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LOMA LINDA UNIVERSITY
School of Medicine
in conjunction with the
Faculty of Graduate Studies

Chronic Hypoxia Induces Epigenetic Modifications in the Fetal Rat Heart

by

Andrew James Grant Patterson

A Dissertation submitted in partial satisfaction of
the requirements for the degree
Doctor of Philosophy in Physiology

December 2011

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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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ABBREVIATIONS

PKCε	Protein Kinase C ε
PKCδ	Protein Kinase C δ
HIF-1α	Hypoxia Inducible Factor-1 alpha
Sp1	Specificity Protein 1
VEGF	Vascular Endothelial Growth Factor
EMSA	Electrophoretic Mobility Shift Assay
ChIP	chromatin immunoprecipitation
CpG	cytosine-phosphate-guanine
ERα, β	estrogen receptor alpha, beta
MTF1	metal regulatory transcription factor 1
Egr1	early growth response protein 1
ROS	Reactive Oxygen Species
NAC	N-acetylcytesine
Tempol	4-hydoxy-tempo

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ABSTRACT OF THE DISSERTATION

Chronic Hypoxia Induces Epigenetic Modifications In The
Fetal Rat Heart

by
Andrew James Grant Patterson

Doctor of Philosophy, Graduate Program in Physiology
Loma Linda University, December 2011
Dr. Lubo Zhang, Chairperson

Heart disease remains the leading cause of death worldwide. As a result of studies done by Barker and associates, our awareness of the significance of stress during gestation as a risk factor for heart diseases has expanded. We now know that events *in utero* can significantly alter gene expression patterns in heart tissue leading to increase susceptibility to ischemia reperfusion injury in adulthood. The focus of this project was to elucidate the role of chronic hypoxia in the programming of the cardioprotective gene, Protein Kinase C epsilon (PKCε) in fetal rat heart. We used an animal, organ base, and cell culturing with the rat embryonic cell line H9c2 to determine the molecular events underpinning the heightened sensitivity to ischemia reperfusion injury of adult offspring exposed to chronic hypoxia *in utero.* We determined that chronic hypoxia directly represses PKCε expression through increase methylation of CpG dinucleotides for two SP1 binding sites located at proximal region of the PKCε promoter. Previous studies using reporter gene assays concluded the region encompassing both SP1 binding sites played a significant role in the activity of PKCε promoter. Chromatin immunoprecipitation (ChIP) assays further verified the functional significance of methylation for both Sp1 sites in reducing SP1 protein binding. In the presence of the DNA methylation inhibitor, 5-aza-2-deoxycytidine, binding of SP1 to PKCε promoter,

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promoter methylation, and PKCε protein and mRNA were restored to control levels. Connecting epigenetics to chronic hypoxia *in utero* led us to further investigate the underlining mechanism of hypoxia-induced methylation of PKCε promoter. The dominant pathway of cellular adaptation to hypoxic stress involves the stabilization of the Hypoxia-Inducible-Factor 1 alpha (HIF-1α). We found blockade of nuclear accumulation of HIF-1α did not restore PKCε mRNA to control values. Next, we found the ROS Scavengers N-acetylcysteine and 4-hydroxy Tempo protect against hypoxia-induced repression of PKCε gene activity, which linked oxidative stress to PKCε repression in fetal hearts. This project has demonstrated that chronic hypoxia directly regulates PKCε gene expression through ROS mediated epigenetic repression of PKCε promoter, which leads to long term programming of the fetal heart.

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CHAPTER 1

BACKGROUND

Introduction

According to the World Health Organization (WHO), cardiovascular disease, namely, ischemic heart disease and cerebral vascular disease are the leading cause of death worldwide (WHO, 2004). For many years, environmental, heredity and lifestyle factors were seen as major contributors to cardiovascular diseases. While changes in adult dietary practices and lifestyles in recent years have contributed to the increase in incidence of coronary heart disease, it does not predict who will or will not develop cardiovascular disease. Recent epidemiological studies have correlated an adverse intrauterine environment with an increase predisposition for developing cardiovascular-related illnesses in adulthood. Most notably, published studies by Barker and colleagues showed that regions in England with higher infant mortality rates and low birthweight had the highest incidence of coronary heart disease decades later (Barker et al., 1986 & 1989). Similarly, The Dutch Family Cohort study found that offspring of pregnant women exposed to the Dutch famine of 1944 had increased rates of cardiovascular disease (Roseboom, 2001). These studies led to what is known as the Fetal Origins hypothesis, which proposes that adaptations made by the fetus in response to undernutrition causes permanent changes in tissue structure and function. This programming *in utero* predisposes the fetus to cardiovascular disease later in life (Lucas, 1991; Barker DJ 1990, 1994 & 1997). *In utero* stress has been expanded to include

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stressors such as prenatal exposure of hypoxia, nicotine, drug use, elevated steroid hormones, and high altitude (Zhang, 2005; Meyer, 2007; Seckl, 2008; Mao, 2010). The focus of this study was to examine the initial molecular events that occur in response to chronic hypoxia in the developing heart that predispose offspring to increased susceptibility to ischemic reperfusion injury later in life.

Hypoxia

Oxygen is an essential substrate for cell survival. It acts as a final electron acceptor in the electron transport chain (ETC). In humans, oxygen tension varies from 100 mm Hg in alveolar arterioles to between 40 and 20 mm Hg in systemic tissues (Webster and Abela 2007). When oxygen is scarce, the ETC is compromised. Since the ETC is coupled to oxidative phosphorylation, ATP (Adensosine Triphosphate) levels drop significantly creating energy disparities within the cell. Hypoxia ensues when the oxygen tension is lower than physiological levels and the demand for oxygen exceeds the supply available. Hypoxia in non-reproductive tissues, as identified by direct measurement of pO_2 values and/or significant induction of hypoxia-inducible genes, suggest oxygen tension between ~20 and ~7 mm Hg as physiological hypoxia [Gross et al. 1995; Raleigh et al. 1998; Fueck 2009). Researchers commonly use between 1% (~7 mm Hg) and 3% (~21 mm Hg) oxygen in cell culturing to mimic hypoxia (Ivanovic, 2009). For *in vivo* studies, animals are typically subjected to between 8% and 12% oxygen in order to significantly reduce arterial pO_2 to comparable values (Lawrence et al. 2008; Reynolds et al. 1996; Mitchell and Van Kainen, 1992). By comparison, fetal arterial blood pO_2 ranges from ~30 to ~20 mm Hg (Fisher, 2007), suggesting normal fetal oxygen is close to

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physiological hypoxia in adult tissues. This implies that fetal development exists in a state of relative hypoxia as compared to adult oxygen tension, and also that fetal tissues have a lower threshold to reach a state of oxygen insufficiency. Interestingly, short bouts of hypoxia naturally occur during gestation when the uterine artery contracts or becomes compressed, which reduces the amount of oxygen delivered to the placenta. Additionally, since the placenta serves as the major interface between mother and fetus, its development and oxygen consumption also influences fetal oxygen supply. Changes in maternal pO_2 and/or abnormal placental development or metabolism may reduce fetal arterial pO_2 and result in fetal hypoxia (Jensen et al. 1999; Myatt, 2006). Under hypoxic conditions, the fetus compensates by altering fetal blood flow away from peripheral tissues to vital organs; shifting from aerobic to greater utilization of anaerobic energy pathways; and the induction of hypoxia-dependent genes necessary for survival in a low oxygen environment.

Cardiogenesis involves the formation of the embryonic heart. Once the heart is formed, it undergoes a stage of rapid growth and maturation during fetal development. The formation and subsequent maturation of the heart are tightly regulated processes in which oxygen tension plays a vital role. Interestingly, the fetal heart is more resistant to hypoxia induced cell death than the adult heart due to its enhanced ability to increase glycolytic flux (Ascuitto and Ross-Ascuitto, 1996). There is increasing evidence suggesting that low oxygen tension in the fetus is essential for normal heart formation and maturation. The expressions of hypoxia-induced genes, such as hypoxia inducible factor 1 (HIF-1) and vascular endothelial growth factor (VEGF), correlate with angiogenesis, vasculogenesis and fetal heart remodeling [Compernolle et al. 2003;

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Sugishita et al. 2004a). Although physiologically “normal” hypoxia (lower oxygen tension in the fetus as compared with the adult) may be beneficial in normal heart development, pathophysiological hypoxia (lower than normal fetal oxygen tension) is associated with significant adverse effects that can lead to changes in structure, function, and gene expression in fetal hearts, which may persist throughout adulthood (See Figure 1). The role of hypoxia in fetal heart will be discussed in more detail later.

Beyond fetal development, hypoxia has pathophysiological consequences in adults. For example, when arteriole blood flow is occluded from plaque accumulation, emboli, or vasospasms, the tissue supplied by that vessel become ischemic. Lower perfusion significantly reduces the amount of oxygen, glucose and other essential materials found in blood for that tissue (Reneman, 1998). Thus, extended periods of oxygen deprivation combined with inadequate nutrients may result in tissue death. Tumorigenesis is a further example of a pathophysiological situation where hypoxia plays a vital role. Tumor formation is characterized by clonal expansion of abnormal cells. Initially, the unabated proliferation outgrows nutrient supply provided by blood vessels, thus producing an environment lacking adequate oxygen. Then a subset of the colony adapt to this environment by up regulating anaerobic pathways in order to sustain cellular functions and growth in a hypoxia environment. Eventually the tumor mass recruits vascular supply through angiogenic mechanisms for long-term nourishment and growth (Papetti, 2002). Oxygen availability is vital for both physiological and pathophysiological processes.

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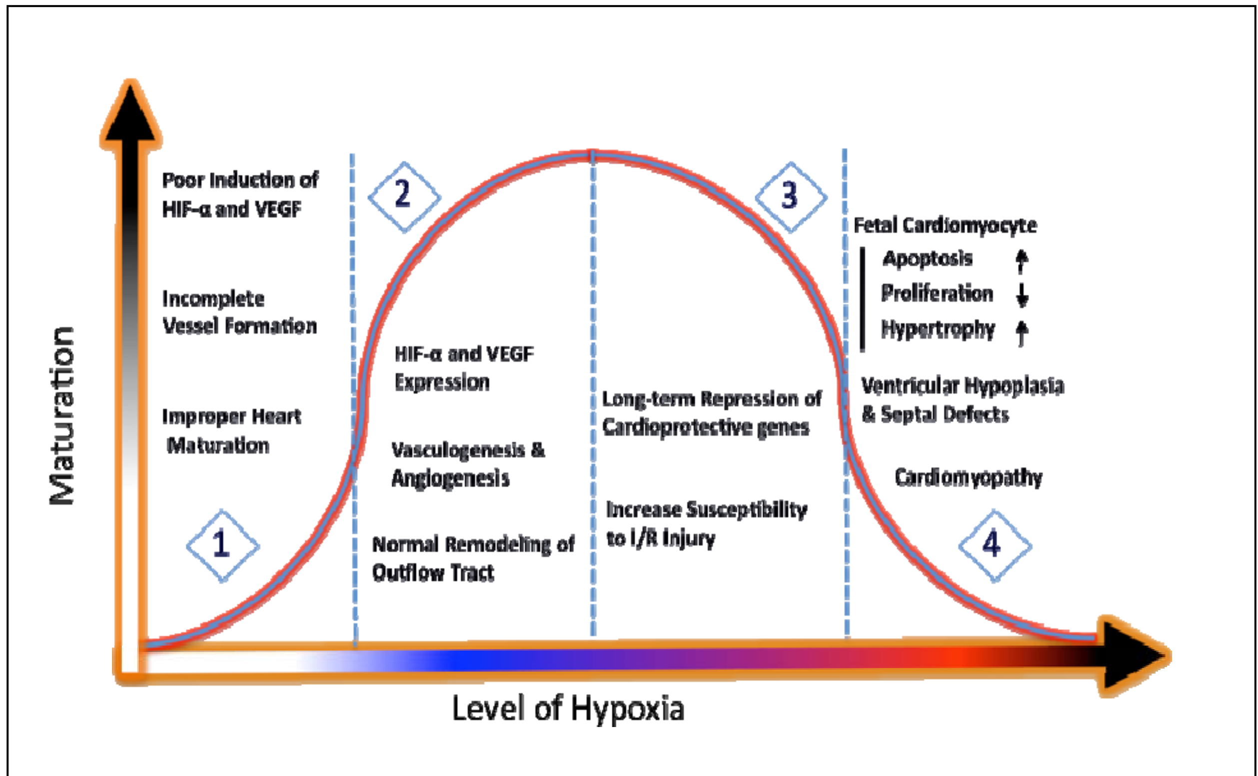

Figure 1. The effect of hypoxia on fetal heart development. **1**. Insufficient exposure to "normal" hypoxia reduces the expression of key genes (*i.e*. HIF-α, VEGF) needed for heart and vessels formation. **2**. Adequate exposure to "normal" hypoxia ensures expression of hypoxia dependent genes needed for vasculogenesis, angiogenesis and fetal heart remodeling. **3**. Chronic exposure to moderate "abnormal" hypoxia can lead to programming of cardioprotective genes, which may decrease the ability of heart to adapt to stresses later in life. **4**. Exposure to more severe "abnormal" hypoxia can significantly affect fetal cardiomyocytes development, which can lead to cardiomyopathy.

