereas the mechanisms underlying the different effects of AT_1R and AT_2R in acute cardiac

ischemic injury remain unclear, it has been know that AT_1R promotes cell growth and proliferation, yet AT_2R mediates antigrowth and apoptosis (Yamada et al., 1996; Matsubara, 1998). These apparent opposite effects provide a congruent functional basis for understanding of the different effects of AT_1R and AT_2R in modulating acute cardiac ischemic injury *vs.* long-term cardiac remodeling. Our previous study suggested that the down-regulation of PKCε in the heart played a role in the increased ischemic susceptibility in adult male offspring of prenatal hypoxia (Xue and Zhang, 2009). Given that AT_1R stimulation and AT_2R blockade activate PKCε and mimic ischemic preconditioning by reducing infarct size (Diaz and Wilson, 1997; Liu et al., 1995; Xu et al., 2000), it is possible that fetal hypoxia-induced programming of the increased AT_2R/AT_1R ratio in the heart of adult males suppresses the PKCε activity leading to the enhanced susceptibility to ischemic injury.

The present investigation provides evidence of fetal programming of up-regulation of the AT_2R/AT_1R ratio in the heart of adult males, linking fetal hypoxia and the increased susceptibility to ischemia and reperfusion injury in the heart of adult male offspring in a sex-dependent manner. Given that hypoxia is one of the most important and clinically relevant stresses to the fetus, and that large epidemiological studies indicate a link between *in utero* adverse stimuli during gestation and an increased risk of ischemic heart disease in the adulthood, the present study provides a mechanistic understanding worthy of investigation in humans.

Acknowledgements

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CHAPTER FIVE

GENERAL DISCUSSION

Hypoxia is one of the most important and clinically stresses which can adversely affect fetal development. Although there is evidence of a link between hypoxia, and fetal intrauterine growth restriction and increased risk of cardiovascular disease in offspring, the mechanism underlying the effect of fetal hypoxia on pulmonary vessels and heart remains unclear. Long-term high-altitude hypoxemia differentially regulates contractility of fetal pulmonary arteries and veins, suggesting a likely common mechanism downstream of NO in fetal pulmonary vessel response to fetal hypoxia. Animal studies have demonstrated that prenatal chronic hypoxia can cause myocardiocyte apoptosis and cardiac hypertrophy in the fetus and adult offspring (Li et al., 2003). Male adult offspring exposed to chronic hypoxia before birth have increased heart susceptibility to I/R injury, which indicates that maternal hypoxia has long term consequences (Li et al., 2003). This increased susceptibility to ischemia injury was correlated with a decrease expression of the cardioprotective gene PKCε and a decrease in HSP70 in the left ventricle of the male rats exposed to chronic hypoxia *in utero* (Li et al., 2003). My research project provided the evidence that (a) fetal hypoxia impaires pulmonary vascular function, (b) resulted in increased heart susceptibility to ischemic injury in male offspring in a sex-dependent manner, and (c) increased heart susceptibility of male offspring is caused by enhanced

AT_2 receptor and reduced PKCε expression in the heart. Additionally, this project also revealed an important role of glucocorticoid in programming of angiotensin II receptor and increased ischemic vulnerability in the heart.

Fetal Hypoixa's Effect on Pulmonary Vessels

Hypoxia in the fetus and newborn is associated with an increased risk of pulmonary hypertension. In the younger animal, vascular resistance seems to reside mainly in both arteries and veins of the lung (Raj and Chen, 1987). When subjected to a variety of stimuli, pulmonary veins exhibit substantial vasoconstriction and contribute a significant portion to total pulmonary vascular resistance (Raj and Anderson, 1990; Zhao et al., 1993). In various species, including human, pulmonary veins show a greater sensitivity than arteries to a number of vasoconstrictor stimuli including hypoxia (Hillier et al., 1997; Sheehan et al., 1992). We have demonstrated that long-term high-altitude hypoxemia differentially regulates contractility and relaxation of both fetal pulmonary arteries and veins. This project has demonstrated a complex relationship between the effects of fetal hypoxia on fetal PA vs. PV and the role of NO. We found that fetal hypoxia significantly increased norepinephrine-induced contractions in PA, but not PV in fetal lambs, suggesting that the site of resistance under conditions of hypoxia in the intact pulmonary circulation is greater in pulmonary arteries than in veins when NO is present. It has been demonstrated that chronic hypoxia produces an increase in α1-adrenoreceptor gene expression and increases pulmonary vascular smooth muscle contractile sensitivity (Lal et al., 1999; Salvi, 1999). In addition, fetal hypoxia decreased norepinephrine-induced contractions in the presence of eNOS inhibitor in PV, indicating that a

compensatory adaptation mechanism in vascular contractility of pulmonary veins to chronic in utero hypoxia.

We also demonstrated that a lack of basal inhibitory effect of eNOS in the regulation of pulmonary arterial contractility in fetal lambs, which is supported by the findings of the minimal eNOS levels and the lack of acetylcholine-induced relaxations in pulmonary arteries. Previous study has shown that calcium ionophore A23187 failed to relax pulmonary arteries in fetal lambs (Irish et al., 1998). Taken together, these studies have demonstrated that the endothelium is not functional in NO- mediated relaxation in pulmonary arteries in near-term fetal lambs. Furthermore, it has been demonstrated that downstream pathway of cGMP-dependent protein kinase is fully functional in fetal pulmonary arteries, supported by the finding that sodium nitroprusside produced concentration-dependent relaxations in pulmonary arteries.

In contrast to pulmonary arteries, pulmonary veins have much higher levels of eNOS in the endothelium and relax to acetylcholine. Additionally, inhibition of eNOS increased norepinephrine-induced contractions, suggesting a significant component of basal eNOS activity in the inhibition of pulmonary vein contractility. We demonstrated that chronic hypoxia decreased both endothelium-dependent and independent relaxation, suggesting a likely mechanism of decreased soluble guanylate cyclase and cGMP in fetal pulmonary vessels in response to fetal hypoxia.

Fetal Hypoxia's Effect on Isolated Working Heart

In chapter 3, we have demonstrated that maternal hypoxia results in the increased sensitivity to I/R injury in male but not female offspring. Previous studies in our lab had

shown a decrease in cardiac PKCε following fetal hypoxia treatment, but the cause and effect relationship of the functional importance of PKCε in the gender dichotomy of increased heart susceptibility to I/R injury in offspring resulting from the fetal hypoxia was not determined. PKCε activation was necessary and sufficient for acute cardioprotection during cardiac I/R injury (Gray et al., 2004). Previous studies have shown a key role of PKCε in cardioprotection against ischemia and reperfusion injury (Dorn et al., 1999; Cross et al., 2002; Gray et al., 2004; Pierre et al., 2007). PKCε activates mitochondrial ATP sensitive K^+ channels which protect cardiomyocytes from apoptosis (Jaburek et al., 2006). Inhibiting PKCε significantly increased ischemic injury and decreased postischemic recovery of left ventricular function in control males, and in the presence of PKCε inhibitor there was no difference in heart susceptibility to I/R injury between the control and hypoxic males. The lack of effect of PKCε inhibitor on ischemic injury of the heart in hypoxic males is consistent with its lack of effect on phospho-PKCε that has already been inhibited in the heart of hypoxic group. In contrast to the males, inhibition of PKCε decreased phospho-PKCε 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 clearly demonstrate a causal effect of PKCε downregulation and increased susceptibility to I/R injury in male offspring resulting from fetal hypoxia.

In chapter 4, we have demonstrated that the acute and direct effects of AT_1R and AT_2R on modulating I/R injury. It has been found that AT_1R antagonist losartan increased, whereas AT_2R antagonist PD 123,319 decreased, the susceptibility to I/R

injury in rats in gender-independent manner. In addition, we also demonstrated that the combination of both AT_1R and the AT_2R blockade indicates an interaction between AT_1R and AT_2R in the heart, suggesting that the ratio of AT_2R to AT_1R is an especially important consideration in the cardiac susceptibility to acute ischemia and reperfusion injury. This is consistent with report that the AT_2R binds directly to the AT_1R and thereby antagonizing its function (AbdAlla et al., 2001). Furthermore, the inhibition of AT_2R rescued the phenotype of increased cardiac ischemic injury observed in adult male offspring exposed to hypoxia before birth, suggesting the cause and effect of increased AT_2R in fetal programming of increased cardiac vulnerability of acute ischemic injury. It has been known that AT1 receptor promotes cell growth and proliferation, however, AT_2R mediates antigrowth and apoptosis (Yamada et al., 1996; Matsubara, 1998), which may explain the different effects of AT_1R and AT_2R on ischemic injury. Previous studies have showed that AT_1R stimulation and AT_2R blockade activate PKCε and mimic ischemic preconditioning by reducing infarct size (Diaz and Wilson, 1997; Liu et al., 1995; Xu et al., 2000), it is possible that fetal hypoxia-induced programming of the increased AT_2R/AT_1R ratio in the heart of adult males suppresses the PKCε activity leading to the enhanced susceptibility to ischemic injury.

Fetal Hypoixa's Effect on AT Receptor

This project focused on AT receptor based on the previous studies implicating a link between fetal insults to differential epigenetic modifications of AT_1R and AT_2R genes in the adrenal and kidney and the resultant alteration of their expression pattern in adult life (Bogdarina et al., 2007; McMullen and Langley-Evans, 2005; Singh et al.,

2007). In chapter 4, we have demonstrated that fetal hypoxia down-regulated $AT_{1b}R$ mRNA and AT_1R protein expression, but had no effect on AT_2R expression in the fetal heart, which was sustained in 3 weeks old male offspring. This is consistent with previous findings showing a remarkable decrease in AT_1R mRNA and protein in neonatal rat brain and murine BV-2 cells exposed to hypoxia (Li et al., 2008). Studies in ovine also reported that maternal nutrient restriction decreased AT_1R, but had no effect on AT_2R in the fetus (Gilbert et al., 2007). Furthermore, fetal corticosterone exposure reduced mRNA expression of $AT_{1b}R$, but not $AT_{1a}R$ and AT_2R, in the kidney of near-term fetal rats (Singh et al., 2007).