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Formation of the Myocardium

Heart Chambers and Vessels

In order to understand the role of hypoxia in normal fetal heart development it is necessary to highlight the key events in normal cardiogenesis. Our understanding of the events that lead to the formation of a functional heart in vertebrates comes primarily from studies done in chicken and mouse models. Although there exists differences between species, the major events are congruent. Arguably, the first significant event in myocardial formation involves the formation of the primitive heart tube (Sedmera et al., 2000). Cardiac progenitor cells from the primary (later become left ventricle) and secondary (later become right ventricle, outflow and inflow tracts) heart fields differentiate into myocardial cells and fuse along the midline to form the heart tube (Fishman and Chien, 1997). It is during this event that peristalic contractions appear along the heart tube. Once initiated, the heterogeneity of the heart chambers to be formed becomes apparent with regions destined to become atrials and ventricles contracting at different rates. Structurally, the primitive heart tube is composed of a myocardial covering that is one or two cells thick, an acellular cardiac jelly, and endocardium (Fishman and Chien, 1997). After the heart tube is formed it undergoes looping and sets the framework in which the chambers can develop. After looping, changes (known as trabeculations) in what will become ventricles emerge within the lumen. Trabeculations are fenestrated sheet-like protrusions into the lumen that initially serve to increase the surface to volume ratio, allowing growth of the myocardium prior to the establishment of the coronary circulation and the separation of blood flow before septation (Sedmera et al., 2000). The heart then undergoes further remodeling with growth primarily from increased cellular proliferation and compaction of trabeculae. At the same time, the

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coronary vasculature is established to meet the increasing demands brought about by the growing myocardium. Angioblasts form the primitive vascular plexus. The vessels undergo extensive remodeling and patterning and mature into the coronary artery tree. Evidence suggests that hypoxia may be the initial signal mediating angioblast invasion of the embryonic heart and subsequent formation of the coronary vasculature (Wikenheiser et al., 2006; Nanka et al., 2006). The establishment of the coronary vessel network ensures adequate nutrients are delivered to the myocardium allowing sustained growth and maturation.

The functional unit of the myocardium is the cardiomyocyte. As previously mentioned, cardiomyocytes originate from cardiac progenitor cells in the primary and secondary heart fields. They differ from adult cardiomyocytes in size, organization of myofibrils, and proliferation capacity. Fetal cardiomyocytes are smaller in diameter and are normally mononucleated, whereas adult heart cells tend to be larger and often display polyploidy (two or more nuclei per cell). Fetal cardiomyocytes have fewer and less organized myofibrils with poorly developed sarcoplasmic reticulum and the T Tubules systems as compared to the numerous and highly organized myofibrils found in adult cardiomyocytes (Hoerter and Vassort, 1982). At early stages of development, the fetal heart grows largely through rapid proliferation of cardiomyocytes (Jonker et al., 2007a). However, towards the end of gestation, cardiac cells become increasingly differentiated and therefore lose the ability to propagate. Immature heart cells lose the ability to proliferate during perinatal periods, with subsequent growth due primarily to enlargement of existing cardiac cells. In comparison, adult mammalian heart cells maintain a permanently differentiated disposition with little or no ability to self-reproduce (Rudolph,

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2000). The change in the proliferation capacity in cardiomyocytes corresponds to the maturation of contractile machinery and the increase in population of poly-nucleated cells. It is likely the highly ordered structure of mature cardiomyocytes, coupled with the continuous contraction necessary for cardiac output and poly-nucleated disposition, preclude self-propagation. In nature, there exists species capable of regenerating heart cells at an adult stage. The adult newt and zebrafish are both capable of regenerating substantial portions of heart tissue (Oberpriller, 1974; Poss et al., 2002). In particular, the adult newt seems to possess the ability to dedifferentiate cardiac cells and proliferate to replace lost or damaged tissue (Bettencourt-Dias et al., 2003). Among other important factors, activation of p38 MAP kinase appears to play an important role in inhibiting cardiomyocyte growth. The expression of p38 MAPK expression is inversely correlated with cardiomyocyte proliferation, while inhibition of p38 isoforms promotes increased expression of genes vital for self-propagation (Engel et al., 2005). These findings imply that mammalian adult heart cells have the capacity to reproduce. Nonetheless, the factors that give the immature heart cell, adult newt and zebrafish the ability to reproduce may shed light that may promote the development of therapies that can assist in the recovery of heart tissue following a coronary event.

Hemodynamic Stress

Functionally, the embryonic heart at an early stage sets itself apart from most organs in that it is called upon to work almost from its inception. As the vascular system forms, there is an increased stress placed on the fetal heart. However, the plastic nature of the developing myocardium gives it the ability to adjust to hemodynamic load brought upon it by an enlarging peripheral vascular system. An increase in afterload, or elevation

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in pressure required to eject blood to systemic tissues, requires compensatory growth of the myocardial wall. Laplace's law explains the relationship between wall thickness and increases in afterload. It states that increases in intraluminal pressure will produce increased wall stress for a given radius. It is compensated by increased wall thickness or decrease internal radius. This phenomenon is seen in individuals with pulmonary hypertension that increases the intraventricular end diastolic pressure resulting in thickening of the right heart wall. In fetal sheep, Barbera et al. (2000) demonstrated that right ventricular wall thickness increases in response to pulmonary artery occlusion, which is an increase in systolic pressure load. The findings from this study suggested that the increase in heart size was primarily due to hyperplasia of cardiomyocytes, although some growth could be attributed to hypertrophy. Indeed, arterial hypertension in fetal sheep increases the weight of hearts, stimulates proliferation, size and binucleation of cardiomyocytes resulting in increased growth and accelerated maturation of the myocardium as a compensatory response to increased afterload (Jonker et al., 2007b). Therefore, ventricular wall mass is influenced by the hemodynamic stress under which it develops.

Hypoxia and the Developing Heart

Methods for Determining Hypoxia In Utero

Hypoxia *in vivo* is a challenging phenomenon to study; yet, several techniques are used to either measure oxygen tension directly or indirectly *in vivo*. The surgical implantation of O_2 sensitive sensors is a method commonly used on larger animals, such as pregnant sheep, to measure fetal pO_2 (Kamitomo et al. 1994). Additionally, the unique metabolic plasticity of the fetal heart allows it to rapidly increase glycolysis under

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conditions of oxygen deprivation (Ascuitto and Ross-Ascuitto, 1996). The switch from aerobic to anaerobic respiration corresponds to increases in glycolytic intermediates, such as lactate, that have been used as an indicator of reduced mitochondrial oxidative activity in response to oxygen insufficiency (Breuer et al. 1968). Beyond surgical implantation and metabolism, Chapman (1979) was the first to suggest the use of nitroimadazole compounds (e.g. EF5, pimonidazole) to study hypoxia. In low oxygen conditions, nitroimadazole compounds are chemically reduced by nitroreductases, which permit covalent binding to intracellular proteins to form adducts (Evans et al. 2007, 2008). Nitroimadazole compounds have been used to study the effects of hypoxia in embryogenesis and tumorgenesis (Wikenheiser et al. 2006; Koch and Evans, 2003).

Tissue hypoxia alters gene expression patterns. Hypoxia stabilizes HIF (1, 2 and 3), a family of transcription factors that play a central role in cellular adaptation to insufficient oxygen. The discovery of HIF sheds new light on the role of hypoxia in fetal heart maturation. HIF is comprised of a α and β subunit, of which the former is oxygen-sensitive and affects HIF stability, and the latter is oxygen-insensitive. HIF is widely known to up-regulate numerous genes associated with external and internal cellular adaptation to hypoxia (Iyer et al. 1998; Semenza, 1998; Ward, 2008). A classic example of HIF associated gene transactivation is the induction of erythropoietin (EPO). Hypoxia promotes HIF-induced up-regulation of EPO (Jiang et al. 1996). EPO stimulates erythropoiesis, inhibits apoptosis, and mobilizes endothelial progenitors for vessel growth by binding to EPO receptors (Arcasoy, 2008; Marzo et al. 2008). HIF and HIF dependent gene expression have become important indicators of tissue hypoxia. Their use has increased our understanding of the role of reduced oxygen in cardiogenesis from a

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standpoint of transcriptional changes. HIF regulation will be discussed in detail later in the introduction.

Outflow Tract Remodeling

Recent studies in avian and mouse models suggest that hypoxia plays a critical role in the formation and development of the heart. Many of these studies were centered on the remodeling of the embryonic outflow track (OFT) and coronary vessel formation. Embryonic OFT remodeling is necessary for the proper transition from single to dual circulation in mammalian and avian hearts (Fisher, 2007). Apoptosis of OFT cardiomyocytes is vital for proper remodeling of OFT (Watanabe et al. 2001; Barbosky et al. 2006). Programmed cell death of OFT cardiomyocytes brings about the shortening and rotation needed for the aorta to join the left ventricle and pulmonary vessels to connect to the right ventricle. At the height of cardiomyocyte apoptosis in the OFT, researchers have observed increases in EF5 (chemical marker of hypoxia) staining as well as increased nuclear accumulation of HIF-1; suggesting hypoxia *via* HIF-1 may be involved in OFT remodeling (Sugishita et al. 2004b; Barbosky et al. 2006). Druyan el al. (2007) demonstrated in chick fetal hearts the up-regulation of hypoxia-regulated genes, heme oxygenase, cardiac troponin T and hypoxia up-regulated protein 1 at specific developmental periods (E7 and E19). Heme oxygenase facilitates the degradation of heme and protects against oxidative stress. Cardiac troponin T is involved in calcium handling and contractions of the heart, and hypoxia up-regulated protein 1 protects against cell death during hypoxia. These finding suggests that as the heart develops under hypoxic conditions, cellular defense pathways are employed in order to sustain normal growth, while protecting against oxidative stress and apoptosis. Ironically,

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cardiomyocytes exhibit increased oxidative stress as a result of hypoxia (Duranteau et al. 1998). Oxidative stress can cause cellular damage, which may lead to cell death. In subsequent experiments, EF5 staining revealed differential levels of oxygen in fetal hearts with areas of low oxygen correlating spatially and temporally with apoptosis-induced myocardial remodeling (Sugishita et al. 2004a & 2004b; Lee et al. 2001). These findings were further substantiated by experiments that demonstrated intense EF5 staining and cell death of OFT cardiomyocytes that were attenuated by hyperoxia resulting in OFT defects. Such defects included the abnormal formation of the right ventricles, which clearly associated oxygen regulation with proper myocardial formation (See Figure 1) (Barbosky et al. 2006). It appears the timing of the hypoxic insult is tightly regulated for proper heart remodeling. Low oxygen was reported in chick at stages 25-32 and around day 13.5 of gestation in mice (Sugishita et al. 2004; Barbosky et al. 2006). Interestingly, the timing and duration of hypoxia plays an important role in the development of other tissues. In the first trimesters, placenta tissues develop under low pO_2 (<20mm Hg), which rises considerably in second trimester, (~60mm Hg) then declines (~40mm Hg) by the third trimester (Rodesch et al. 1992; Jaunaiux et al. 2001; Soothill et al. 1986). Accordingly, studies suggest the fluctuation in pO_2 modulates trophoblast differentiation and invasion, affording concomitant growth of the placenta throughout gestation (Pringle et al. 2010). These findings indicate that hypoxia functions in several tissues to signal changes that promote normal development.

Coronary Vessel Formation

In embryonic tissues, the formation of primary blood vessels from endothelial precursors called angioblasts is known as vasculogenesis. During vasculogenesis,

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angioblasts combine and form the primary capillary plexus, which serves as the framework from which all subsequent vessel formation occurs. After the establishment of the primary capillary plexus, subsequent budding and sprouting of new vessels from preexisting ones is known as angiogenesis. Though proper vessel development and maturation involves input from multiple signals, VEGF signaling plays a vital role throughout vessel formation and growth. The binding of VEGF to receptor tyrosine kinases (e.g. KDR/Fik-1, Fit-1 in endothelial cells) initiates the expression of genes necessary for the recruitment, proliferation, and differentiation needed for vasculogenesis and angiogenesis (Covassin et al. 2006; Nasevicius et al. 2000; Martyn and Schulte-Merker, 2004; Weinstein and Lawson, 2002). For a more thorough review of VEGF signaling, consider the following excellent reviews (Papetti and Herman, 2002; Josko et al. 2006; Roskoski, 2008; Yia-Herttuala et al. 2007). In cardiogenesis, VEGF is essential for vessel growth and the induction of VEGF correlated spatially with coronary vessel patterning (Tomanek et al. 1998, 1999 & 2003). In addition, cardiomyocyte-restricted knockout of Vegfα impaired coronary vessel development, promoted myocardial thinning, depressed basal contractile function, and caused significant dysfunction of beta-adrenergic stimulation (Giordano et al. 2001). Also, mice deficient of receptor tyrosine kinase showed abnormal heart development, primarily ventricular septal defects (Takeuchi et al. 2000). These findings suggest that VEGF-mediated vessel formation promotes adequate growth, remodeling and function of the myocardium.