Nevertheless, the finding that fetal hypoxia resulted in increased AT_2R expression in the heart of adult offspring is intriguing and suggests epigenetic reprogramming of up-regulating a fetal gene of pathophysiologic significance in the heart in a developmental-dependent manner (Papait & Condorelli, 2010; Swynghedauw et al., 2010; Matsubara, 1998). We also demonstrated that fetal hypoxia increased the ratio of AT_2R to AT_1R in male but not female offspring. AT_1R and AT_2R exert antagonistic action on myocardial biology, especially growth, then the relative expression of these receptors and their ratios under different cardiac pathological conditions may be important in determining myocardial function and structure. Cardiac expression of AT_1R and AT_2R is species dependent, and changes in their relative proportion may influence myocardial hypertrophy and fibrosis. Although AT_2R usually are expressed at low levels in adult life, they are up-regulated in adult hearts under pathological conditions (Matsubara, 1998; levy, 2005). The expression of the myocardial AT_2R was increased in experimental myocardial infarction 1 day after infarction (Nio et al., 1995). Consistent with the present

finding in the project, previous studies demonstrated that prenatal low-protein and/or corticosterone exposure caused an up-regulation of AT_2R in the kidney of offspring (McMullen and Langley-Evans, 2005; Singh et al., 2007). Furthermore, there was a higher expression of AT_2R in endocardial, interstitial, perivascular and infarcted regions of the ventricles of patients with end-stage ischemic heart disease or dilated cardiomyopathy, which were related to fibroblast proliferation and collagen deposition (Wharton et al., 1998). Taken together, these findings suggest that the AT_2R plays some role in cardiovascular remodeling in both animals and humans.

Regulation of PKCε and AT Receptor

We have demonstrated that prenatal hypoxia resulted in a decrease in PKCε protein levels in the heart of male adult offspring suggests in utero epigenetic programming of PKCε gene re- pression in the heart. PKCε has previously been demonstrated to be cardioprotective and the role of PKCε in regulating many pathways has been demonstrated (Chen et al. 2001; Murriel and Mochly-Rosen 2003; Ping et al. 2001). 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 physiologic mechanism of gene regulation and an important component of development and tissue differentiation. Alterations in the DNA methylation status of genes either to increase the expression of growth promoters or inhibit expression of tumor suppressors can result in uncontrolled cell growth and be linked to the pathogenesis of many types f cancer (Baylin et al., 2001). Recent study in our lab demonstrated an epigenetic mechanism of DNA

methylation in programming of cardiac PKCε gene repression, linking fetal cocaine exposure and pathophysiological consequences in the heart of adult male offspring (Zhang et al., 2009). In this study, eight transcription factor binding sites, which contain CpG dinucleotides in their core binding sites, were identified at the promoter of PKCε 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 PKCε promoter and PKCε gene repression in the heart of male offspring. However, increased methylation was observed only at SP1 binding site of -268 in female, which did not change PKCε gene expression in the heart. Recent study in our lab demonstrated that in hearts of both fetuses and adult offspring, hypoxia-increased methylation of SP1 sites was significantly greater in males than in females, and decreased PKCε mRNA was seen only in males.

The expression of AT_1R and AT_2R are regulated by glucocorticoids (Matsubara, 1998). It has been suggested in rats that glucocorticoids play an important role in fetal programming of AT_1R and AT_2R expression pattern in offspring (McCullen S and Langley-Evans, 2005a, 2005b). It has been known that there are GREs in both $AT_{1a}R$ and $AT_{1b}R$ gene promoters in rodents previously (Guo et al., 1995, Bogdarina et al., 2009). $AT_{1a}R$ promoter harbors positive GREs and $AT_{1b}R$ contains negative GREs. Although it has been suggested that glucocorticoids inhibit AT_2R gene expression, GREs at AT_2R gene promoter have not been fully studied. We cloned the sequence of the rat AT_2R promoter region. Each sequence indicated the presence of a putative TATAA box. We demonstrated that deletion of the TATAA box region reduced the promoter activity suggesting an important role of the TATAA element in the transcription of AT_2R gene.

We also have identified and characterized eight GREs at rat AT_2R gene promoter. These include GRE1 (-1853), GRE2 (-1674), GRE3 (-1526), GRE4 (-1159), GRE5 (-945), GRE6 (-676), GRE7 (-107), GRE8 (+13). The site-specific deletions increased the AT_2R promoter activity independently, indicating an inhibitory role of these GREs. Using electrophoretic mobility shift assay (EMSA), we have demonstrated GR nuclear proteins bind to the putative GREs of AT_2R promoter. Immunoblot analysis confirmed the presence of GRs in the nuclear extracts used in EMSA.

The intact fetal heart model provided further evidence of negatively regulated GREs at the AT_2R promoter. The dexamethasone treatment of isolated E17 fetal rat hearts caused a dose-dependent down-regulation of AT_2R protein and mRNA expression. Treating the hearts with GR blocker RU486 during dexamethasone exposure block dexamethasone induced downregulation of AT_2R, indicating a GR-mediated response. Similar findings of glucocorticoid-mediated inhibition of AT_2R expression were obtained previously in PC12 cells (Kijima et al., 1995). The finding of multiple negatively regulated GREs at the AT_2R promoter suggests an important physiological function of glucocorticoids in the down-regulation of AT_2R gene expression in the postnatal development. It has been known that glucocorticoid increases after birth. Furthermore, we demonstrated that fetal hypoxia caused significant decreases in both total cellular and nuclear GR protein abundance in the heart of adult offspring, providing a mechanism that may contribute to the reverse of glucocorticoid-dependent developmental down-regulation of AT_2R in the heart. Indeed, fetal hypoxia caused about 50% decreases in GR bindings to the GREs at the AT_2R promoter in offspring hearts, which were similar to the extent of decrease in nuclear GR protein abundance. The finding that the binding affinity of

nuclear extracts to the GREs in the hearts was not significantly different between control and fetal hypoxic offspring suggests that the decreased GR binding to the GREs at rat AT_2R promoter is mainly mediated by the reduced GR density.

In chapter 4, we also have demonstrated that fetal hypoxia increased AT_1R protein abundance in the heart of female adult offspring, which is mainly mediated by an up-regulation of $AT_{1b}R$ gene expression. Maternal protein restriction increased the expression of AT1b in the adrenal, which may due to the promoter of the $AT_{1b}R$ gene was significantly undermethylated, suggesting a link between fetal insults to epigenetic modification of genes and the resultant alteration of gene expression in adult life leading to the development of hypertension (Bogdarina et al., 2007). To a less extent, $AT_{1b}R$ gene is also negatively regulated by glucocorticoids (Bogdarina I et al., 2009). Although the cause of the gender difference in programming of AT_1R and AT_2R expression pattern in the heart remains unclear, it has been shown in rats that fetal corticosterone exposure results in increased AT_2R in the kidney of both male and female offspring, but increased AT_1R only in females (Singh et al., 2007).

Sex Dichotomy

This project has clearly demonstrated a sex dichotomy in manifestation of increased cardiac vulnerability to ischemia and reperfusion injury in adult offspring resulting from fetal hypoxia, which is consistent with the previous study that demonstrated 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). All these findings suggest organ specificity, tissue specificity, or both of sex-dependent programming induced by intrauterine insults.

In chapter 3 and 4, we have demonstrated that fetal hypoxia alters the expression of PKCε and AT receptor in a sex dependent manner. In the ovine fetus, it has been shown that there is a sex difference in the ontogeny of gene expression in the RAS (Gilbert et al., 2007). AT_1R protein was increased from middle gestation to late gestation in male but not female fetuses. However, AT_2R protein decreased in the female but not male fetuses from middle to late gestation. Although the origin and the mechanism of these sex differences in fetal protein expression remain unclear, it should be considered that these observations simply reflect different trajectories of fetal development between the sexes. Additionally, sex differences are observed in the response to maternal nutrient restriction (MNR) between male and female fetuses near term (Cox et al., 2008). Compared with females, that gene expression of key components of the RAS is down-regulated in MNR males. Study has shown that maternal global caloric restriction impairs nephrogenesis and alters AT_1, AT_2, and renin expression in gender-specific mmaner (Gilbert et al., 2007)). It has also been found that only male offspring of these MNR ewes are hypertensive (Gilbert et al., 2005). The mechanisms by which MNR alters gene expression are not fully understood, which (Lillycrop et al. 2005, 2007, 2008) may be due to the alteration of gene methylation patterns. Furthermore, recent clinical studies show that females are more responsive to the effects of ACE inhibition than men in an estrogen-independent manner (Pretorius et al., 2005). It seems plausible that there are

many potential avenues, from embryonic life to adulthood, through which sex differences may interact with developmental programming stimuli or programmed adaptations to result in sex-specific cardiorenal disease susceptibility.

Future Studies

This research has yielded several interesting findings which are warrant further investigation. The gender dichotomy in the effect of fetal hypoxia to heart is an intriguing finding that has been seen in other models (Bae et al., 2005). It is not clear at present whether the gender-specific effect of prenatal hypoxia on the heart of offspring are primarily mediated by the differences in sex steroid hormones developed postnatally or by the differences exist between genetically male (XY) and female (XX) cells in determining an "programming-sensitive" phenotype. The mechanisms for the sexual dimorphism in cardiac programming in vulnerability to I/R injury remains unclear. Major differences in plasma levels of sex steroids between males and females are not seen until after puberty. However, the effect of estrogen may be gender dependent due to the possible difference in estrogen receptor expression. The sex dichotomy independent of sex hormones may be due to dysfunction of the X chromosome gene in male, which results in the more sensitive to fetal hypoxia, or the effect of Y chromosome gene expression. However, studies of ovariectomized rats and estrogen replacement have suggested that estrogen plays an important role in the cardioprotection of global I/R injury in female hearts (Zhai et al., 2000). Future study can test whether fetal hypoxia can increase the susceptibility to I/R injury in ovariectomized rats and whether estrogen

replacement can abolish the difference in I/R injury in ovariectomized rats. In brief, Time-dated pregnant rats will be raised and divided between normoxic and hypoxic (10.5% O2 on days 15-21 of gestation). After birth, the pups will be raised under the normoxic condition. Some of 2-month-old female rats will be ovariectomized with or without estrogen replacement treatment. Hearts from control, ovariectomized rats, and rats treated with estrogen replacement will be isolated and subjected to I/R injury in a langendorff preparation.