Hypoxic stress increases vessel formation in fetal heart tissue, while hyperoxia delays vessel growth (Yue and Tomanek, 1999). Studies have confirmed that hypoxia is a major stimulus for vessel growth in fetal development and tumorgenesis (Yue and

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Tomanek, 1999; Lainakis and Bamias, 2008; Hu et al. 2003). Hypoxia is known to stabilize HIF-1& 2, which preferentially up-regulates VEGF and its receptor (Hu et al. 2003; Forsythe et al. 1996). However, studies conducted by Kotch et al. (1999) showed that VEGF mRNA increased modestly (compared to controls in HIF-1α null mice), suggesting additional factors may regulate VEGF induction in fetal heart tissue. For example, induction of VEGF in the absences of HIF-1α may be explained by the presence of HIF-2α (which is known to up-regulate VEGF and VEGF receptors), or by the increased stability of VEGF mRNA in hypoxic conditions. Furthermore, other growth factors such as platelet-derived growth factor (PDGF) have been shown to induce VEGF expression (Edelberg et al. 1998), suggesting VEGF can be induced independent of HIF-1 activity. However, other findings have linked hypoxic stimulus through HIF-1 stabilization with significant induction of VEGF and VEGF receptor in cardiogenesis (Lee et al. 2001; Liu and Fisher, 2008). The central role of hypoxia-dependent genes during cardiogenesis suggests that oxygen regulation at the molecular level plays a major role in fetal heart formation.

The Role of HIF in Cardiac Formation and Maturation

Oxygen Regulation

The molecular signals underpinning hypoxia's role in cardiac formation and development involves numerous genes. By far, the HIF family of genes is the most studied in cardiac development. HIF is a heterodimeric transcription factor that plays a pivotal role in cellular sensing and response to low oxygen tension. HIF belongs to the basic helix-loop-helix (bHLH)/Per-ARNT-Sim (PAS) domain family of transcription factors and is composed of an oxygen-sensitive α subunit and constitutively expressed β

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subunit (Semenza, 1998; Jiang et al. 1996a; Ward, 2008). It is known to regulate numerous functions during hypoxia such as energy metabolism, erythropoiesis, cell survival and death, vascularization, angiogenesis, and differentiation (Semenza et al. 1999; Adelman et al. 2000; Cowden et al. 2005; Covello et al. 2006; Galanis et al. 2008). Although HIF stability and activity is influence by a wide range of physiological factors (i.e. Insulin like Growth Factor 1 & 2), the effect of oxygen is most understood. Oxygen tension regulates HIF-1 expression *via* prolyl hydroxylase (PHDs) activity. Under normoxic conditions, the HIF-1α subunit is recognized and hydoxylated at proline residues 402 and 564 by PHDs. Hydroxylation allows recruitment and binding of the von Hippel-Lindau protein, an E3 ubiquitin protein ligase, which primes HIF-1α for subsequent proteasome degradation. In addition, the transcriptional activity of HIF-1 is also regulated by hydroxylation. Hydroxylation of arginine 803 by Factor inhibiting HIF-1 (FIH) prevents the association of HIF-1 and CREB-binding protein (CBP)/p300, precluding the transactivation of HIF-1 dependent genes (Mahon et al. 2001). When cellular oxygen levels fall, the activity of PHDs and FIH are reduced, allowing stability of the HIF-1α subunit. Interestingly, HIF-1 stability is inversely proportional to oxygen concentration within the cell (Jiang et al. 1996b). Stable HIF-1α subunit translocates into the nucleus where it dimerizes with HIF-1β and transactivates HIF-1 genes that possess hypoxic response elements (HRE) (short sequences of DNA that include 5'-CGTGC/T-3').

HIF and the Developing Heart

Both HIF-1αβ & 2 αβ are expressed in the cardiac tissue (Wiesener et al. 2003; Stroka et al. 2001). Little is known about the role of HIF-2 in heart formation and

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development, although HIF-2α knockout impairs vascular development as well as altered cardiac rhythm due to the deregulation of catecholamine release (Peng et al. 2000). Though it is not fully understood how HIF-1 dependent mechanisms coordinate in cardiogenesis, it is known that HIF-1 expression is vital for proper myocardial remodeling and coronary vessel formation. Elevated HIF-1 expression is found in fetal hearts exposed to hypoxia (Sugishita et al. 2004; Bae et al. 2003). HIF-1 was also found in the nuclei of OFT cardiomyocytes undergoing apoptosis as well as those that are not (Sugishita et al. 2004). This implies HIF-1 activity is involved in orchestrating OFT cardiomyocyte fate. The importance of hypoxic-dependent signaling in myocardial development is further supported by studies using mice deficient in HIF-1α. Global knockout of HIF-1α resulted in arrested development by day E9 and embryonic lethality by day E11 with significant cardiovascular irregularities including cardiac bifida, abnormal cardiac looping, abnormal remodeling of the aortic outflow tract and cephalic blood vessels, and mesenchymal cell death (Compernollo et al. 2003; Iyer et al. 1998). When HIF-1α null mice were placed in hyperoxia, partial recovery of the embryos was observed, suggesting that adaptation to hypoxic conditions requires HIF-1 signaling in the developing heart (Compernollo et al. 2003). HIF-1 is known to up-regulate, either directly, through binding to HRE sites, or indirectly, by influencing other transcription factors that promote prosurvival and apoptotic genes. In the fetal heart, HIF-dependent activation of VEGF, stromal cell-derived factor-1 and EPO receptor expression promote the vessel formation (Ladoux and Frelin, 1997; Tilmanns et al. 2008; Maloyan et al. 2005). In addition, survival signals in the myocardium are augmented by HIF-dependent direct activation of glycolytic genes such as Glucose transporter 1 (Glut1), aldolase A,

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enolase 1 (EN01), lactate dehydrogcnasc A, and phosphoglyceratc kinase 1(PGK1), which facilitate the shift from aerobic to anaerobic respiration (Wenger and Gassmann, 1997; Gordan and Simon, 2007). Paradoxically, HIF-1 has been shown to up-regulate proapoptic genes BNIP3 and Bax in cardiomyocytes (Graham et al. 2004). This dual role of HIF is also seen in cerebral ischemia models where HIF-1 up-regulates prosurvival (EPO, GLUT1, VEGF) and death genes (BNIP3, caspase 3, stabilization of p53) depending on the extent and duration of the ischemic insult (Chen et al. 2009). It is likely that complex mechanisms contribute to the duality of HIF-1 signaling in cardiogenesis. Presumably the ability of HIF-1 to bind HRE sites and thus influence gene activity changes depending on the timing and duration of hypoxic insult. This twin nature of HIF may influence HIF-dependent cardiomyocyte survival or death, and possibly play a role in long-term programming of fetal cardiomyocytes during chronic hypoxia.

Fetal Hypoxia and Abnormal Heart Development

Though fetal hearts show remarkable ability to survive and function under low oxygen, chronically pathophysiological hypoxia is associated with numerous complications that have both short and long-term effects. Some of the most striking data illustrating the effects of hypoxia on fetal development originate from high altitude studies. About 140 million people live in high altitude environment worldwide, of which some 400,000 live in the United States (Moore et al. 2001). High altitude is considered to be elevations above 2500 meters (8000ft) (Moore et al. 2001). Pregnancies at higher elevations may result in significantly depressed maternal arterial pO_2 and changes in placental growth when compared to the sea level (Zamudio, 2003). Epidemiological

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studies have indicated that high altitude pregnancies increase the risk of intrauterine growth restriction (IUGR) and low birth weight (Jensen and Moore, 1997; Moore et al. 2001; Moore, 2003). These factors are known to cause premature birth, infant mortality, and an increased risk of developing cardiovascular related diseases (Barker et al. 1989; Barker, 1994; Barker, 1997; Zhang, 2005). Other factors that may contribute to hypoxia *in utero* include pre-existing maternal illness, pre-eclampsia, cord compression, smoking, pollution, hemoglobinopathy, and aberrant placenta development. Lowered maternal arterial pO_2 or incomplete delivery of oxygen to fetal tissues induces hypoxemia and sustained tissue hypoxia *in utero*, resulting in significant changes in fetal development (Blackburn, 2007; Lueder et al. 1995).

The developing heart, more than any other organ, is susceptible to hypoxic stress due to its enhanced metabolic demand. Numerous studies, primarily in animals, have shown that hypoxia causes incomplete development of the heart. One of the earliest studies demonstrating the adverse effects of simulated high altitude on pregnant rats found that hypoxia caused ventricle septal defects in rat offspring (Clemmer, 1966). More recent studies have found that insufficient oxygen *in utero* produces myocardial thinning, ventricle dilation, and epicardium detachment. It also slows fetal heart maturation in both chicken and mouse (Ream et al. 2008; Sharma et al. 2006). Other studies demonstrated cardiomyocyte hypertrophy and myocardial hypoplasia in fetal hearts subjected to chronic hypoxia (Val'kovich et al. 1986; Martin et al. 1998; Bae et al. 2003; Ream et al. 2008). It is likely that the increase in size of cardiomyocyte is compensatory for reduced proliferation of myocyte. Interestingly, studies have found that prenatal hypoxia increases the heart to body mass ratio (Martin et al. 1998; Xiao et al. 2000; Bae et al. 2003),

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suggesting either reduced growth of nonessential organs or heart enlargement. The reduction in cardiomyocyte number is likely influenced by either increased program cell death and/or reduced cellular proliferation during critical periods of development. Hypoxia-mediated increase in apoptosis is supported by studies that indicate that prenatal hypoxia increased death signaling *via* elevated caspase 3 activity and Fas mRNA, and also suppressed survival pathways *via* depressed Bcl-2 and Hsp70 expression in fetal hearts (Bae et al. 2003). Conversely, the reduction in cardiomyocyte may be attributed to premature exit of cardiomyocytes from the cell cycle. Bae et al. (2003) reported that prenatal hypoxia increased the percentage and size of binucleated cardiomyocytes in fetal rat hearts. Binucleated cardiomyocytes are terminally differentiated cells that are no longer capable of division. Taken together, prolonged insufficient oxygen alters fetal heart growth resulting in abnormalities in heart structure. These abnormalities likely involve sustained reduction in cardiomyocyte proliferation and increased apoptosis.

Intrauterine stress *via* hypoxia induces not only changes in fetal heart morphology, but also in function. In humans, changes in fetal heart rate have long been observed in fetuses in response to intrauterine stress (Thornburg, 1991). Animal studies have also confirmed the dysfunction of the myocardium in response to hypoxia. Sedmera et al. (2002) demonstrated that the rate of recovery from anoxia/reoxygenation declines from the loop tubular heart to the septated trabeculated heart, suggesting oxygen dependence increases with the development. In a study examining the significance of catecholamines in development, researchers reported hypoxia decreased the heart rate of fetal mice by 35-40% in culture and by 20% *in utero* when compared to wild-type hearts (Portbury et al. 2003). High altitude Sheep models also present with altered cardiac

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function in response to prenatal hypoxia as demonstrated by decreased cardiac output and lowered contractility studies (Kamitomo et al. 1994 & 2002; Gilbert, 1998; Browne et al. 1997). Possible explanations for the depressed cardiac function relate to altered calcium homeostasis and reduced ATP availability due to decreased Mg^{2+}-activated myofibrillar ATPase activity (Browne et al. 1997; Kamitomo et al. 2002; Onishi et al. 2004). In avian models, poor cardiac performance was observed as demonstrated by decreased maximum ventricular +dP/dt and peak pressure, increased ventricular end-systolic volume, elevated after-load, and decreased left ventricular ejection fractions (Sharma et al. 2006; Tintu, 2009). Whether hypoxia directly mediates or indirectly facilitates these changes is not clear. Studies suggest that cardiac defects such as cardiac bifida and looping defects may be mediated *via* A1 adenosine receptor signaling in chicken hearts (Ghatpande et al. 2008). Sarre et al. (2006) demonstrated in an *in vitro* model arrhythmias in 4-day-old isolated embryonic hearts subjected to anoxia/reoxygenation. Graf et al. (2006) observed an increased rate of contractions and decreased sensitivity to norepinephrine of cultured rat cardiomyocytes exposed to hypobaric hypoxia during organogenesis. *In utero* stress may cause an increase in circulating stress hormones, which may explain reduced cardiac performance. In addition, structural changes in the myocardium may contribute to depressed cardiac function. It should be noted that many hypoxic models involve maternal exposure to reduced room oxygen. More studies demonstrating the direct effect of hypoxia on fetal hearts are warranted. Taken together, prolong exposure to hypoxia *in utero* alters heart structure and function and these changes may persist into adulthood.

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Hypoxia and Intrauterine Programming of the Heart

Many studies have correlated an adverse intrauterine environment with an increase predisposition for developing cardiovascular-related diseases in adulthood. Most notably, epidemiological studies by Barker and colleagues (Barker, 1989; Barker and Osmond 1986) were the first to correlate undernutrition and low birthweight with increased incidence of coronary heart disease in adulthood. Hypoxia *in utero* is known to cause depressed cardiac performance and cardiomyopathies that persist into adulthood (Tintu et al. 2009). It is less clear, however, the extent to which hypoxia mediates programming of genes that alter structure, function and survival that are not apparent until later in life.