We has demonstrated that the dexamethasone treatment in isolated E17 fetal rat hearts caused a dose-dependent down-regulation of AT_2R expression and up-regulation of AT_1R expression, which can be reversed by RU486. Glucocorticoids play an important role in the response to stress, influencing the regulation of blood pressure, inflammation, immune function and cellular energy metabolism (Sapolsky et al., 2000). The cardioprotective effects of glucocorticoids in acute I/R injury have been demonstrated in animals (Valen et al., 2000; Varga et al., 2004; Skyschally et al., 2004) and human (Giugliano et al., 2003). Glucocorticoids have the ability to limit the acute inflammatory response associated with acute myocardial infarction. However, the mechanism underlying the dexamethasone induced cardiac protection from I/R injury remains unclear. The GR positively and negatively regulate gene expression by distinct mechanisms (Pascual and Glass, 2006). Our preliminary data showed that dexamethasone (1mg/kg for 5 days, intraperitoneal injection) increased AT_1R protein expression, decreased AT_2R protein expression and decreased GR expression in adult male rats (data not shown). We have demonstrated the presence of negatively regulated GREs at the AT_2R promoter. In addition, previous studies have demonstrated that $AT_{1a}R$ promoter

harbors positive GREs and $AT_{1b}R$ contains negative GREs. Furthermore, we have demonstrated that AT_1R antagonist decreased, whereas AT_2R antagonist enhanced, the post-ischemic recovery of left ventricle function in rats. All these findings suggest that it is possible that the glucocorticoid protect hearts from I/R injury due to the alteration of ATR. To test the hypothesis, hearts will be isolated from both control and dexamethasone treatment rats, and be subjected to I/R injury in a langendorff preparation with and without combination of both AT_1R and AT_2R blockers. We will confirm the binding of GR to GRE at the promoter of AT_1R and AT_2R gene by EMSA and Chromatin Immunoprecipitation (ChIP).

Conclusions

This project has demonstrated heterogeneity of fetal pulmonary arteries and veins in response to long-term high-altitude hypoxia and suggested a likely common mechanism downstream of NO in fetal pulmonary vessel response to chronic hypoxia *in utero*. In addition, our finding also suggest that prenatal hypoxia causes an increase in heart susceptibility to ischemia and reperfusion injury in offspring in a sex-dependent manner, which is due to enhanced AT_2R and reduced PKCε expression in the heart. Furthermore, in adult hearts, there is a significant decrease in the abundance of total cellular and nuclear GR in the offspring with prenatal hypoxia. Our studies have revealed an important role of glucocorticoid in programming of angiotensin II receptor and PKCε expression pattern and increased ischemic vulnerability in the heart. These findings provide a mechanistic understanding worth of investigation in human in fetal origins of cardiovascular disease.

REFERENCES

ABDALLA, S., H. LOTHER, A. M. ABDEL-TAWAB and U. QUITTERER, 2001 The angiotensin II AT2 receptor is an AT1 receptor antagonist. J Biol Chem **276:** 39721-39726.

ABMAN, S. H., B. A. CHATFIELD, S. L. HALL and I. F. MCMURTRY, 1990 Role of endothelium-derived relaxing factor during transition of pulmonary circulation at birth. Am J Physiol **259:** H1921-1927.

AKISHITA, M., M. IWAI, L. WU, L. ZHANG, Y. OUCHI *et al.*, 2000 Inhibitory effect of angiotensin II type 2 receptor on coronary arterial remodeling after aortic banding in mice. Circulation **102:** 1684-1689.

ARRIGONI, F. I., A. A. HISLOP, S. G. HAWORTH and J. A. MITCHELL, 1999 Newborn intrapulmonary veins are more reactive than arteries in normal and hypertensive piglets. Am J Physiol **277:** L887-892.

BAE, S., R. D. GILBERT, C. A. DUCSAY and L. ZHANG, 2005 Prenatal cocaine exposure increases heart susceptibility to ischaemia-reperfusion injury in adult male but not female rats. J Physiol **565:** 149-158.

BAE, S., Y. XIAO, G. LI, C. A. CASIANO and L. ZHANG, 2003 Effect of maternal chronic hypoxic exposure during gestation on apoptosis in fetal rat heart. Am J Physiol Heart Circ Physiol **285:** H983-990.

BAE, S., and L. ZHANG, 2005 Gender differences in cardioprotection against ischemia/reperfusion injury in adult rat hearts: focus on Akt and protein kinase C signaling. J Pharmacol Exp Ther **315:** 1125-1135.

BALLARD, P. L., 1987 Glucocorticoid regulation of lung maturation. Mead Johnson Symp Perinat Dev Med: 22-27.

BARKER, D. J., 1997a Fetal nutrition and cardiovascular disease in later life. Br Med Bull **53:** 96-108.

BARKER, D. J., 1997b Intra-uterine programming of the adult cardiovascular system. Curr Opin Nephrol Hypertens **6:** 106-110.

BARKER, D. J., 1999 Fetal origins of cardiovascular disease. Ann Med **31 Suppl 1:** 3-6.

BARKER, D. J., 2000 In utero programming of cardiovascular disease. Theriogenology **53:** 555-574.

BARKER, D. J., 2007 The origins of the developmental origins theory. J Intern Med **261:** 412-417.

BARKER, D. J., P. D. GLUCKMAN, K. M. GODFREY, J. E. HARDING, J. A. OWENS *et al.*, 1993 Fetal nutrition and cardiovascular disease in adult life. Lancet **341:** 938-941.

BARKER, D. J., C. OSMOND, J. GOLDING, D. KUH and M. E. WADSWORTH, 1989 Growth in utero, blood pressure in childhood and adult life, and mortality from cardiovascular disease. BMJ **298:** 564-567.

BATESON, P., D. BARKER, T. CLUTTON-BROCK, D. DEB, B. D'UDINE *et al.*, 2004 Developmental plasticity and human health. Nature **430:** 419-421.

BAYLIN, S., 2001 DNA methylation and epigenetic mechanisms of carcinogenesis. Dev Biol (Basel) **106:** 85-87; discussion 143-160.

BEATO, M., 1989 Gene regulation by steroid hormones. Cell **56:** 335-344.

BEATO, M., G. CHALEPAKIS, M. SCHAUER and E. P. SLATER, 1989 DNA regulatory elements for steroid hormones. J Steroid Biochem **32:** 737-747.

BENITZ, W. E., J. D. COULSON, D. S. LESSLER and M. BERNFIELD, 1986 Hypoxia inhibits proliferation of fetal pulmonary arterial smooth muscle cells in vitro. Pediatr Res **20:** 966-972.

BILLET, S., F. AGUILAR, C. BAUDRY and E. CLAUSER, 2008 Role of angiotensin II AT1 receptor activation in cardiovascular diseases. Kidney Int **74:** 1379-1384.

BLACK, S. M., M. J. JOHENGEN, Z. D. MA, J. BRISTOW and S. J. SOIFER, 1997 Ventilation and oxygenation induce endothelial nitric oxide synthase gene expression in the lungs of fetal lambs. J Clin Invest **100:** 1448-1458.

BLOOM, S. L., J. S. SHEFFIELD, D. D. MCINTIRE and K. J. LEVENO, 2001 Antenatal dexamethasone and decreased birth weight. Obstet Gynecol **97:** 485-490.

BOGDARINA, I., S. WELHAM, P. J. KING, S. P. BURNS and A. J. CLARK, 2007 Epigenetic modification of the renin-angiotensin system in the fetal programming of hypertension. Circ Res **100:** 520-526.

BOGDARINA, I. G., P. J. KING and A. J. CLARK, 2009 Characterization of the angiotensin (AT1b) receptor promoter and its regulation by glucocorticoids. J Mol Endocrinol **43:** 73-80.

BOLLI, R., 2001 Cardioprotective function of inducible nitric oxide synthase and role of nitric oxide in myocardial ischemia and preconditioning: an overview of a decade of research. J Mol Cell Cardiol **33:** 1897-1918.

BOLLI, R., M. O. JEROUDI, B. S. PATEL, C. M. DUBOSE, E. K. LAI *et al.*, 1989 Direct evidence that oxygen-derived free radicals contribute to postischemic myocardial dysfunction in the intact dog. Proc Natl Acad Sci U S A **86:** 4695-4699.

BOOZ, G. W., 2004 Cardiac angiotensin AT2 receptor: what exactly does it do? Hypertension **43:** 1162-1163.

BOUWMAN, R. A., K. SALIC, F. G. PADDING, E. C. ERINGA, B. J. VAN BEEK-HARMSEN *et al.*, 2006 Cardioprotection via activation of protein kinase C-delta depends on modulation of the reverse mode of the Na+/Ca2+ exchanger. Circulation **114:** I226-232.

BRAWLEY, L., S. ITOH, C. TORRENS, A. BARKER, C. BERTRAM *et al.*, 2003 Dietary protein restriction in pregnancy induces hypertension and vascular defects in rat male offspring. Pediatr Res **54:** 83-90.

BRAWLEY, L., C. TORRENS, F. W. ANTHONY, S. ITOH, T. WHEELER *et al.*, 2004 Glycine rectifies vascular dysfunction induced by dietary protein imbalance during pregnancy. J Physiol **554:** 497-504.

BREDE, M., W. ROELL, O. RITTER, F. WIESMANN, R. JAHNS *et al.*, 2003 Cardiac hypertrophy is associated with decreased eNOS expression in angiotensin AT2 receptor-deficient mice. Hypertension **42:** 1177-1182.

BROWNE, V. A., V. M. STIFFEL, W. J. PEARCE, L. D. LONGO and R. D. GILBERT, 1997 Activator calcium and myocardial contractility in fetal sheep exposed to long-term high-altitude hypoxia. Am J Physiol **272:** H1196-1204.

BUTKUS, A., A. ALBISTON, D. ALCORN, M. GILES, J. MCCAUSLAND *et al.*, 1997 Ontogeny of angiotensin II receptors, types 1 and 2, in ovine mesonephros and metanephros. Kidney Int **52:** 628-636.