Recently, researchers have used animal models to elucidate the role of hypoxia in intrauterine programming. Li and colleagues demonstrated that 6-month-old male rat hearts exposed to prenatal hypoxia responded less favorably than control animals when subjected to simulated ischemia and reperfusion (I/R) injury (Li et al. 2003). The hypoxic animals exhibited a persistent decrease in postischemic recovery, an increase in myocardial infarction (MI), and fewer but larger cardiomyocytes. In addition, the hearts of these animals had elevated caspase 3 activity and decreased levels of Hsp70 and eNOS when compared to control animals. While both Hsp70 and eNOS play important roles in cardioprotection against I/R injury, caspase 3 belongs to a family of proteases that perform critical cellular functions to facilitate programmed cell death (Nicholson et al. 1995; Okamura et al. 2000; Snoekx et al. 2001; Sharp et al. 2002). Xu and colleagues also observed lower levels of metalloproteinase-2 in 4-month-old rats (Xu et al. 2006). Moreover, prenatal hypoxia abolishes the protective affects afforded by heat stress

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against I/R injury and significantly reduces HSP70 and PKCε content in the left ventricles (Li et al. 2004). Furthermore, Xue and colleagues showed that prenatal hypoxia caused a decrease in I/R recovery in 3-month-old male, but not female, rat offspring (Xue and Zhang, 2009). When normoxic offspring were exposed to simulated I/R in the presence of PKCε translocation inhibitor peptide, those animals also displayed reduced I/R recovery; suggesting that programming of decreased PKCε gene expression is key to the observed increase vulnerability to I/R injury in males (Xue and Zhang, 2009). This finding is supported by studies involving PKCε over-expression, PKCε null animals, and PKCε activating peptide, which confirm the pivotal role of PKCε in I/R preconditioning and myocardial protection (Saurin et al., 2002; Ping et al. 2002; Inagaki et al., 2005 & 2006).

Conversely, Netuka et al. (2006) reported no difference in MI in prenatally hypoxic rats of either sex, but noted that normoxic females had less MI than normoxic males when exposed to I/R. They also noted that prenatal hypoxia protected against ischemic-induced arrhythmias of female rats, but was deleterious in male rats. The disparate results reported by Li (2003), Xu (2006) & Xue (2009) and Netuka's (2006) studies might be explained by differences in strain, age, and experimental models. Whereas the Xue (2009), Xu (2006) and Li (2003) studies used 3, 4 & 6-month-old Sprawley Dawley rats respectively exposed to hypoxia only *in utero*, Netuka (2006) used 3-month-old Wistar rats exposed to intermittent hypobaric hypoxia *in utero* and 10 days postgestation. In addition, Li et al. (2003) used simulated I/R insult with hearts removed from the animal, while Netuka et al. (2006) performed open chest I/R insult. These reasons may explain the differing results; nonetheless, both groups drew similar

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conclusions in that prenatal hypoxia causes programming of the fetal heart. It is also clear that prenatal hypoxia has sex dependent effects, as demonstrated by poor recovery of male rats, which likely involves altered programming of cardioprotective genes, namely PKCε.

The long-term effect of hypoxia *in utero* is not restricted to the cardiovascular system but also produces programming affects in other tissues. Pregnant sheep exposed to chronic high altitude conditions had significant reduction in kidney to body weight size, larger Bowman's capsule in nephrons and alterations in angiotensin I and II expression (Mao et al. 2009). Long-term hypoxia alters the expression of CYP17 and CYP11A1 in fetal sheep adrenal glands (Myers et al. 2005). CYP17 and CYP11A1 are two key enzymes involved in steroidogenesis, suggesting that high altitude exerts stress on animals such that elevated levels of stress hormone may be measured in fetal tissues. Whether this expression pattern is maintained into adult stages is not known. Furthermore, the direct effects of hypoxia are not clear. The maternal hypoxic model is capable of significantly lowering fetal oxygen status. However, maternal hypoxia elicits other systemic effects that may reflect the observed effects. For example, maternal hypoxia increases circulating glucocortoids that may cause abnormal development of the fetus (Levitt et al. 1996). Identifying the direct effects of hypoxia on fetal heart development is critical to understanding the underlying mechanisms involved in hypoxia-induced programming of fetal hearts.

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Hypoxia and Oxidative Stress

The majority of ATP synthesizes occurs in the mitochondria through oxidative phosphorylation. Oxidative phosphorylation utilizes proton concentration differences across the inner membrane of the mitochondria to create an electrochemical gradient. The electrochemical gradient is important in several functions including the transport of metabolites across the membrane, the import of proteins from the cytosol and the synthesis of ATP by ATP synthase. During this process, the majority of oxygen is reduced to water. However, between 1-4% of oxygen is only partially reduced to superoxide ($O_2\bullet^-$). $O_2\bullet^-$ belongs to a family of compounds called Reactive Oxygen Species (ROS). ROS can oxidize and damage nucleic acids, lipids, and proteins resulting in significant cellular damage. Typically, superoxide is eliminated by endogenous antioxidant defense systems. For example, Mitochondrial Superoxide Dismutase (mtSOD) converts superoxide to hydrogen peroxide (H_2O_2). H_2O_2 is further broken down into H_2O and O_2 by glutathione peroxidase or by catalases found in peroxisomes. In the presence of reduce metals, H_2O_2 is converted to the highly reactive hydroxyl ion (•OH). A significant endogenous antioxidant is the reduced form of glutathione. Glutathione reductase and NADP regenerate Glutathione. When antioxidant defense systems are unable to restore cellular redox balance, Glutathione levels fall promoting a state of oxidative stress. In cardiomyogenesis, oxidative stress plays an important role in signaling events that regulate cardiomyocyte differentiation (Sauer and Wartenberg, 2005).

Hypoxia is a unique phenomenon in that it paradoxically increases intracellular ROS (Chandel et al., 1998; Mansfield 2005; Liu et al., 2008). Interestingly, Chandel et

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al., (1998) reported hypoxia increases ROS; influences hypoxic dependent gene expression and that the primary source of hypoxia-induced ROS is the mitochondria. Furthermore, mitochondrial complex III was identified as the major site of mtROS productions (Guzy & Schumacker, 2006). Hypoxia increases probability of free radicals interacting with oxygen to produce superoxide along the electron transport chain. Interestingly, studies have suggested that hypoxia-derived ROS *via* mitochondrial complex III initiates the events necessary for HIF-1α stabilization (Chandel et al., 2000). The change in intracellular redox state reduces the levels of $Fe2+$, a necessary cofactor for prolyl hydroxylases hydroxylation of HIF-1α. Reduced prolyl hydroxylases activity allows nuclear accumulation of HIF-1α. While this theory is most commonly accepted, a recent study contends that oxygen availability instead of ROS production is the main stimulus altering prolyl hydroxylase activity and therefore HIF-1α stabilization (Chua et al., 2010). ROS is known to alter gene expression patterns. Studies have shown moderate hypoxia induces intracellular ROS production that initiates the Integrated Stress Response (ISR), which involves PERK activation, eIF2α phosphorylation and ATF4-mediated stress gene induction (Liu et al., 2008). These events alter global protein and mRNA synthesis, which is a likely response to reduce energy production. These findings suggest oxidative stress generated by the mitochondria as a result of hypoxia may increase HIF-1α nuclear accumulation as well as influence gene expression.

ROS causes extensive DNA damage that can lead to genomic mutations and tumor formation, but researches have sought to determine the link between oxidative stress and epigenetic modifications. In one elegant study, researchers demonstrated oxidative stress decreased E-cadherin expression in hepatocellular carcinoma cells (Lim

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et al., 2008). Reduced E-cadherin expression is associated with tissue-type transitions, metastasis and poor outcomes in hepatocellular carcinoma (Endo et al., 2000). Lim et al., (2008) showed ROS increases Snail expression, which then recruits DNA methyltransferase 1 (DNMT1) and histone deacetylase 1 (HDAC1). The interaction between Snail and epigenetic modifiers caused increased methylation of E-caherin promoter and reduced E-cadherin expression. It is therefore conceivable that hypoxia-induced ROS may mediate epigenetic changes in the promoters of cardioprotective genes, resulting in long-term down regulation of cardioprotective genes in fetal cardiomyocytes. (See figure 16).

PKCε and Cardioprotection

PKCε belongs to a group of phospholipid-dependent kinases. PKC isoforms share common domains in the regulatory region such as pseudosubtrate domains that binds the substrate recognition site while the kinase is inactive; C1a and C1b domains for diacylglcerol, phosphatidylserine, and phorbol ester binding; and, C2 region for receptor activated C kinase (RACK) interaction (Newton, 1995). PKC isoforms contain significant homology within the kinase or catalytic domain. The kinase domain is the region where ATP and substrate binding occurs. PKC isoforms are divided into three groups based on their primary structure. Conventional isoforms (α, βI, βII, γ) are responsive to diacylglcerol (DAG) and Ca^{2+}. Novel isoforms (δ, ε, η, θ) are DAG sensitive and Ca^{2+} insensitive. Atypical isoforms (ζ, ι/λ) are insensitive to both DAG and Ca^{2+}, and are responsive to lipid-derived molecules (Newton, 1995). Compounds that mimic DAG binding to C1 domain such as tumor promoting phorbol ester can also

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activate PKCε. Moreover, lipid-derived molecules such as arachidonic acid can activate PKCε. Interestingly, PKC isoforms must be primed through phosphorylation at three sites termed; the activation loop, hydrophobic motif and turn motif; in order to fully respond to allosteric regulators (Barnette, 2007). Phosphorylation is initiated either by 3-phosphoinositide-dependent protein kinase-1 (PDK1) or through autophosphorylation mechanisms. Once activated, PKC translocates to subcellular compartment via its interaction with RACK proteins. PKC then influences a wide array of cellular functions including muscle contraction, gene expression, differentiation, cell growth and ischemic preconditioning (Bogoyevitch & Sugden, 1993; Newton, 1995).

PKCε is highly expressed in heart tissue and is activated during ischemic preconditioning (IPC) (Bogoyevitch & Sugden, 1993; Gray et al., 1997). Knockout studies indicate PKCε is not critical for development, but plays a critical role in ischemic reperfusion preconditioning (Saurin et al., 2002). Studies using PKCε translocation inhibitory peptide (PKCε-TIP) and PKCε activating peptide provide further evidence of PKCε involvement in myocardial protection from ischemic reperfusion injury (Ping et al., 2002; Inagaki et al., 2004; Xue et al., 2009). The mechanism whereby PKCε protects hearts from ischemia reperfusion injury has not been fully explained. However, ischemia reperfusion insult increases the levels of intracellular ROS, which is known to stimulate PKCε activity. ROS may activate PKC causing its translocation to the mitochondria. Studies have shown PKCε interacts with mitochondrial proteins that potentially may mediate cytoprotection (Ardehali, 2006). Studies have shown PKCε enhances phosphorylation of BAD proteins, causing its inactivation and attenuating its ability to induce apoptosis (Bertolotto et al, 2000). In addition, phosphorylation of mitochondrial

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K_{ATP} by PKCε during ischemic stress causes the opening of mitoK_{ATP} allowing K+ into the inner matrix and thus hyperpolarizing and stabilizing the mitochondria. (Takano et al., 2003; Das et al., 2003; Hausenloy et al., 2003; Ardehali H, 2006; Jaburek et al., 2006; Kabir et al., 2006).

Epigenetics: A Plausible Mechanism

The mechanisms underlying the changes in function and gene expression in fetal hearts are not fully understood and are complex; however, epigenetic modifications are likely present. Major epigenetic modifications include methylation of cytosine in CpG dinucleotide, and post-translation modification of histone proteins (e.g. acetylation). DNA methylation plays a critical role in gene silencing and has been implicated in processes such as imprinting and embryogenesis (Hirst, 2009). Methylated Cytosines recruit methyl binding proteins that act to restrict binding of transcription factors to promoter regions. Methylation of CpG is normally conserved during somatic replication. Modifications of histone proteins can either increase or reduce the association of DNA with histones thereby regulating access of transcription machinery for selected genes. These modifications are dynamic and are influenced by a range of factors including the expression of cofactors, phase of cell cycle (i.e. G_1, S, mitosis), and environmental stimuli (Nafee, 2008). Major histone modification include acetylation of lysine residues via histone acetyltransferases that are associated with transcription activity; deacetylation of lysine residues via histone deacetylases that are commonly associated with transcription repression; methylation of lysine 4 on histone H3 is generally associated with transcriptional active promoters while multiple methylation of histone restricts

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transcription (Hirst, 2009). Further modifications include phosphorylation, ubiquitousation, hydoxlation, and sumolation. Histone modification not only influences transcription, but DNA replication, repair, and condensation.

Epigenetics plays an important role in embryonic development. In embryogenesis, developmental changes involve a progressive specialization of gene expression patterns that incorporate epigenetic mechanisms such as DNA methylation, chromatin remodeling, and/or histone posttranslational modification (Wu and Sun, 2006). Aberrant epigenetic silencing or enhancing of genes that influences cell cycle, death, and metabolism is commonly found in tumoriogenesis (Mckenna and Roberts, 2009; Hirst and Marra, 2009; Delcuve et al., 2009). Epigenetic modifications alter gene expression pattern in the long-term. In a related model, studies have demonstrated that maternal cocaine injections increases DNA methylation at Sp1 binding sites in PKCε promoter in fetal hearts (Zhang et al., 2009; Meyer et al., 2009B). Maternal cocaine injections during gestation induce intrauterine stress that results in increased susceptibility to I/R injury and abolition of protection afforded by preconditioning in the hearts of male offspring (Bae et al, 2005; Meyer et al., 2009A). In addition, reduced PKCε and phospho-PKCε expression was noted in the left ventricles of male offspring exposed to maternal cocaine, suggesting altered programming of the PKCε gene (Meyer et al., 2009A). Zhang et al. (2009) showed that cocaine exposure *in utero* caused hypermethylation of CpG dinucleotides of Sp1 binding sites for PKCε gene in left ventricles of 3-month-old rats. Supporting this discovery was the finding that Sp1 binding sites on the proximal promoter played a significant role in PKCε gene transcription (Zhang et al., 2009). Meyer et al. (2009A) used DNA methylation inhibitors 5-aza-2-deoxycytine and procainamide,

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to block cocaine-mediated down-regulation of PKCε. These findings clearly link epigenetics *via* DNA methylation *in utero* with the increased susceptibility to coronary heart disease later in life. The similarities between prenatal hypoxia and cocaine exposure are striking, and it is possible that the underlying mechanisms for both are concurrent. It is well known that cocaine acts as a potent vasoconstrictor, which may alter blood flow to the uterus resulting in oxygen insufficiency *in utero*. Furthermore, both hypoxia and cocaine cause an increase in oxidative stress. How prenatal hypoxia predisposes fetal hearts to I/R injury, and whether the mechanisms mirrors that of cocaine, are fascinating questions that remain to be elucidated.

HIF and Epigenetics

To date, little is known about HIF-1 in epigenetics and whether it plays a role in long-term gene silencing in immature cardiomyocytes. Since HIF activity is central to cellular adaptation to hypoxic stress, it is plausible HIF may play a role in hypoxia-induced fetal programming of cardiomyocytes. Hypoxia through HIF plays an important role in influencing transcription of multiple genes in embryogenesis. Epigenetic mechanisms play an important role in the process of both cases. An example of the influence of hypoxia in embryogenesis is seen in stem cell differentiation. Studies have demonstrated that normal placental development requires HIF-1 induction for proper trophoblast differentiation (Adelman et al., 2000; Cowden et al., 2005; Maltepe et al., 2005). One fascinating study demonstrated that HIF-2 preferentially up-regulated Oct-4 (Wiesener et al., 2003). Oct-4 plays an important role in maintaining a dedifferentiated cell disposition in embryonic stem cells, primordial germ cells and embryonic epiblast

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(Scholer et al., 1990; Suzuki et al., 1990; Nichols et al., 1998). Furthermore, reports have shown the involvement of HIF-1 in tumor formation and irregular gene expression silencing via DNA methylation (Kondo et al., 2002; Gordan et al., 2008). HIF-1 has also been shown to interact directly with epigenetic modulators. Studies done by Beyer (2008) and Wellmann et al. (2008) identified a HRE site in the proximal promoter of the histone demethylase Jumonji domain containing 1A and 2B (JMJD1A, JMJD2B). JMJD1A and JMJD2b demethylate H3K9 residues of histones, which are usually associated with reduced acetylation and gene repression [Beyer et al., 2008; Chen et al., 2006). In renal cell carcinoma, von Hippel Lindau activity is lost leading to aberrant expression of HIF-1 & 2, as well as increased expression and activity of histone demethylases JMJD1A and JMJD2b (Kondo et al., 2002; Gordan et al., 2008; Krieg et al., 2000). In addition, studies suggest that HIF-1 interacts directly with histone deacetylases 7 (HDAC7) (Maltepe et al., 2005; Granger et al., 2008). The interaction seems to highlight HDAC7 ability to enhance HIF activity by forming a complex with HIF and p300 and influencing gene transcription. Histone deacetylases remove acetyl groups from histone residues, causing structural changes that preclude the binding of transcription factors and RNA polymerase II to gene targets. HIF-1 is known to influence cellular adaptation in response to acute and chronic hypoxia; yet HIF-1 may also mediate long-term changes in gene expression through direct modulation of epigenetic effectors.

Recently, studies have linked c-myc activity with HIF-1 and HIF-2. C-myc is a proto-oncogene involved in cell proliferation and enhancement of cellular metabolism. There is evidence that c-myc can modulate epigenetic mechanisms. Work done by He et al. (2009) demonstrated a link between overexpression of c-myc and hypermethylation of

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CpG sites in the tumor suppressor gene's retinoic acid receptor beta (RARbeta) and PDLIM4 in a prostate cancer cell line. Brenner et al, (2005) found that c-myc is able to direct DNA methyltransferase 3a (DNMT3a) to the p21Cip1 promoter through direct binding with DNMT3a and Miz-1, identifying a potential mechanism for epigenetic silencing in cancer. In cancer cell models, HIF-1 actively competes with c-myc causing inhibition of gene targets, while HIF-2 promotes c-myc activity (Gordan et al., 2008; Koshiji et al., 2004). These findings suggest HIF may also indirectly influence epigenetic patterns through interaction with c-myc or other transcription factors. Undoubtedly, HIF-1 plays an important role in normal fetal heart development, and may also play a key role in fetal programming of the myocardium through modulation of epigenetic effectors.

Central Hypothesis

Our hypothesis is that hypoxia directly affects PKCε expression in fetal hearts through epigenetic modifications of the PKCε promoter; and, hypoxia-induced HIF-1α stabilization and/or oxidative stress play a role in this process.

Significance

Increasing evidence suggests programming of protective genes *in utero* can predispose offspring to developing coronary disease later in life. Particularly, PKCε expression is known to play a vital role in cardioprotection against ischemia reperfusion injury; however prenatal hypoxia reduces PKCε expression in adult hearts increasing the susceptibility to I/R injury. Although hypoxia is known to cause certain cardiomyopathies, little is known about the mechanism through which hypoxia mediates

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programming of cardioprotective genes and whether it involves epigenetics. Since hypoxic stress is a common insult during development and very little is known about the role of epigenetics in hypoxia-induced down-regulation of cardioprotective genes, we expect findings from our study to reveal a novel role of hypoxia and hypoxia-induced gene expression in fetal programming of cardiomyocytes. We further anticipate our findings to have relevance in areas beyond cardiogenesis; including embryogenesis and tumorigenesis, where normal and aberrant gene programming as a result of epigenetic modifications are common. Our findings will provide a greater understanding of how adaptations in response to intrauterine stress, namely hypoxia, can influence cellular responses to stress later in life.

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CHAPTER 2

CHRONIC PRENATAL HYPOXIA INDUCES EPIGENETIC PROGRAMMING OF PKCε GENE REPRESSION IN RAT HEARTS

by

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Abstract

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

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

Methods and Results: Hypoxic treatment of pregnant rats from day 15 to 21 of gestation resulted in significant decreases in PKCε protein and mRNA in fetal hearts. Similar results were obtained in *ex vivo* hypoxic treatment of isolated fetal hearts and rat embryonic ventricular myocyte cell line H9c2. Increased methylation of PKCε promoter at SP1 binding sites, -346 and -268, were demonstrated in both fetal hearts of maternal hypoxia and H9c2 cells treated with 1% O_2 for 24 h. Whereas hypoxia had no significant effect on the binding affinity of SP1 to the unmethylated sites in H9c2 cells, hearts of fetuses and adult offspring, methylation of both SP1 sites reduced SP1 binding. The addition of 5-aza-2'-deoxycytidine blocked the hypoxia-induced increase in methylation of both SP1 binding sites and restored PKCε mRNA and protein to the control levels. In hearts of both fetuses and adult offspring, hypoxia-induced methylation of SP1 sites was significantly greater in males than in females, and decreased PKCε mRNA was seen only in males. In fetal hearts, there was significantly higher abundance of estrogen receptor α (ERα) and β (ERβ) isoforms in females than in males. Both ERα and ERβ interacted

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with the SP1 binding sites in the fetal heart, which may explain the gender differences in SP1 methylation in the fetal heart. Additionally, selective activation of PKCε restored the hypoxia-induced cardiac vulnerability to ischemic injury in offspring.

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

Keywords: Fetal heart, PKCε, hypoxia, epigenetics, DNA methylation

Introduction

Heart disease is the leading cause of death in the United States. In addition to other risk factors, recent epidemiological and animal studies have shown a clear association of adverse intrauterine environment with an increased risk of hypertension and ischemic heart disease in adulthood.[1-4] Hypoxia is a common form of intrauterine stress, and the fetus may experience prolonged hypoxic stress under a variety of conditions, including pregnancy at high altitude, pregnancy with anemia, placental insufficiency, cord compression, preeclampsia, heart, lung and kidney disease, or with hemoglobinopathy. Animal studies suggest a possible link between fetal hypoxia and increased risk of cardiovascular disease in offspring.[5-13] Studies in rats have demonstrated that maternal hypoxia results in an increase in cardiac vulnerability to ischemia and reperfusion injury in male offspring.[10,14,15] In addition, it has been demonstrated that down-regulation of protein kinase C epsilon (PKCε) protein expression in the hearts of

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adult offspring is a mechanism for the increased heart susceptibility to ischemia and reperfusion injury in the animals exposed to hypoxia before birth.[14]

Among other mechanisms, PKCε plays a pivotal role of cardioprotection in heart ischemia and reperfusion injury.[16-18] The study in a PKCε knock-out mouse model has demonstrated that PKCε expression is not required for cardiac function under normal physiological conditions, but PKCε activation is necessary for acute cardioprotection during cardiac ischemia and reperfusion.[19] The finding that fetal hypoxia resulted in a decrease in PKCε protein expression in the heart of adult offspring[10,14] suggests that an epigenetic mechanism may explain PKCε gene repression in the heart. Epigenetic mechanisms are essential for development and differentiation, and allow an organism to respond to the environment through changes in gene expression patterns.[20-22] DNA methylation is a chief mechanism for epigenetic modification of gene expression patterns, and occurs at cytosines in the CpG dinucleotide sequence.[23] Methylation in promoter regions is generally associated with repression of transcription, leading to a long-term shutdown of the associated gene. Methylation of CpG islands in gene promoter regions alters chromatin structure and transcription. Similarly, methylation of CpG dinucleotides within transcription factor binding sites generally represses transcription.[20,24,25] The present study tested the hypothesis that fetal hypoxia-induced methylation of CpG dinucleotides at the PKCε promoter contributes to PKCε gene repression in the heart of adult offspring. Herein, we present evidence that hypoxia has a direct effect on PKCε expression in the fetal heart, and demonstrate in a cell model that hypoxia causes an increase in methylation of CpG dinucleotides at the proximal promoter region of PKCε

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gene, resulting in decreased binding of sequence-specific transcription factors to the promoter and reduced PKCε gene expression.

Methods

An expanded Methods section is available in the data supplement.

Experimental Animals

Pregnant rats were randomly divided into two groups: 1) normoxic control; and 2) hypoxic treatment of 10.5% O_2 from day 15 to 21 of gestation.[14] Hearts were isolated from near-term (21 d) fetuses and 3 months old offspring. To study the direct effect of hypoxia on the fetal heart, hearts were isolated from day 17 fetal rats and cultured at 37 °C in 95% air/5% CO_2 and 1% O_2 for 48 h, as reported previously.[24]

Cell Culture

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

Western Blot Analysis

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

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Real-Time RT-PCR

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

Quantitative Methylation-Specific PCR

DNA collected from H9c2 cells and hearts of fetuses and adult offspring was treated with sodium bisulfite at 55 °C for 16 h. Bisulfite-treated DNA was used as a template for real-time fluorogenic methylation-specific PCR (MSP) using primers created to amplify promoter binding sites containing possible methylation sites based on our previous sequencing of rat PKCε promoter.[25]

Electrophoretic Mobility Shift Assay (EMSA)

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

Chromatin Immunoprecipitation (ChIP)

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

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Hearts Subjected to Ischemia and Reperfusion

Isolated hearts from 3 months old male offspring were subjected to 20 minutes of global ischemia followed by 30 minutes of reperfusion in the absence or presence of a PKCε activator peptide ψ-εRACK (0.5 μM, KAE-1, KAI Pharmaceuticals) in a Langendorff preparation, as previously described.[14, 27] Post-ischemic recovery of left ventricular function and lactate dehydrogenase (LDH) release were determined.[14]

Statistical Analysis

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

Results

Effect of Hypoxia on PKC Protein and mRNA in Fetal Cardiomyocytes

Maternal hypoxia resulted in significant decreases in both protein and mRNA abundance of PKCε in fetal rat hearts (Figure 2A). Similar findings were obtained in isolated fetal hearts treated *ex vivo* with 1% O_2 (Figure 2A), demonstrating a direct effect of hypoxia on PKCε expression in the heart. Further study demonstrated in rat embryonic ventricular myocyte cell line H9c2 that 1% O_2, but not 3% or 10.5% O_2, significantly decreased PKCε mRNA and protein levels as compared to 21% O_2 (Figure 2B).