BUTLER, T. G., J. SCHWARTZ and I. C. MCMILLEN, 2002 Differential effects of the early and late intrauterine environment on corticotrophic cell development. J Clin Invest **110:** 783-791.

CHASSAGNE, C., S. EDDAHIBI, C. ADAMY, D. RIDEAU, F. MAROTTE *et al.*, 2000 Modulation of angiotensin II receptor expression during development and regression of hypoxic pulmonary hypertension. Am J Respir Cell Mol Biol **22:** 323-332.

CHAZOVA, I., J. E. LOYD, V. S. ZHDANOV, J. H. NEWMAN, Y. BELENKOV *et al.*, 1995 Pulmonary artery adventitial changes and venous involvement in primary pulmonary hypertension. Am J Pathol **146:** 389-397.

CHEN, L., H. HAHN, G. WU, C. H. CHEN, T. LIRON *et al.*, 2001 Opposing cardioprotective actions and parallel hypertrophic effects of delta PKC and epsilon PKC. Proc Natl Acad Sci U S A **98:** 11114-11119.

CHUNG, O., H. KUHL, M. STOLL and T. UNGER, 1998 Physiological and pharmacological implications of AT1 versus AT2 receptors. Kidney Int Suppl **67:** S95-99.

CORNFIELD, D. N., B. A. CHATFIELD, J. A. MCQUESTON, I. F. MCMURTRY and S. H. ABMAN, 1992 Effects of birth-related stimuli on L-arginine-dependent pulmonary vasodilation in ovine fetus. Am J Physiol **262:** H1474-1481.

CORNFIELD, D. N., H. L. REEVE, S. TOLAROVA, E. K. WEIR and S. ARCHER, 1996 Oxygen causes fetal pulmonary vasodilation through activation of a calcium-dependent potassium channel. Proc Natl Acad Sci U S A **93:** 8089-8094.

CORSTIUS, H. B., M. A. ZIMANYI, N. MAKA, T. HERATH, W. THOMAS *et al.*, 2005 Effect of intrauterine growth restriction on the number of cardiomyocytes in rat hearts. Pediatr Res **57:** 796-800.

COX, B. E., X. T. LIU, S. J. FLUHARTY and C. R. ROSENFELD, 2005 Vessel-specific regulation of angiotensin II receptor subtypes during ovine development. Pediatr Res **57:** 124-132.

CROSS, H. R., E. MURPHY, R. BOLLI, P. PING and C. STEENBERGEN, 2002 Expression of activated PKC epsilon (PKC epsilon) protects the ischemic heart, without attenuating ischemic H(+) production. J Mol Cell Cardiol **34:** 361-367.

DAEMEN, M. J., D. M. LOMBARDI, F. T. BOSMAN and S. M. SCHWARTZ, 1991 Angiotensin II induces smooth muscle cell proliferation in the normal and injured rat arterial wall. Circ Res **68:** 450-456.

DAVIS, D. R., K. WILSON, M. J. SAM, S. E. KENNEDY, N. MACKMAN *et al.*, 2007 The development of cardiac fibrosis in low tissue factor mice is gender-dependent and is associated with differential regulation of urokinase plasminogen activator. J Mol Cell Cardiol **42:** 559-571.

DHALLA, N. S., M. R. DENT, P. S. TAPPIA, R. SETHI, J. BARTA *et al.*, 2006 Subcellular remodeling as a viable target for the treatment of congestive heart failure. J Cardiovasc Pharmacol Ther **11:** 31-45.

DHALLA, N. S., H. K. SAINI, P. S. TAPPIA, R. SETHI, S. A. MENGI *et al.*, 2007 Potential role and mechanisms of subcellular remodeling in cardiac dysfunction due to ischemic heart disease. J Cardiovasc Med (Hagerstown) **8:** 238-250.

DIAZ, R. J., and G. J. WILSON, 1997 Selective blockade of AT1 angiotensin II receptors abolishes ischemic preconditioning in isolated rabbit hearts. J Mol Cell Cardiol **29:** 129-139.

DIEP, Q. N., J. S. LI and E. L. SCHIFFRIN, 1999 In vivo study of AT(1) and AT(2) angiotensin receptors in apoptosis in rat blood vessels. Hypertension **34:** 617-624.

DO CARMO PINHO FRANCO, M., D. NIGRO, Z. B. FORTES, R. C. TOSTES, M. H. CARVALHO *et al.*, 2003 Intrauterine undernutrition--renal and vascular origin of hypertension. Cardiovasc Res **60:** 228-234.

DONG, F., S. P. FORD, C. X. FANG, M. J. NIJLAND, P. W. NATHANIELSZ *et al.*, 2005 Maternal nutrient restriction during early to mid gestation up-regulates cardiac insulin-like growth factor (IGF) receptors associated with enlarged ventricular size in fetal sheep. Growth Horm IGF Res **15:** 291-299.

DORN, G. W., 2ND, and D. MOCHLY-ROSEN, 2002 Intracellular transport mechanisms of signal transducers. Annu Rev Physiol **64:** 407-429.

DORN, G. W., 2ND, M. C. SOUROUJON, T. LIRON, C. H. CHEN, M. O. GRAY *et al.*, 1999 Sustained in vivo cardiac protection by a rationally designed peptide that causes epsilon protein kinase C translocation. Proc Natl Acad Sci U S A **96:** 12798-12803.

DRAKE, A. J., and B. R. WALKER, 2004 The intergenerational effects of fetal programming: non-genomic mechanisms for the inheritance of low birth weight and cardiovascular risk. J Endocrinol **180:** 1-16.

DUDLEY, D. T., R. L. PANEK, T. C. MAJOR, G. H. LU, R. F. BRUNS *et al.*, 1990 Subclasses of angiotensin II binding sites and their functional significance. Mol Pharmacol **38:** 370-377.

DUMITRASCU, R., N. WEISSMANN, H. A. GHOFRANI, E. DONY, K. BEUERLEIN *et al.*, 2006 Activation of soluble guanylate cyclase reverses experimental pulmonary hypertension and vascular remodeling. Circulation **113:** 286-295.

EISEN, A., E. Z. FISMAN, M. RUBENFIRE, D. FREIMARK, R. MCKECHNIE *et al.*, 2004 Ischemic preconditioning: nearly two decades of research. A comprehensive review. Atherosclerosis **172:** 201-210.

ERIKSSON, J. G., T. FORSEN, J. TUOMILEHTO, P. D. WINTER, C. OSMOND *et al.*, 1999 Catch-up growth in childhood and death from coronary heart disease: longitudinal study. BMJ **318:** 427-431.

EVERETT, A. D., A. FISHER, A. TUFRO-MCREDDIE and M. HARRIS, 1997 Developmental regulation of angiotensin type 1 and 2 receptor gene expression and heart growth. J Mol Cell Cardiol **29:** 141-148.

FERNANDEZ-TWINN, D. S., S. EKIZOGLOU, A. WAYMAN, C. J. PETRY and S. E. OZANNE, 2006 Maternal low-protein diet programs cardiac beta-adrenergic response and signaling in 3-mo-old male offspring. Am J Physiol Regul Integr Comp Physiol **291:** R429-436.

FINEMAN, J. R., S. J. SOIFER and M. A. HEYMANN, 1995 Regulation of pulmonary vascular tone in the perinatal period. Annu Rev Physiol **57:** 115-134.

FLAHERTY, J. T., B. PITT, J. W. GRUBER, R. R. HEUSER, D. A. ROTHBAUM *et al.*, 1994 Recombinant human superoxide dismutase (h-SOD) fails to improve recovery of

ventricular function in patients undergoing coronary angioplasty for acute myocardial infarction. Circulation **89:** 1982-1991.

FORD, W. R., A. S. CLANACHAN and B. I. JUGDUTT, 1996 Opposite effects of angiotensin AT1 and AT2 receptor antagonists on recovery of mechanical function after ischemia-reperfusion in isolated working rat hearts. Circulation **94:** 3087-3089.

FORD, W. R., A. S. CLANACHAN, G. D. LOPASCHUK, R. SCHULZ and B. I. JUGDUTT, 1998 Intrinsic ANG II type 1 receptor stimulation contributes to recovery of postischemic mechanical function. Am J Physiol **274:** H1524-1531.

FRANCO MDO, C., A. P. DANTAS, E. H. AKAMINE, E. M. KAWAMOTO, Z. B. FORTES *et al.*, 2002 Enhanced oxidative stress as a potential mechanism underlying the programming of hypertension in utero. J Cardiovasc Pharmacol **40:** 501-509.

FRENCH, N. P., R. HAGAN, S. F. EVANS, M. GODFREY and J. P. NEWNHAM, 1999 Repeated antenatal corticosteroids: size at birth and subsequent development. Am J Obstet Gynecol **180:** 114-121.

GAO, Y., S. DHANAKOTI, E. M. TREVINO, F. C. SANDER, A. M. PORTUGAL *et al.*, 2003 Effect of oxygen on cyclic GMP-dependent protein kinase-mediated relaxation in ovine fetal pulmonary arteries and veins. Am J Physiol Lung Cell Mol Physiol **285:** L611-618.

GAO, Y., A. D. PORTUGAL, J. LIU, S. NEGASH, W. ZHOU *et al.*, 2008 Preservation of cGMP-induced relaxation of pulmonary veins of fetal lambs exposed to chronic high altitude hypoxia: role of PKG and Rho kinase. Am J Physiol Lung Cell Mol Physiol **295:** L889-896.

GAO, Y., and J. U. RAJ, 2005 Role of veins in regulation of pulmonary circulation. Am J Physiol Lung Cell Mol Physiol **288:** L213-226.

GAO, Y., H. ZHOU and J. U. RAJ, 1995 Heterogeneity in role of endothelium-derived NO in pulmonary arteries and veins of full-term fetal lambs. Am J Physiol **268:** H1586-1592.

GEGGEL, R. L., M. J. ARONOVITZ and L. M. REID, 1986 Effects of chronic in utero hypoxemia on rat neonatal pulmonary arterial structure. J Pediatr **108:** 756-759.

GILBERT, J. S., L. A. COX, G. MITCHELL and M. J. NIJLAND, 2006 Nutrient-restricted fetus and the cardio-renal connection in hypertensive offspring. Expert Rev Cardiovasc Ther **4:** 227-237.