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Methylation of SP1 Binding Sites at the PKC Promoter

Eight putative transcription binding sites containing CpG dinucleotides at rat PKCε promoter were previously identified.[25] Treatment of H9c2 cells with 1% O_2 for 24 h resulted in a significant increase in methylation of SP1 binding sites at -346 and -268, but decreased methylation of MTF1 binding site at -168, as compared to 21% O_2 control (Figure 3). Hypoxia did not change significantly the methylation status of Stra13, PPARG, E2F, Egr1, and MTF1 at -603. Consistent with the findings in H9c2 cells, maternal hypoxic treatment revealed a similar pattern of increased methylation of the two SP1 binding sites, -346 and -268, at the PKCε promoter in the fetal hearts (Figure 3). In addition, *in vivo* hypoxia resulted in an increase in methylation of the putative Egr1 binding site in the fetal heart (Figure 3).

Methylation Inhibits SP1 Binding

H9c2 cells were used to further determine the role of methylation in PKCε down-regulation. Given the previous finding that deletions of the regions containing the putative Egr1 site and MTF1 site at -168 had no significant effects on the PKCε promoter activity,[25] our further investigation focused on the two SP1 binding sites. SP1 binding and the functional significance of the SP1 binding sites in the regulation of PKCε gene activity was demonstrated previously.[25] To determine if methylation of the SP1 sites inhibits SP1 binding from nuclear extracts of H9c2 cells, EMSA was performed with methylated and unmethylated oligonucleotide probes containing the SP1 sites at -346 and -268. As shown in Figure 4, nuclear extracts from H9c2 cells bound and shifted the double-stranded unmethylated SP1 oligonucleotides at both sites, but failed to cause a shift

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of the methylated SP1 oligonucleotides. This is consistent with the previous findings in fetal rat hearts showing a loss of binding of the nuclear extracts to the methylated SP1 oligonucleotides.[24]

Effect of Hypoxia on SP1 Abundance and Binding Affinity

Western blots revealed that hypoxia caused a significant increase in nuclear SP1 abundance in H9c2 cells (Figure S1, available in the data supplement). In contrast, there was no significant difference in SP1 abundance either in fetal hearts between control and maternal hypoxic treatments, or in the hearts of both male and female offspring between the control and prenatally hypoxic animals (Online Figure I). The binding affinity of SP1 to the unmethylated SP1 binding sites was determined in competition studies performed in pooled nuclear extracts with the increasing ratio of unlabeled/labeled oligonucleotides encompassing the SP1 sites at -346 and -268, respectively. Hypoxia had no significant effect on the binding of nuclear extracts to either SP1 sites at -346 or -268 in H9c2 cells, fetal hearts, or the hearts of both male and female offspring (Online Figure II).

Inhibition of DNA Methylation Restored PKCε Expression

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

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restoration of PKCε protein (Figure 5). The inhibition of hypoxia-induced methylation of the two SP1 binding sites by 5-aza-2'-deoxycytodine was demonstrated (Figure 6A). To confirm that hypoxia-induced methylation alters SP1 binding to the PKCε promoter in the context of intact chromatin, ChIP assays were performed using a SP1 antibody. Figure 6B shows that hypoxia caused significant decreases in the SP1 binding to both SP1 sites at -346 and -268, respectively, in H9c2 cells. However, in the presence of 5-aza-2-deoxycytine, there were no significant differences in SP1 binding to the either sites between hypoxic and normoxic samples (Figure 6B).

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

We further investigated the potential sex differences in hypoxia-induced methylation of the SP1 binding sites and PKCε transcription. Maternal hypoxia caused a minimal but significant increase in methylation at both SP1 binding sites in the hearts of female fetuses, but induced significantly greater methylation in male fetal hearts (Figure 7A). PKCε mRNA was significantly decreased in the hearts of male but not female fetuses (Figure 7B). The similar pattern of sex differences in the hypoxia-induced SP1 methylation and PKCε transcription was demonstrated in the hearts of male and female offspring (Figure 7A, B). The sex difference observed in the fetal hearts was intriguing given that male and female fetuses were likely exposed to the similar concentrations of steroid hormones *in utero*. However, both ERα and ERβ abundance was significantly higher in the hearts of female, as compared with male fetuses (Figure 8A). Chromatin immunoprecipitation assay demonstrated the PCR products of the two SP1 binding sites in the DNA sequences pulled-down by both ERα and ERβ antibodies in the fetal hearts

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(Figure 8B), suggesting an interaction between estrogen receptors and the SP1 binding sites at the PKCε promoter.

PKCε Activation Restored Hypoxia-Induced Cardiac Vulnerability to Ischemic Injury

The cause-and-effect evidence of the functional importance of PKCε in hypoxia-mediated, sex-dependent programming of increased heart vulnerability to ischemia and reperfusion injury in adult male offspring has been previously demonstrated by selective inhibition of PKCε with a PKCε translocation inhibitor peptide.[14] To further demonstrate that decreased PKCε is an important factor in the hypoxia-induced increase in cardiac ischemic susceptibility in male offspring, additional studies were performed in the hearts in a Langendorff preparation using a selective PKCε activator peptide ψ-εRACK obtained from KAI Pharmaceuticals.[16, 27-29] There were no significant differences in left ventricle developed pressure (LVDP), heart rate (HR), dP/dt_{max}, dP/dt_{min} and coronary flow rate at the baseline among all groups (Online Table I). In the absence of ψ-εRACK, fetal hypoxia caused a significant decrease in post-ischemic recovery of LVDP and increases in left ventricle end diastolic pressure (LVEDP) and lactate dehydrogenase (LDH) release (Figure 8), as well as decreases in the recovery of dP/dt_{max} and dP/dt_{min} (Figure S3), as previously reported.[14] ψ-εRACK increased post-ischemic recovery of left ventricular function as shown in the previous studies[27, 29] and abolished the hypoxic effects (Figure 9 and Online Figure III).

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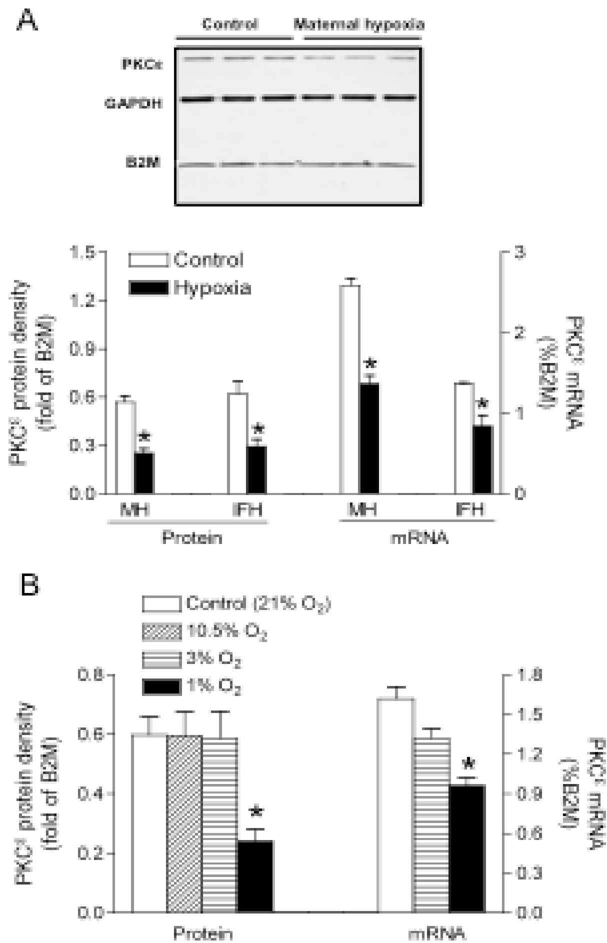

Figure 2. Effect of hypoxia on PKCε protein and mRNA. (A) Fetal hearts of maternal hypoxia (MH) and isolated fetal hearts (IFH) treated with 1% (hypoxia) and 21% O_2 (control) for 48 hours. (B) H9c2 cells treated with 1%, 3%, 10.5%, and 21% O_2 for 24 hours. Data are means ± SEM. * $P < 0.05$ *vs*. control (n = 5-15)

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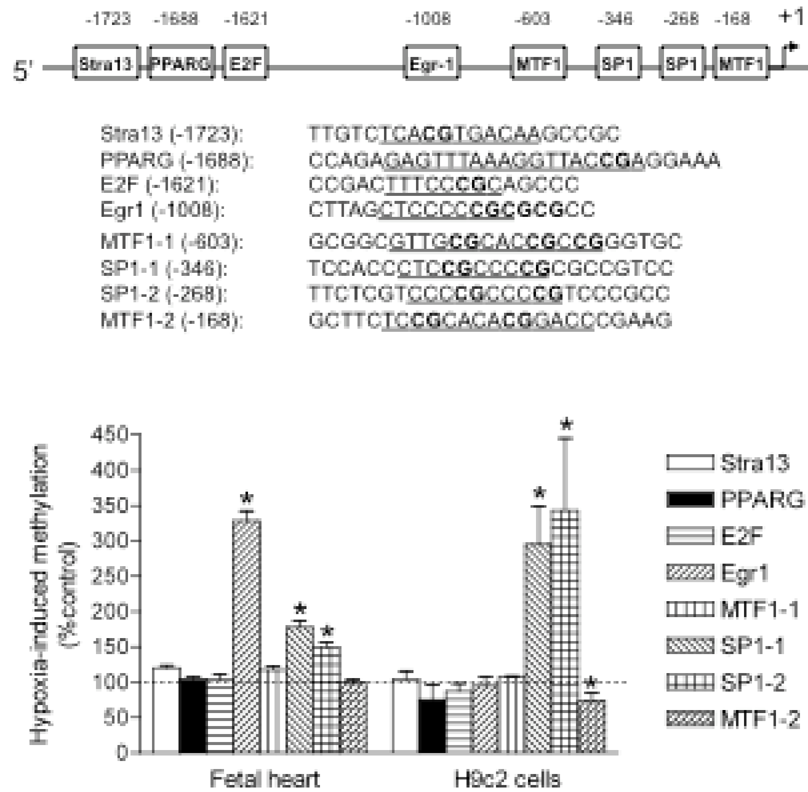

Figure 3. Effect of hypoxia on methylation of the PKCε promoter. Methylation patterns were determined in fetal hearts of maternal hypoxia and H9c2 cells treated with 1% O_2 *vs*. 21% O_2 for 24 hours. Data are means ± SEM. * $P < 0.05$ *vs*. control (n = 5-10)

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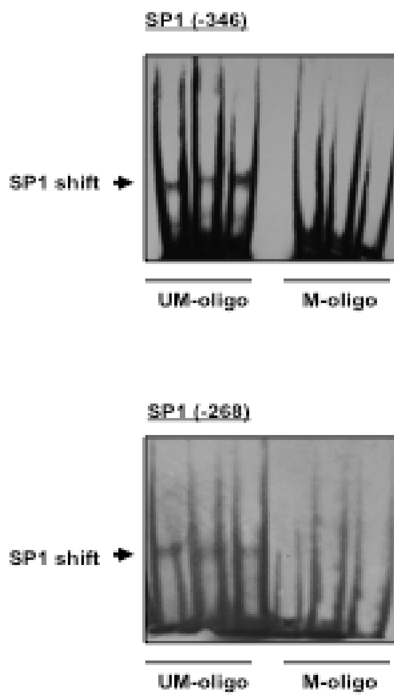

Figure 4. Effect of methylation on SP1 binding. Nuclear extracts of H9c2 cells were incubated with unmethylated (UM-oligo) or methylated (M-oligo) oligonucleotides containing SP1 consensus sequence at sites -346 and -268.

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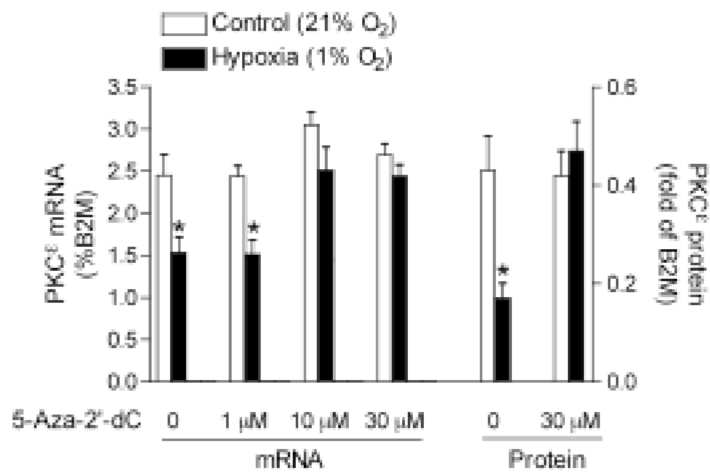

Figure 5. Effect of 5-aza-2'-deoxycytidine on PKCε mRNA and protein. H9c2 cells were treated with 1% O_2 and 21% O_2 for 24 hours in the absence or presence of increasing concentration of 5-aza-2'-deoxycytidine (5-Aza-2'-dC). Data are means ± SEM. * $P < 0.05$ *vs*. control (n = 4-17).