GILBERT, J. S., S. P. FORD, A. L. LANG, L. R. PAHL, M. C. DRUMHILLER *et al.*, 2007 Nutrient restriction impairs nephrogenesis in a gender-specific manner in the ovine fetus. Pediatr Res **61:** 42-47.

GILBERT, R. D., 1998 Fetal myocardial responses to long-term hypoxemia. Comp Biochem Physiol A Mol Integr Physiol **119:** 669-674.

GIUGLIANO, G. R., R. P. GIUGLIANO, C. M. GIBSON and R. E. KUNTZ, 2003 Meta-analysis of corticosteroid treatment in acute myocardial infarction. Am J Cardiol **91:** 1055-1059.

GIUSSANI, D. A., P. S. PHILLIPS, S. ANSTEE and D. J. BARKER, 2001 Effects of altitude versus economic status on birth weight and body shape at birth. Pediatr Res **49:** 490-494.

GLUCKMAN, P. D., M. A. HANSON, C. COOPER and K. L. THORNBURG, 2008 Effect of in utero and early-life conditions on adult health and disease. N Engl J Med **359:** 61-73.

GODFREY, K. M., and D. J. BARKER, 2000 Fetal nutrition and adult disease. Am J Clin Nutr **71:** 1344S-1352S.

GOPALAKRISHNAN, G. S., D. S. GARDNER, J. DANDREA, S. C. LANGLEY-EVANS, S. PEARCE *et al.*, 2005 Influence of maternal pre-pregnancy body composition and diet during early-mid pregnancy on cardiovascular function and nephron number in juvenile sheep. Br J Nutr **94:** 938-947.

GOULD, A. B., and S. A. GOODMAN, 1970 The effect of hypoxia on the renin-angiotensinogen system. Lab Invest **22:** 443-447.

GRAY, M. O., J. S. KARLINER and D. MOCHLY-ROSEN, 1997 A selective epsilon-protein kinase C antagonist inhibits protection of cardiac myocytes from hypoxia-induced cell death. J Biol Chem **272:** 30945-30951.

GRAY, M. O., H. Z. ZHOU, I. SCHAFHALTER-ZOPPOTH, P. ZHU, D. MOCHLY-ROSEN *et al.*, 2004 Preservation of base-line hemodynamic function and loss of inducible cardioprotection in adult mice lacking protein kinase C epsilon. J Biol Chem **279:** 3596-3604.

GROVER, G. J., P. G. SLEPH and S. DZWONCZYK, 1992 Role of myocardial ATP-sensitive potassium channels in mediating preconditioning in the dog heart and their possible interaction with adenosine A1-receptors. Circulation **86:** 1310-1316.

GUO, D. F., S. UNO and T. INAGAMI, 1995a Steroid hormones upregulate rat angiotensin II type 1A receptor gene: role of glucocorticoid responsive elements in rat angiotensin II type 1A promoter. J Steroid Biochem Mol Biol **53:** 69-73.

GUO, D. F., S. UNO, A. ISHIHATA, N. NAKAMURA and T. INAGAMI, 1995b Identification of a cis-acting glucocorticoid responsive element in the rat angiotensin II type 1A promoter. Circ Res **77:** 249-257.

GUTIERREZ-ADAN, A., M. OTER, B. MARTINEZ-MADRID, B. PINTADO and J. DE LA FUENTE, 2000 Differential expression of two genes located on the X chromosome between male and female in vitro-produced bovine embryos at the blastocyst stage. Mol Reprod Dev **55:** 146-151.

HADOKE, P. W., R. S. LINDSAY, J. R. SECKL, B. R. WALKER and C. J. KENYON, 2006 Altered vascular contractility in adult female rats with hypertension programmed by prenatal glucocorticoid exposure. J Endocrinol **188:** 435-442.

HAN, H. C., K. J. AUSTIN, P. W. NATHANIELSZ, S. P. FORD, M. J. NIJLAND *et al.*, 2004 Maternal nutrient restriction alters gene expression in the ovine fetal heart. J Physiol **558:** 111-121.

HARDING, J. E., 2001 The nutritional basis of the fetal origins of adult disease. Int J Epidemiol **30:** 15-23.

HASSOUN, P. M., G. FILIPPOV, M. FOGEL, C. DONALDSON, U. S. KAYYALI *et al.*, 2004 Hypoxia decreases expression of soluble guanylate cyclase in cultured rat pulmonary artery smooth muscle cells. Am J Respir Cell Mol Biol **30:** 908-913.

HERRERA, E. A., V. M. PULGAR, R. A. RIQUELME, E. M. SANHUEZA, R. V. REYES *et al.*, 2007 High-altitude chronic hypoxia during gestation and after birth modifies cardiovascular responses in newborn sheep. Am J Physiol Regul Integr Comp Physiol **292:** R2234-2240.

HEYDECK, D., J. ROIGAS, C. ROIGAS, B. PAPIES and A. LUN, 1994 The catecholamine sensitivity of adult rats is enhanced after prenatal hypoxia. Biol Neonate **66:** 106-111.

HILLIER, S. C., J. A. GRAHAM, C. C. HANGER, P. S. GODBEY, R. W. GLENNY *et al.*, 1997 Hypoxic vasoconstriction in pulmonary arterioles and venules. J Appl Physiol **82:** 1084-1090.

HO, S. M., and W. Y. TANG, 2007 Techniques used in studies of epigenome dysregulation due to aberrant DNA methylation: an emphasis on fetal-based adult diseases. Reprod Toxicol **23:** 267-282.

HORIUCHI, M., M. AKISHITA and V. J. DZAU, 1999 Recent progress in angiotensin II type 2 receptor research in the cardiovascular system. Hypertension **33:** 613-621.

HUNTER, J. C., J. C. KOSTYAK, J. L. NOVOTNY, A. M. SIMPSON and D. H. KORZICK, 2007 Estrogen deficiency decreases ischemic tolerance in the aged rat heart: Roles of PKCdelta, PKCepsilon, Akt, and GSK3beta. Am J Physiol Regul Integr Comp Physiol **292:** R800-809.

ICHIKI, T., Y. KAMBAYASHI and T. INAGAMI, 1996 Transcription of the rat angiotensin II type 2 receptor gene. Biochem Biophys Res Commun **222:** 566-571.

IKEGAMI, M., A. H. JOBE, J. NEWNHAM, D. H. POLK, K. E. WILLET *et al.*, 1997 Repetitive prenatal glucocorticoids improve lung function and decrease growth in preterm lambs. Am J Respir Crit Care Med **156:** 178-184.

INAGAKI, K., E. CHURCHILL and D. MOCHLY-ROSEN, 2006 Epsilon protein kinase C as a potential therapeutic target for the ischemic heart. Cardiovasc Res **70:** 222-230.

INAGAKI, K., H. S. HAHN, G. W. DORN, 2ND and D. MOCHLY-ROSEN, 2003 Additive protection of the ischemic heart ex vivo by combined treatment with delta-protein kinase C inhibitor and epsilon-protein kinase C activator. Circulation **108:** 869-875.

IRISH, M. S., P. L. GLICK, J. RUSSELL, P. KAPUR, D. A. BAMBINI *et al.*, 1998 Contractile properties of intralobar pulmonary arteries and veins in the fetal lamb model of congenital diaphragmatic hernia. J Pediatr Surg **33:** 921-928.

JAENISCH, R., and A. BIRD, 2003 Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. Nat Genet **33 Suppl:** 245-254.

JAMES, T. N., 1998 Normal and abnormal consequences of apoptosis in the human heart. Annu Rev Physiol **60:** 309-325.

JEFFERY, T. K., and J. C. WANSTALL, 2001 Pulmonary vascular remodeling: a target for therapeutic intervention in pulmonary hypertension. Pharmacol Ther **92:** 1-20.

JENSEN, G. M., and L. G. MOORE, 1997 The effect of high altitude and other risk factors on birthweight: independent or interactive effects? Am J Public Health **87:** 1003-1007.

JONES, P. A., and S. B. BAYLIN, 2002 The fundamental role of epigenetic events in cancer. Nat Rev Genet **3:** 415-428.

JONES, P. A., and P. W. LAIRD, 1999 Cancer epigenetics comes of age. Nat Genet **21:** 163-167.

KAMITOMO, M., L. D. LONGO and R. D. GILBERT, 1992 Right and left ventricular function in fetal sheep exposed to long-term high-altitude hypoxemia. Am J Physiol **262:** H399-405.

KAMITOMO, M., J. ONISHI, I. GUTIERREZ, V. M. STIFFEL and R. D. GILBERT, 2002 Effects of long-term hypoxia and development on cardiac contractile proteins in fetal and adult sheep. J Soc Gynecol Investig **9:** 335-341.

KAWAMURA, M., H. ITOH, S. YURA, H. MOGAMI, S. SUGA *et al.*, 2007 Undernutrition in utero augments systolic blood pressure and cardiac remodeling in adult mouse offspring: possible involvement of local cardiac angiotensin system in developmental origins of cardiovascular disease. Endocrinology **148:** 1218-1225.

KAWAMURA, S., K. YOSHIDA, T. MIURA, Y. MIZUKAMI and M. MATSUZAKI, 1998 Ischemic preconditioning translocates PKC-delta and -epsilon, which mediate functional protection in isolated rat heart. Am J Physiol **275:** H2266-2271.

KIJIMA, K., H. MATSUBARA, S. MURASAWA, K. MARUYAMA, Y. MORI *et al.*, 1995 Gene transcription of angiotensin II type 2 receptor is repressed by growth factors and glucocorticoids in PC12 cells. Biochem Biophys Res Commun **216:** 359-366.

KOIKE, G., E. S. WINER, M. HORIUCHI, D. M. BROWN, C. SZPIRER *et al.*, 1995 Cloning, characterization, and genetic mapping of the rat type 2 angiotensin II receptor gene. Hypertension **26:** 998-1002.

LAL, H., K. I. WILLIAMS and B. WOODWARD, 1999 Chronic hypoxia differentially alters the responses of pulmonary arteries and veins to endothelin-1 and other agents. Eur J Pharmacol **371:** 11-21.

LAMBERS, D. S., and K. E. CLARK, 1996 The maternal and fetal physiologic effects of nicotine. Semin Perinatol **20:** 115-126.

LANGDOWN, M. L., M. J. HOLNESS and M. C. SUGDEN, 2001 Early growth retardation induced by excessive exposure to glucocorticoids in utero selectively increases cardiac GLUT1 protein expression and Akt/protein kinase B activity in adulthood. J Endocrinol **169:** 11-22.

LANGDOWN, M. L., M. J. HOLNESS and M. C. SUGDEN, 2003 Effects of prenatal glucocorticoid exposure on cardiac calreticulin and calsequestrin protein expression during early development and in adulthood. Biochem J **371:** 61-69.

LANGLEY-EVANS, S. C., and A. A. JACKSON, 1995 Captopril normalises systolic blood pressure in rats with hypertension induced by fetal exposure to maternal low protein diets. Comp Biochem Physiol A Physiol **110:** 223-228.

LANGLEY-EVANS, S. C., and D. V. SCULLEY, 2005 Programming of hepatic antioxidant capacity and oxidative injury in the ageing rat. Mech Ageing Dev **126:** 804-812.

LANGLEY-EVANS, S. C., S. J. WELHAM and A. A. JACKSON, 1999 Fetal exposure to a maternal low protein diet impairs nephrogenesis and promotes hypertension in the rat. Life Sci **64:** 965-974.

LAWRENCE, J., D. XIAO, Q. XUE, M. REJALI, S. YANG *et al.*, 2008 Prenatal nicotine exposure increases heart susceptibility to ischemia/reperfusion injury in adult offspring. J Pharmacol Exp Ther **324:** 331-341.

LEESON, C. P., P. H. WHINCUP, D. G. COOK, A. E. DONALD, O. PAPACOSTA *et al.*, 1997 Flow-mediated dilation in 9- to 11-year-old children: the influence of intrauterine and childhood factors. Circulation **96:** 2233-2238.

LEITGES, M., M. MAYR, U. BRAUN, U. MAYR, C. LI *et al.*, 2001 Exacerbated vein graft arteriosclerosis in protein kinase Cdelta-null mice. J Clin Invest **108:** 1505-1512.

LEVY, B. I., 2004 Can angiotensin II type 2 receptors have deleterious effects in cardiovascular disease? Implications for therapeutic blockade of the renin-angiotensin system. Circulation **109:** 8-13.

LI, G., S. BAE and L. ZHANG, 2004 Effect of prenatal hypoxia on heat stress-mediated cardioprotection in adult rat heart. Am J Physiol Heart Circ Physiol **286:** H1712-1719.

LI, G., Y. XIAO, J. L. ESTRELLA, C. A. DUCSAY, R. D. GILBERT *et al.*, 2003 Effect of fetal hypoxia on heart susceptibility to ischemia and reperfusion injury in the adult rat. J Soc Gynecol Investig **10:** 265-274.

LILLYCROP, K. A., E. S. PHILLIPS, A. A. JACKSON, M. A. HANSON and G. C. BURDGE, 2005 Dietary protein restriction of pregnant rats induces and folic acid supplementation prevents epigenetic modification of hepatic gene expression in the offspring. J Nutr **135:** 1382-1386.

LILLYCROP, K. A., E. S. PHILLIPS, C. TORRENS, M. A. HANSON, A. A. JACKSON *et al.*, 2008 Feeding pregnant rats a protein-restricted diet persistently alters the methylation of specific cytosines in the hepatic PPAR alpha promoter of the offspring. Br J Nutr **100:** 278-282.

LILLYCROP, K. A., J. L. SLATER-JEFFERIES, M. A. HANSON, K. M. GODFREY, A. A. JACKSON *et al.*, 2007 Induction of altered epigenetic regulation of the hepatic glucocorticoid receptor in the offspring of rats fed a protein-restricted diet during pregnancy suggests that reduced DNA methyltransferase-1 expression is involved in impaired DNA methylation and changes in histone modifications. Br J Nutr **97:** 1064-1073.

LIM, K., M. A. ZIMANYI and M. J. BLACK, 2006 Effect of maternal protein restriction in rats on cardiac fibrosis and capillarization in adulthood. Pediatr Res **60:** 83-87.

LIU, Y., A. TSUCHIDA, M. V. COHEN and J. M. DOWNEY, 1995 Pretreatment with angiotensin II activates protein kinase C and limits myocardial infarction in isolated rabbit hearts. J Mol Cell Cardiol **27:** 883-892.

LOPEZ BERNAL, A., A. B. ANDERSON, D. M. PARRY and A. C. TURNBULL, 1980 Evidence that fetal membranes are not involved in cortisol metabolism: study of dichorionic twin pregnancies. Am J Obstet Gynecol **138:** 1168-1172.

LOPEZ BERNAL, A., and I. L. CRAFT, 1981 Corticosteroid metabolism in vitro by human placenta, fetal membranes and decidua in early and late gestation. Placenta **2:** 279-285.

LUO, Z. C., W. D. FRASER, P. JULIEN, C. L. DEAL, F. AUDIBERT *et al.*, 2006 Tracing the origins of "fetal origins" of adult diseases: programming by oxidative stress? Med Hypotheses **66:** 38-44.

MACLENNAN, N. K., S. J. JAMES, S. MELNYK, A. PIROOZI, S. JERNIGAN *et al.*, 2004 Uteroplacental insufficiency alters DNA methylation, one-carbon metabolism, and histone acetylation in IUGR rats. Physiol Genomics **18:** 43-50.

MAJUMDER, P. K., N. C. MISHRA, X. SUN, A. BHARTI, S. KHARBANDA *et al.*, 2001 Targeting of protein kinase C delta to mitochondria in the oxidative stress response. Cell Growth Differ **12:** 465-470.

MATSUBARA, H., 1998 Pathophysiological role of angiotensin II type 2 receptor in cardiovascular and renal diseases. Circ Res **83:** 1182-1191.

MCMILLEN, I. C., and J. S. ROBINSON, 2005 Developmental origins of the metabolic syndrome: prediction, plasticity, and programming. Physiol Rev **85:** 571-633.

MCMULLEN, S., and S. C. LANGLEY-EVANS, 2005a Maternal low-protein diet in rat pregnancy programs blood pressure through sex-specific mechanisms. Am J Physiol Regul Integr Comp Physiol **288:** R85-90.

MCMULLEN, S., and S. C. LANGLEY-EVANS, 2005b Sex-specific effects of prenatal low-protein and carbenoxolone exposure on renal angiotensin receptor expression in rats. Hypertension **46:** 1374-1380.

MEYER, K., H. ZHANG and L. ZHANG, 2009 Direct effect of cocaine on epigenetic regulation of PKCepsilon gene repression in the fetal rat heart. J Mol Cell Cardiol **47:** 504-511.

MILLER, J. A., L. A. ANACTA and D. C. CATTRAN, 1999 Impact of gender on the renal response to angiotensin II. Kidney Int **55:** 278-285.

MONE, S. M., M. W. GILLMAN, T. L. MILLER, E. H. HERMAN and S. E. LIPSHULTZ, 2004 Effects of environmental exposures on the cardiovascular system: prenatal period through adolescence. Pediatrics **113:** 1058-1069.

MOORE, L. G., 2003 Fetal growth restriction and maternal oxygen transport during high altitude pregnancy. High Alt Med Biol **4:** 141-156.

MOORE, L. G., M. SHRIVER, L. BEMIS, B. HICKLER, M. WILSON *et al.*, 2004 Maternal adaptation to high-altitude pregnancy: an experiment of nature--a review. Placenta **25 Suppl A:** S60-71.

MOORE, L. G., S. ZAMUDIO, J. ZHUANG, S. SUN and T. DROMA, 2001 Oxygen transport in tibetan women during pregnancy at 3,658 m. Am J Phys Anthropol **114:** 42-53.

MUKOYAMA, M., M. NAKAJIMA, M. HORIUCHI, H. SASAMURA, R. E. PRATT *et al.*, 1993 Expression cloning of type 2 angiotensin II receptor reveals a unique class of seven-transmembrane receptors. J Biol Chem **268:** 24539-24542.

MUROTSUKI, J., J. R. CHALLIS, V. K. HAN, L. J. FRAHER and R. GAGNON, 1997 Chronic fetal placental embolization and hypoxemia cause hypertension and myocardial hypertrophy in fetal sheep. Am J Physiol **272:** R201-207.

MURPHY, J. D., M. J. ARONOVITZ and L. M. REID, 1986 Effects of chronic in utero hypoxia on the pulmonary vasculature of the newborn guinea pig. Pediatr Res **20:** 292-295.

MURRIEL, C. L., and D. MOCHLY-ROSEN, 2003 Opposing roles of delta and epsilonPKC in cardiac ischemia and reperfusion: targeting the apoptotic machinery. Arch Biochem Biophys **420:** 246-254.

NEWNHAM, J. P., S. F. EVANS, M. GODFREY, W. HUANG, M. IKEGAMI *et al.*, 1999 Maternal, but not fetal, administration of corticosteroids restricts fetal growth. J Matern Fetal Med **8:** 81-87.

NIERMEYER, S., 2007 Going to high altitude with a newborn infant. High Alt Med Biol **8:** 117-123.

NORTH, A. J., K. S. LAU, T. S. BRANNON, L. C. WU, L. B. WELLS *et al.*, 1996 Oxygen upregulates nitric oxide synthase gene expression in ovine fetal pulmonary artery endothelial cells. Am J Physiol **270:** L643-649.

NUWAYHID, B., C. R. BRINKMAN, 3RD, C. SU, J. A. BEVAN and I. N. ASSALI, 1975 Systemic and pulmonary hemodynamic responses to adrenergic and cholinergic agonists during fetal development. Biol Neonate **26:** 301-317.

NYIRENDA, M. J., R. S. LINDSAY, C. J. KENYON, A. BURCHELL and J. R. SECKL, 1998 Glucocorticoid exposure in late gestation permanently programs rat hepatic phosphoenolpyruvate carboxykinase and glucocorticoid receptor expression and causes glucose intolerance in adult offspring. J Clin Invest **101:** 2174-2181.