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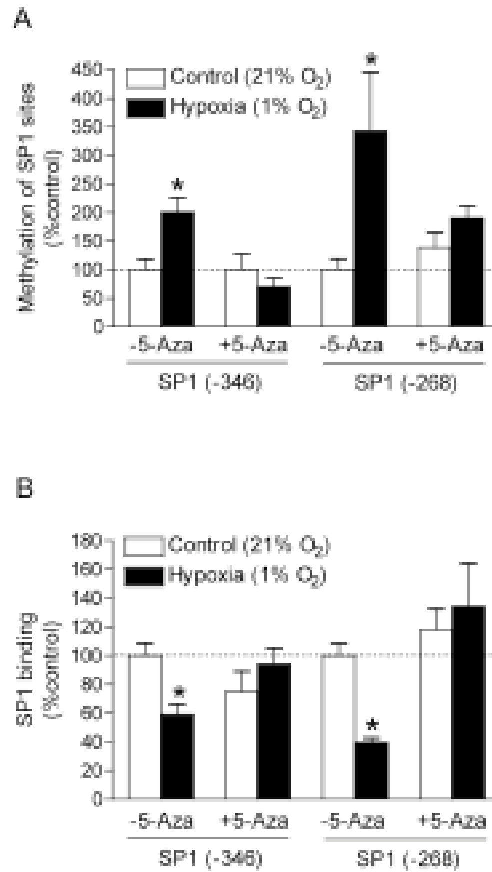

Figure 6. Effect of 5-aza-2'-deoxycytidine on methylation of SP1 sites and SP1 binding. H9c2 cells were treated with 1% O_2 and 21% O_2 for 24 h in the absence or presence of 30 μM 5-aza-2'-deoxycytidine (5-Aza). (A) Methylation of SP1 binding sites was determined by methylation-specific PCR. (B) SP1 binding was determined by ChIP assays. Data are means ± SEM. * $P < 0.05$ *vs*. control (n = 4-9).

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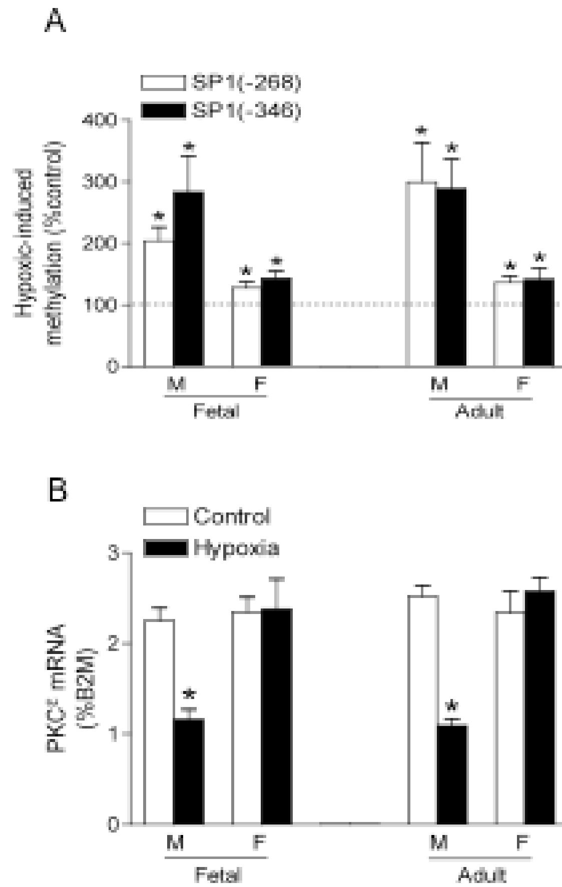

Figure 7. Effect of hypoxia on SP1 methylation and PKCε mRNA. Pregnant rats were treated with hypoxia, and hearts were isolated from near-term fetuses or 3 months old offspring. M, male; F, female. (A) Methylation of SP1 binding sites at the PKCε promoter. (B) PKCε mRNA. Data are means ± SEM. * $P < 0.05$ *vs*. control (n = 5)

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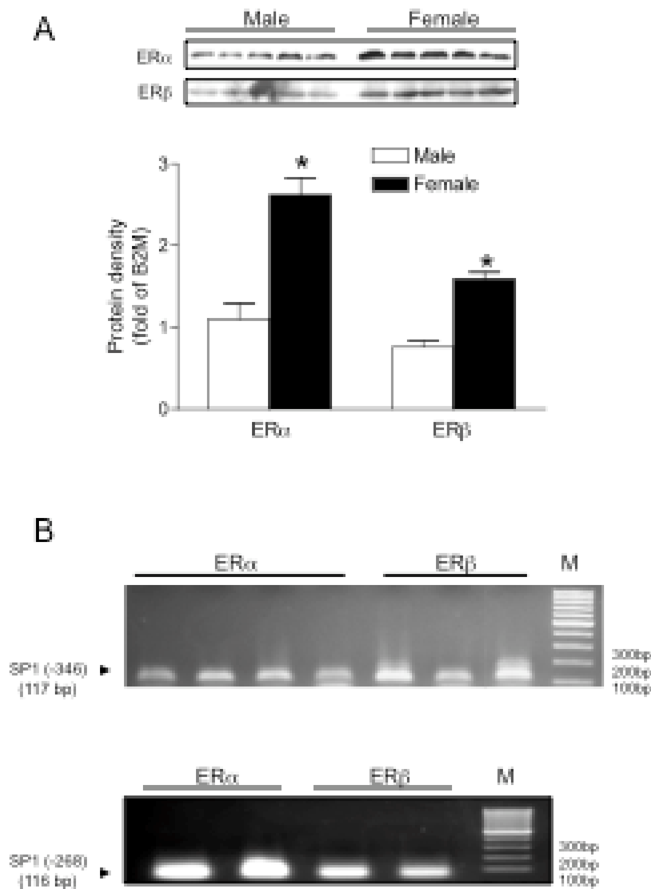

Figure 8. Estrogen receptors and SP1 binding sites in the fetal heart. Hearts were isolated from near-term fetuses. (A) Protein abundance of estrogen receptor alpha (ERα) and beta (ERβ) subtypes. Data are mean ± SEM. * $P < 0.05$ *vs*. male (n = 5). (B) PCR products of the SP1 binding sites (-346 and -268) following chromatin immunoprecipitation (ChIP) with ERα and ERβ antibodies, respectively, in the fetal heart. M, markers.

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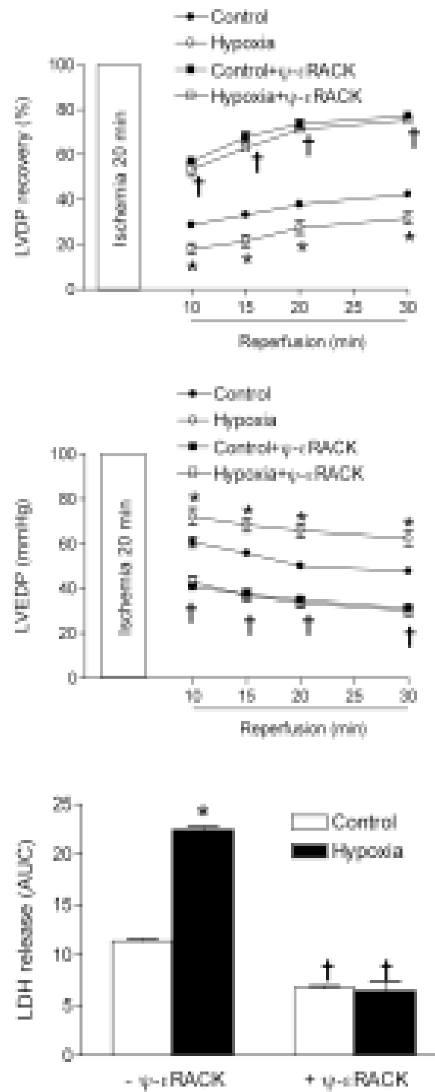

Figure 9. Effect of PKCε activation on cardiac ischemia and reperfusion injury. Hearts were isolated from 3-month-old male offspring that had been exposed to normoxia (control) or hypoxia before birth, and were treated in the absence or presence of the PKCε activator ψ-εRACK (0.5 μM) for 10 minutes before subjecting to 20 minutes of ischemia and 30 minutes of reperfusion in a Langendorff preparation. Post-ischemic recovery of left ventricular developed pressure (LVDP) and end diastolic pressure (LVEDP) were determined. Lactate dehydrogenase (LDH) release over 30 minutes of reperfusion was measured as area under curve (AUC). Data are mean ± SEM. * $P < 0.05$, hypoxia vs. control; † $P < 0.05$, + ψ-εRACK vs. -ψ-εRACK. n = 5.

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Discussion

The present study demonstrates that hypoxia has a direct effect on fetal cardiomyocytes causing a reduction in PKCε protein and mRNA. This is correlated with increased methylation of CpG dinucleotides in the two SP1 binding sites at the proximal promoter region of PKCε gene, resulting in decreased binding of SP1 to the promoter. The causal effect of methylation in the hypoxia-induced PKCε gene repression is demonstrated through the use of a DNA methylation inhibitor, which blocks the methylation and restores PKCε mRNA and protein expression to normal.

Previous studies demonstrated the long-term adverse effect of maternal hypoxia on cardiac vulnerability to ischemia and reperfusion injury in adult male offspring in a sex-dependent manner.[10,14,15] Particularly, the decreased expression of cardioprotective genes, namely PKCε in hearts of adult male offspring are of significant interest.[14] Acute hypoxia and reactive oxygen species increase the activity of PKCε in the heart, which plays an important role in cardioprotection during ischemia and reperfusion injury.[30] The present study demonstrated that chronic hypoxia during gestation down-regulated PKCε expression in the developing heart through an epigenetic modification. This suggests differential regulations of acute hypoxia and chronic hypoxia on PKCε activity and gene expression in the heart. The causal role of reduced PKCε in sex-dependent programming of increased heart vulnerability to ischemia and reperfusion injury in adult male offspring has been demonstrated by selective inhibition of PKCε with a PKCε translocation inhibitor peptide.[14, 31] The present study demonstrated that a selective PKCε activator peptide[16, 27-29] restored the hypoxia-induced cardiac vulnerability to ischemic injury, providing further evidence of cause-and-effect role of decreased PKCε in the hypoxia-

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mediated, sex-dependent programming of increased heart ischemic vulnerability in male offspring. This is consistent with other studies showing that PKCε expression is necessary for acute cardioprotection during cardiac ischemia and reperfusion.[19] The present finding of decreased PKCε protein and mRNA in fetal hearts caused by maternal hypoxia suggests that the reduction in PKCε expression observed in adult rats[10,14] originates *in utero*. Previous studies in the same animal model demonstrated that maternal hypoxia increased hypoxia-inducible factor 1α (HIF-1α) protein in fetal hearts, suggesting tissue hypoxia in the fetal heart.[32] The similar results of isolated hearts treated *ex vivo* with hypoxia indicate that hypoxia is necessary and sufficient to impact the PKCε expression in fetal hearts. Whether this decreased PKCε expression in the heart is a protective mechanism that increases fetal survival is not clear at present. However, the finding that the down-regulation of PKCε resulted in increased heart ischemic vulnerability in offspring would suggest that it is a maladaptive response.

The present study used the embryonic rat ventricular myocyte cell line H9c2[33] to investigate the underlying mechanisms. H9c2 cell line has been widely used in a variety of myocardiocyte studies, including those investigating apoptosis, differentiation and ischemia and reperfusion injury.[34-38] H9c2 cells retain many electrophysical properties found in freshly isolated cardiomyocytes,[33] albeit they do not spontaneously contract and are capable of continuous growth.[39] In the present study, the hypoxia-induced decrease in PKCε expression observed in fetal hearts *in vivo* and *ex vivo* mirrored that found in H9c2 cells, suggesting a congruent underlying mechanism for each model and providing a comparable model of H9c2 cells in the study of PKCε gene regulation. Similar to the

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present finding, it has been reported that PKCε protein expression is decreased in H9c2 cells exposed to 2 and 8 h of hypoxia in the presence of low and high glucose.[40, 41]

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

The finding of similar pattern of increased methylation of the two SP1 binding sites at the PKCε promoter between H9c2 cells and the fetal hearts of maternal hypoxic treatment provides further evidence supporting the model of H9c2 cells in the present study. Whereas the differences in methylation patterns in the putative Egr1 and MTF1 (-168) binding sites observed in fetal hearts and H9c2 cells are intriguing, the functional significance of this finding is not clear given that deletions of the regions containing the Egr1 and MTF1 (-168) sites had no significant effects on the PKCε promoter activity.[25] In contrast, deletion of the confirmed binding sites for both SP1 (-346) and SP1 (-268) significantly decreased the PKCε promoter activity in H9c2 cells.[25] In the present study, we demonstrated by EMSA that methylation of CpG dinucleotides at the core of both

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SP1 binding sites abolished SP1 binding in H9c2 cells. Previous studies with site-directed methylation of PKCε promoter-luciferase constructs selectively at SP1 binding sites at -268 and -346, demonstrated that mutation of $C^{m}G$ at either SP1 site -268 or -346 alone had no significant effect on the promoter activity, but mutation of $C^{m}G$ at the both SP1 binding sites significantly reduced the promoter activity in H9c2 cells.[24] Although increases in nuclear SP1 protein abundance in hypoxic H9c2 cells may serve as a compensatory mechanism, this increase is relatively ineffective because increased methylation of SP1 binding sites caused an even more significant decrease in SP1 binding to the PKCε promoter in the intact chromatin as demonstrated by ChIP assays. The finding that SP1 abundance was not significantly affected by hypoxia in fetal hearts, as well as in the hearts of either male or female adult offspring, together with the finding that hypoxia had no significant effect on SP1 binding affinity in H9c2 cells, fetal and adult hearts, reinforces a primary role of methylation in programming of PKCε gene repression.