ORTIZ, L. A., A. QUAN, A. WEINBERG and M. BAUM, 2001 Effect of prenatal dexamethasone on rat renal development. Kidney Int **59:** 1663-1669.

ORTIZ, L. A., A. QUAN, F. ZARZAR, A. WEINBERG and M. BAUM, 2003 Prenatal dexamethasone programs hypertension and renal injury in the rat. Hypertension **41:** 328-334.

OSMOND, C., and D. J. BARKER, 2000 Fetal, infant, and childhood growth are predictors of coronary heart disease, diabetes, and hypertension in adult men and women. Environ Health Perspect **108 Suppl 3:** 545-553.

PAPAIT, R., and G. CONDORELLI, 2010 Epigenetics in heart failure. Ann N Y Acad Sci **1188:** 159-164.

PASCUAL, G., and C. K. GLASS, 2006 Nuclear receptors versus inflammation: mechanisms of transrepression. Trends Endocrinol Metab **17:** 321-327.

PEIPPO, J., A. FARAZMAND, M. KURKILAHTI, M. MARKKULA, P. K. BASRUR *et al.*, 2002 Sex-chromosome linked gene expression in in-vitro produced bovine embryos. Mol Hum Reprod **8:** 923-929.

PETRY, C. J., M. W. DORLING, C. L. WANG, D. B. PAWLAK and S. E. OZANNE, 2000 Catecholamine levels and receptor expression in low protein rat offspring. Diabet Med **17:** 848-853.

PEYRONNET, J., Y. DALMAZ, M. EHRSTROM, J. MAMET, J. C. ROUX *et al.*, 2002 Long-lasting adverse effects of prenatal hypoxia on developing autonomic nervous system and cardiovascular parameters in rats. Pflugers Arch **443:** 858-865.

PIERRE, S. V., C. YANG, Z. YUAN, J. SEMINERIO, C. MOUAS *et al.*, 2007 Ouabain triggers preconditioning through activation of the Na+,K+-ATPase signaling cascade in rat hearts. Cardiovasc Res **73:** 488-496.

PING, P., C. SONG, J. ZHANG, Y. GUO, X. CAO *et al.*, 2002 Formation of protein kinase C(epsilon)-Lck signaling modules confers cardioprotection. J Clin Invest **109:** 499-507.

PLOTSKY, P. M., and M. J. MEANEY, 1993 Early, postnatal experience alters hypothalamic corticotropin-releasing factor (CRF) mRNA, median eminence CRF content and stress-induced release in adult rats. Brain Res Mol Brain Res **18:** 195-200.

PORT, J. D., and M. R. BRISTOW, 2001 Altered beta-adrenergic receptor gene regulation and signaling in chronic heart failure. J Mol Cell Cardiol **33:** 887-905.

POSTON, L., 2007 Influences of maternal nutritional status on vascular function in the offspring. Curr Drug Targets **8:** 914-922.

PRETORIUS, M., J. M. LUTHER, L. J. MURPHEY, D. E. VAUGHAN and N. J. BROWN, 2005 Angiotensin-converting enzyme inhibition increases basal vascular tissue plasminogen activator release in women but not in men. Arterioscler Thromb Vasc Biol **25:** 2435-2440.

PRZYKLENK, K., G. LI, B. Z. SIMKHOVICH and R. A. KLONER, 2003 Mechanisms of myocardial ischemic preconditioning are age related: PKC-epsilon does not play a requisite role in old rabbits. J Appl Physiol **95:** 2563-2569.

RAJ, J. U., and P. CHEN, 1987 Role of eicosanoids in hypoxic vasoconstriction in isolated lamb lungs. Am J Physiol **253:** H626-633.

RANKI, H. J., G. R. BUDAS, R. M. CRAWFORD and A. JOVANOVIC, 2001 Gender-specific difference in cardiac ATP-sensitive K(+) channels. J Am Coll Cardiol **38:** 906-915.

RECKELHOFF, J. F., 2001 Gender differences in the regulation of blood pressure. Hypertension **37:** 1199-1208.

REIK, W., M. CONSTANCIA, A. FOWDEN, N. ANDERSON, W. DEAN *et al.*, 2003 Regulation of supply and demand for maternal nutrients in mammals by imprinted genes. J Physiol **547:** 35-44.

REIK, W., W. DEAN and J. WALTER, 2001 Epigenetic reprogramming in mammalian development. Science **293:** 1089-1093.

RINAUDO, P. F., and J. LAMB, 2008 Fetal origins of perinatal morbidity and/or adult disease. Semin Reprod Med **26:** 436-445.

ROBILLARD, J. E., W. V. PAGE, M. S. MATHEWS, B. C. SCHUTTE, A. M. NUYT *et al.*, 1995 Differential gene expression and regulation of renal angiotensin II receptor subtypes (AT1 and AT2) during fetal life in sheep. Pediatr Res **38:** 896-904.

ROUWET, E. V., A. N. TINTU, M. W. SCHELLINGS, M. VAN BILSEN, E. LUTGENS *et al.*, 2002 Hypoxia induces aortic hypertrophic growth, left ventricular dysfunction, and sympathetic hyperinnervation of peripheral arteries in the chick embryo. Circulation **105:** 2791-2796.

RUIZ-ORTEGA, M., J. RODRIGUEZ-VITA, E. SANCHEZ-LOPEZ, G. CARVAJAL and J. EGIDO, 2007 TGF-beta signaling in vascular fibrosis. Cardiovasc Res **74:** 196-206.

RUNO, J. R., and J. E. LOYD, 2003 Primary pulmonary hypertension. Lancet **361:** 1533-1544.

RUSH, J. W., and C. D. AULTMAN, 2008 Vascular biology of angiotensin and the impact of physical activity. Appl Physiol Nutr Metab **33:** 162-172.

SALVI, S. S., 1999 Alpha1-adrenergic hypothesis for pulmonary hypertension. Chest **115:** 1708-1719.

SAMYN, M. E., J. A. PETERSHACK, K. A. BEDELL, M. S. MATHEWS and J. L. SEGAR, 1998 Ontogeny and regulation of cardiac angiotensin types 1 and 2 receptors during fetal life in sheep. Pediatr Res **44:** 323-329.

SAPOLSKY, R. M., L. M. ROMERO and A. U. MUNCK, 2000 How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocr Rev **21:** 55-89.

SAYER, A. A., C. COOPER and D. J. BARKER, 1997 Is lifespan determined in utero? Arch Dis Child Fetal Neonatal Ed **77:** F162-164.

SCHENKER, S., Y. YANG, R. F. JOHNSON, J. W. DOWNING, R. S. SCHENKEN *et al.*, 1993 The transfer of cocaine and its metabolites across the term human placenta. Clin Pharmacol Ther **53:** 329-339.

SCHNEIDER, M. D., and B. H. LORELL, 2001 AT(2), judgment day: which angiotensin receptor is the culprit in cardiac hypertrophy? Circulation **104:** 247-248.

SCHWERTZ, D. W., V. VIZGIRDA, R. J. SOLARO, M. R. PIANO and C. RYJEWSKI, 1999 Sexual dimorphism in rat left atrial function and response to adrenergic stimulation. Mol Cell Biochem **200:** 143-153.

SECHI, L. A., E. F. GRADY, C. A. GRIFFIN, J. E. KALINYAK and M. SCHAMBELAN, 1992a Distribution of angiotensin II receptor subtypes in rat and human kidney. Am J Physiol **262:** F236-240.

SECHI, L. A., C. A. GRIFFIN, E. F. GRADY, J. E. KALINYAK and M. SCHAMBELAN, 1992b Characterization of angiotensin II receptor subtypes in rat heart. Circ Res **71:** 1482-1489.

SECKL, J. R., 2004 Prenatal glucocorticoids and long-term programming. Eur J Endocrinol **151 Suppl 3:** U49-62.

SEMENZA, G. L., 2000 Cellular and molecular dissection of reperfusion injury: ROS within and without. Circ Res **86:** 117-118.

SENBONMATSU, T., S. ICHIHARA, E. PRICE, JR., F. A. GAFFNEY and T. INAGAMI, 2000 Evidence for angiotensin II type 2 receptor-mediated cardiac myocyte enlargement during in vivo pressure overload. J Clin Invest **106:** R1-5.

SHAMS, M., M. D. KILBY, D. A. SOMERSET, A. J. HOWIE, A. GUPTA *et al.*, 1998 11Beta-hydroxysteroid dehydrogenase type 2 in human pregnancy and reduced expression in intrauterine growth restriction. Hum Reprod **13:** 799-804.

SHANMUGAM, S., P. CORVOL and J. M. GASC, 1996 Angiotensin II type 2 receptor mRNA expression in the developing cardiopulmonary system of the rat. Hypertension **28:** 91-97.

SHEEHAN, D. W., L. E. FARHI and J. A. RUSSELL, 1992 Prolonged lobar hypoxia in vivo enhances the responsivity of isolated pulmonary veins to hypoxia. Am Rev Respir Dis **145:** 640-645.

SHERMAN, R. C., and S. C. LANGLEY-EVANS, 1998 Early administration of angiotensin-converting enzyme inhibitor captopril, prevents the development of hypertension programmed by intrauterine exposure to a maternal low-protein diet in the rat. Clin Sci (Lond) **94:** 373-381.

SINGH, R. R., L. A. CULLEN-MCEWEN, M. M. KETT, W. M. BOON, J. DOWLING *et al.*, 2007 Prenatal corticosterone exposure results in altered AT1/AT2, nephron deficit and hypertension in the rat offspring. J Physiol **579:** 503-513.

SKYSCHALLY, A., M. HAUDE, H. DORGE, M. THIELMANN, A. DUSCHIN *et al.*, 2004 Glucocorticoid treatment prevents progressive myocardial dysfunction resulting from experimental coronary microembolization. Circulation **109:** 2337-2342.

SLOTKIN, T. A., 1998 Fetal nicotine or cocaine exposure: which one is worse? J Pharmacol Exp Ther **285:** 931-945.

SLOTKIN, T. A., J. L. SALEH, E. C. MCCOOK and F. J. SEIDLER, 1997 Impaired cardiac function during postnatal hypoxia in rats exposed to nicotine prenatally: implications for perinatal morbidity and mortality, and for sudden infant death syndrome. Teratology **55:** 177-184.

SNYDER, D. L., P. GAYHEART, M. D. JOHNSON, H. GURDAL and J. ROBERTS, 1995 Prenatal cocaine exposure alters norepinephrine release from cardiac adrenergic nerve terminals. Life Sci **56:** 1475-1484.

SOOD, R., J. L. ZEHNDER, M. L. DRUZIN and P. O. BROWN, 2006 Gene expression patterns in human placenta. Proc Natl Acad Sci U S A **103:** 5478-5483.

STEINHORN, R. H., F. C. MORIN, 3RD, S. F. GUGINO, E. C. GIESE and J. A. RUSSELL, 1993 Developmental differences in endothelium-dependent responses in isolated ovine pulmonary arteries and veins. Am J Physiol **264:** H2162-2167.

STOLL, M., and T. UNGER, 2001 Angiotensin and its AT2 receptor: new insights into an old system. Regul Pept **99:** 175-182.

SWYNGHEDAUW, B., C. DELCAYRE, J. L. SAMUEL, A. MEBAZAA and A. COHEN-SOLAL, 2010 Molecular mechanisms in evolutionary cardiology failure. Ann N Y Acad Sci **1188:** 58-67.

TAKAHASHI, H., S. SOMA, M. MURAMATSU, M. OKA and Y. FUKUCHI, 2001 Upregulation of ET-1 and its receptors and remodeling in small pulmonary veins under hypoxic conditions. Am J Physiol Lung Cell Mol Physiol **280:** L1104-1114.

TAPPIA, P. S., M. S. NIJJAR, A. MAHAY, N. AROUTIOUNOVA and N. S. DHALLA, 2005 Phospholipid profile of developing heart of rats exposed to low-protein diet in pregnancy. Am J Physiol Regul Integr Comp Physiol **289:** R1400-1406.

TAPPIA, P. S., H. SANDHU, T. ABBI and N. AROUTIOUNOVA, 2009 Alterations in the expression of myocardial calcium cycling genes in rats fed a low protein diet in utero. Mol Cell Biochem **324:** 93-99.

THOMASSIN, H., M. FLAVIN, M. L. ESPINAS and T. GRANGE, 2001 Glucocorticoid-induced DNA demethylation and gene memory during development. EMBO J **20:** 1974-1983.

THOMPSON, L. P., and C. P. WEINER, 1993 Endothelium-derived relaxing factor inhibits norepinephrine contraction of fetal guinea pig arteries. Am J Physiol **264:** H1139-1145.

TIKTINSKY, M. H., J. J. CUMMINGS and F. C. MORIN, 3RD, 1992 Acetylcholine increases pulmonary blood flow in intact fetuses via endothelium-dependent vasodilation. Am J Physiol **262:** H406-410.

TUFRO-MCREDDIE, A., and R. A. GOMEZ, 1993 Ontogeny of the renin-angiotensin system. Semin Nephrol **13:** 519-530.

TZAO, C., P. A. NICKERSON, J. A. RUSSELL, S. F. GUGINO and R. H. STEINHORN, 2001 Pulmonary hypertension alters soluble guanylate cyclase activity and expression in pulmonary arteries isolated from fetal lambs. Pediatr Pulmonol **31:** 97-105.

VALEN, G., T. KAWAKAMI, P. TAHEPOLD, A. DUMITRESCU, C. LOWBEER *et al.*, 2000 Glucocorticoid pretreatment protects cardiac function and induces cardiac heat shock protein 72. Am J Physiol Heart Circ Physiol **279:** H836-843.

VARGA, E., N. NAGY, J. LAZAR, G. CZIFRA, I. BAK *et al.*, 2004 Inhibition of ischemia/reperfusion-induced damage by dexamethasone in isolated working rat hearts: the role of cytochrome c release. Life Sci **75:** 2411-2423.

VERMEERSCH, P., E. BUYS, P. POKREISZ, G. MARSBOOM, F. ICHINOSE *et al.*, 2007 Soluble guanylate cyclase-alpha1 deficiency selectively inhibits the pulmonary vasodilator response to nitric oxide and increases the pulmonary vascular remodeling response to chronic hypoxia. Circulation **116:** 936-943.

WAKSCHLAG, L. S., K. E. PICKETT, E. COOK, JR., N. L. BENOWITZ and B. L. LEVENTHAL, 2002 Maternal smoking during pregnancy and severe antisocial behavior in offspring: a review. Am J Public Health **92:** 966-974.

WATERLAND, R. A., D. C. DOLINOY, J. R. LIN, C. A. SMITH, X. SHI *et al.*, 2006 Maternal methyl supplements increase offspring DNA methylation at Axin Fused. Genesis **44:** 401-406.

WHARTON, J., K. MORGAN, R. A. RUTHERFORD, J. D. CATRAVAS, A. CHESTER *et al.*, 1998 Differential distribution of angiotensin AT2 receptors in the normal and failing human heart. J Pharmacol Exp Ther **284:** 323-336.

WIINBERG, N., A. HOEGHOLM, H. R. CHRISTENSEN, L. E. BANG, K. L. MIKKELSEN *et al.*, 1995 24-h ambulatory blood pressure in 352 normal Danish subjects, related to age and gender. Am J Hypertens **8:** 978-986.

WILLIAMS, J. M., C. R. WHITE, M. M. CHANG, E. R. INJETI, L. ZHANG *et al.*, 2006 Chronic hypoxic decreases in soluble guanylate cyclase protein and enzyme activity are age dependent in fetal and adult ovine carotid arteries. J Appl Physiol **100:** 1857-1866.

WINTOUR, E. M., K. M. MORITZ, K. JOHNSON, S. RICARDO, C. S. SAMUEL *et al.*, 2003 Reduced nephron number in adult sheep, hypertensive as a result of prenatal glucocorticoid treatment. J Physiol **549:** 929-935.

WOODALL, S. M., B. M. JOHNSTON, B. H. BREIER and P. D. GLUCKMAN, 1996 Chronic maternal undernutrition in the rat leads to delayed postnatal growth and elevated blood pressure of offspring. Pediatr Res **40:** 438-443.

WU, G., F. W. BAZER, T. A. CUDD, C. J. MEININGER and T. E. SPENCER, 2004 Maternal nutrition and fetal development. J Nutr **134:** 2169-2172.

XIAO, D., I. M. BIRD, R. R. MAGNESS, L. D. LONGO and L. ZHANG, 2001 Upregulation of eNOS in pregnant ovine uterine arteries by chronic hypoxia. Am J Physiol Heart Circ Physiol **280:** H812-820.

XIAO, D., C. A. DUCSAY and L. ZHANG, 2000 Chronic hypoxia and developmental regulation of cytochrome c expression in rats. J Soc Gynecol Investig **7:** 279-283.

XIAO, D., X. HUANG, J. LAWRENCE, S. YANG and L. ZHANG, 2007 Fetal and neonatal nicotine exposure differentially regulates vascular contractility in adult male and female offspring. J Pharmacol Exp Ther **320:** 654-661.

XU, F., C. MAO, C. RUI, Z. XU and L. ZHANG, 2009 Cardiovascular Effects of Losartan and Its Relevant Clinical Application. Curr Med Chem.

XU, Y., A. S. CLANACHAN and B. I. JUGDUTT, 2000 Enhanced expression of angiotensin II type 2 receptor, inositol 1,4, 5-trisphosphate receptor, and protein kinase cepsilon during cardioprotection induced by angiotensin II type 2 receptor blockade. Hypertension **36:** 506-510.

XU, Y., S. J. WILLIAMS, D. O'BRIEN and S. T. DAVIDGE, 2006 Hypoxia or nutrient restriction during pregnancy in rats leads to progressive cardiac remodeling and impairs postischemic recovery in adult male offspring. FASEB J **20:** 1251-1253.

XUE, Q., and L. ZHANG, 2009 Prenatal hypoxia causes a sex-dependent increase in heart susceptibility to ischemia and reperfusion injury in adult male offspring: role of protein kinase C epsilon. J Pharmacol Exp Ther **330:** 624-632.

YAJNIK, C. S., and U. S. DESHMUKH, 2008 Maternal nutrition, intrauterine programming and consequential risks in the offspring. Rev Endocr Metab Disord **9:** 203-211.

YAMADA, T., M. HORIUCHI and V. J. DZAU, 1996 Angiotensin II type 2 receptor mediates programmed cell death. Proc Natl Acad Sci U S A **93:** 156-160.

YAMAMOTO, K. R., 1985 Steroid receptor regulated transcription of specific genes and gene networks. Annu Rev Genet **19:** 209-252.

ZHAI, P., T. E. EURELL, R. COTTHAUS, E. H. JEFFERY, J. M. BAHR *et al.*, 2000 Effect of estrogen on global myocardial ischemia-reperfusion injury in female rats. Am J Physiol Heart Circ Physiol **279:** H2766-2775.

ZHANG, H., A. DARWANTO, T. A. LINKHART, L. C. SOWERS and L. ZHANG, 2007 Maternal cocaine administration causes an epigenetic modification of protein kinase Cepsilon gene expression in fetal rat heart. Mol Pharmacol **71:** 1319-1328.

ZHANG, H., K. D. MEYER and L. ZHANG, 2009 Fetal exposure to cocaine causes programming of Prkce gene repression in the left ventricle of adult rat offspring. Biol Reprod **80:** 440-448.

ZHANG, L., 2005 Prenatal hypoxia and cardiac programming. J Soc Gynecol Investig **12:** 2-13.

ZHAO, J., O. RENNER, L. WIGHTMAN, P. H. SUGDEN, L. STEWART *et al.*, 1998 The expression of constitutively active isotypes of protein kinase C to investigate preconditioning. J Biol Chem **273:** 23072-23079.

ZHAO, Y., C. S. PACKER and R. A. RHOADES, 1993 Pulmonary vein contracts in response to hypoxia. Am J Physiol **265:** L87-92.

ZHOU, H. Z., J. S. KARLINER and M. O. GRAY, 2002 Moderate alcohol consumption induces sustained cardiac protection by activating PKC-epsilon and Akt. Am J Physiol Heart Circ Physiol **283:** H165-174.