The causal effect of increased methylation in hypoxia-induced PKCε gene repression was further demonstrated with a DNA methylation inhibitor 5-aza-2'-deoxycytodine in the present study. 5-Aza-2'-deoxycytodine binds to DNA methyltransferase 1 causing its depletion and preventing DNA methylation,[45] and has been widely used to inhibit DNA methylation.[46-49] In the present study, we demonstrated that 5-aza-2'-deoxycytodine concentration-dependently inhibited hypoxia-induced down-regulation of PKCε mRNA expression. This is caused by inhibition of the hypoxia-induced methylation of both SP1 binding sites resulting in recovered SP1 binding to the PKCε promoter in the intact chromatin. Whereas the study of 5-aza-2'-deoxycytodine in

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H9c2 cells is limited, relatively high concentrations of 5-aza-cytidine (a close derivative of 5-aza-2-deoxycytidine) have been shown to rescue luciferase activity in transfected H9c2 cells in excess of 250 μM,[50] which may reflect cell-type specific response to 5-aza-2-deoxycytidine and derivatives. The ability of 5-aza-2-deoxycytidine to restore PKCε mRNA and protein expression in cardiomyocytes in the presence of a stressor is consistent with earlier studies that showed that 5-aza-2-deoxycytidine blocked cocaine-mediated repression of PKCε expression in fetal rat hearts.[24]

Previous studies demonstrated that prenatal hypoxia caused an increase in heart susceptibility to ischemia and reperfusion injury in a sex-dependent manner, which was due to fetal programming of PKCε gene repression resulting in downregulation of PKCε function in the heart of adult male offspring.[14] In the present study, no significant differences were found in SP1 methylation and PKCε mRNA abundance between male and female control groups in both fetuses and offspring. This is consistent with the previous findings of no significant difference in PKCε protein abundance in the heart between control males and females.[14, 51] However, the sex differences in hypoxia-induced changes in methylation of the SP1 binding sites and PKCε transcription were demonstrated in both fetal and offspring hearts. The finding that the hypoxia-induced methylation was significantly greater in the hearts of male fetuses is intriguing given that male and female fetuses are likely exposed to the similar concentrations of steroid hormones *in utero*. Whereas the mechanisms are not clear at present, the sex difference observed may be caused in part by the greater expression of ERα and ERβ in the hearts of female fetuses. The finding that both ERα and ERβ interacted with the SP1 binding sites at the PKCε promoter in intact chromatin in the fetal heart suggests a possible

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mechanism for the increased protection of SP1 binding sites and PKCε transcription in the female hearts in response to hypoxic stress. It has been demonstrated that both ERα and ERβ can activate transcription of the retinoic acid receptor α1 (RAR-1) gene by the formation of an ER-SP1 complex on the SP1 sites in the RAR-1 promoter.[52] It is not clear at present whether the presence of phytoestrogens in the diet of soy-based chows used in the present study might contribute to the gender difference, though the pregnancy is a stage of high estrogen concentrations. This remains as an intriguing area for the future investigation using a casein-based diet. The similar pattern of sex differences in the hypoxia-induced SP1 methylation and PKCε transcription demonstrated in the hearts of male and female offspring supports the notion that fetal hypoxia-induced methylation of CpG dinucleotides at the PKCε promoter contributes to PKCε gene repression in the heart of adult offspring.

The present investigation provides evidence of a novel mechanism of methylation in non-CpG island, sequence-specific transcription factor binding sites in subtle epigenetic modifications of gene expression pattern in fetal programming of cardiac function in response to adverse intrauterine environment. Although it may be difficult to translate the present findings directly into the humans, the possibility that fetal hypoxia may result in programming of a specific gene in the offspring with a consequence of increased cardiac vulnerability provides a mechanistic understanding worthy of investigation in humans. This is because hypoxia is one of the most important and clinically relevant stresses to the fetus, and because large epidemiological studies indicate a link between *in utero* adverse stimuli during gestation and an increased risk of ischemic heart disease in the adulthood.

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References

1. Barker DJ, Osmond C. Infant mortality, childhood nutrition, and ischaemic heart disease in England and Wales. *Lancet.* 1986;1(8489):1077-1081.

2. Bateson P, Barker D, Clutton-Brock T, Deb D, D'Udine B, Foley RA, Gluckman P, Godfrey K, Kirkwood T, Lahr MM, McNamara J, Metcalfe NB, Monaghan P, Spencer HG, Sultan SE. Developmental plasticity and human health. *Nature.* 2004;430(6998):419-421.

3. Gluckman PD, Hanson MA, Cooper C, Thornburg KL. Effect of in utero and early-life conditions on adult health and disease. *N Engl J Med.* 2008;359(1):61-73.

4. McMillen IC, Robinson JS. Developmental origins of the metabolic syndrome: prediction, plasticity, and programming. *Physiol Rev.* 2005;85(2):571-633.

5. Heydeck D RJ, Roigas C, Papies B, Lun A. The catecholamine sensitivity of adult rats is enhanced after prenatal hypoxia. *Biol Neonate.* 1994;66(2-3):106-111.

6. Roigas J, Roigas C, Heydeck D, Papies B. Prenatal hypoxia alters the postnatal development of beta-adrenoceptors in the rat myocardium. *Biol Neonate.* 1996;69(6):383-388.

7. Butler TG, Schwartz J, McMillen IC. Differential effects of the early and late intrauterine environment on corticotrophic cell development. *J Clin Invest.* 2002;110(6):783-791.

8. Peyronnet J, Dalmaz Y, Ehrstrom M, Mamet J, Roux JC, Pequignot JM, Thoren HP, Lagercrantz H. Long-lasting adverse effects of prenatal hypoxia on developing autonomic nervous system and cardiovascular parameters in rats. *Pflugers Arch.* 2002;443(5-6):858-865.

9. Davis L RJ, Thornburg KL, Shokry M, Hohimer AR, Giraud GD. Augmentation of coronary conductance in adult sheep made anaemic during fetal life. *J Physiol.* 2003;547:53-59.

10. Li G XY, Estrella JL, Ducsay CA, Gilbert RD, Zhang L. Effect of fetal hypoxia on heart susceptibility to ischemia and reperfusion injury in the adult rat. *J Soc Gynecol Investig.* 2003;10(5):265-274.

11. Jones RD MA, Emery CJ. Effects of perinatal exposure to hypoxia upon the pulmonary circulation of the adult rat. *Physiol Res.* 2004;53(1):11-17.

12. Mone SM GM, Miller TL, Herman EH, Lipshultz SE. Effects of environmental exposures on the cardiovascular system: prenatal period through adolescence. *Pediatrics.* 2004;113(4 suppl):1058-1069.

المنارة للاستشارات
www.manaraa.com

13. Zhang L. Prenatal hypoxia and cardiac programming. *J Soc Gynecol Investig.* 2005;12(1):2-13.

14. Xue Q, Zhang L. Prenatal hypoxia causes a sex-dependent increase in heart susceptibility to ischemia and reperfusion injury in adult male offspring: Role of PKCε. *J Pharmacol Exp Ther.* 2009;330(2):624-632.

15. Xu Y, Williams SJ, O'Brien D, Davidge ST. Hypoxia or nutrient restriction during pregnancy in rats leads to progressive cardiac remodeling and impairs postischemic recovery in adult male offspring. *FASEB J.* 2006;20(8):1251-1253.

16. Chen L, Hahn H, Wu G, Chen CH, Liron T, Schechtman D, Cavallaro G, Banci L, Guo Y, Bolli R, Dorn GW, 2nd, Mochly-Rosen D. Opposing cardioprotective actions and parallel hypertrophic effects of delta PKC and epsilon PKC. *Proc Natl Acad Sci U S A.* 2001;98(20):11114-11119.**18.**

17. Ping P, Zhang J, Pierce WM, Jr., Bolli R. Functional proteomic analysis of protein kinase C epsilon signaling complexes in the normal heart and during cardioprotection. *Circ Res.* 2001;88(1):59-62.

18. Murriel CL, Mochly-Rosen D. Opposing roles of delta and epsilonPKC in cardiac ischemia and reperfusion: targeting the apoptotic machinery. *Arch Biochem Biophys.* 2003;420(2):246-254.

19. Gray MO, Zhou HZ, Schafhalter-Zoppoth I, Zhu P, Mochly-Rosen D, Messing RO. Preservation of base-line hemodynamic function and loss of inducible cardioprotection in adult mice lacking protein kinase C epsilon. *J Biol Chem.* 2004;279(5):3596-3604.

20. Jaenisch R BA. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nat Genet.* 2003;33:245-254.

21. Bogdarina I, Welham S, King PJ, Burns SP, Clark AJ. Epigenetic modification of the renin-angiotensin system in the fetal programming of hypertension. *Circ Res.* 2007;100(4):520-526.

22. Gluckman PD HM, Buklijas T, Low FM, Beedle AS. Epigenetic mechanisms that underpin metabolic and cardiovascular diseases. *Nat Rev Endocrinol.* 2009;5(7):401-408.

23. Jones PA, Takai D. The role of DNA methylation in mammalian epigenetics. *Science.* 2001;293(5532):1068-1070.

24. Meyer K, Zhang H, Zhang L. Direct effect of cocaine on epigenetic regulation of PKCε gene repression in the fetal rat heart. *J Mol Cell Cardiol.* 2009;47(4):504-511.

المنارة للاستشارات
www.manaraa.com

25. Zhang H, Meyer KD, Zhang L. Fetal exposure to cocaine causes programming of Prkce gene repression in the left ventricle of adult rat offspring. *Biol Reprod.* 2009;80(3):440-448.

26 Foldager CB, Munir S, Ulrik-Vinther M, Soballe K, Bunger C, Lind M. Validation of suitable house keeping genes for hypoxia-cultured human chondrocytes. *BMC Mol Biol.* 2009;10:94.

27. Inagaki K, Hahn HS, Dorn GW, 2nd, Mochly-Rosen D. Additive protection of the ischemic heart ex vivo by combined treatment with delta-protein kinase C inhibitor and epsilon-protein kinase C activator. *Circulation.* 2003;108(7):869-875.

28. Inagaki K, Begley R, Ikeno F, Mochly-Rosen D. Cardioprotection by epsilon-protein kinase C activation from ischemia: continuous delivery and antiarrhythmic effect of an epsilon-protein kinase C-activating peptide. *Circulation.* 2005;111(1):44-50.

29. Shinmura K, Nagai M, Tamaki K, Bolli R. Loss of ischaemic preconditioning in ovariectomized rat hearts: possible involvement of impaired protein kinase C epsilon phosphorylation. *Cardiovasc Res.* 2008;79(3):387-394.

30. Kabir AM, Clark JE, Tanno M, Cao X, Hothersall JS, Dashnyam S, Gorog DA, Bellahcene M, Shattock MJ, Marber MS. Cardioprotection initiated by reactive oxygen species is dependent on activation of PKCepsilon. *Am J Physiol Heart Circ Physiol.* 2006;291(4):H1893-H1899.

31. Meyer K, Zhang H, Zhang L. Prenatal cocaine exposure abolished ischemic preconditioning- induced protection in adult male rat hearts: role of PKCε. *Am J Physiol Heart Circ Physiol.* 2009;296:H1566-H1576.

32. Bae S, Xiao YH, Li G, Casiano C, Zhang L. Effect of maternal chronic hypoxic exposure during gestation on apoptosis in fetal rat heart. *Am J Physiol Heart Circ Physiol.* 2003;285:H983-H990.

33. Hescheler J, Meyer R, Plant S, Krautwurst D, Rosenthal W, Schultz G. Morphological, biochemical, and electrophysiological characterization of a clonal cell (H9c2) line from rat heart. *Circ Res.* 1991;69(6):1476-1486.

34. Hamid T, Gu Y, Ortines RV, Bhattacharya C, Wang G, Xuan YT, Prabhu SD. Divergent tumor necrosis factor receptor-related remodeling responses in heart failure: role of nuclear factor-kappaB and inflammatory activation. *Circulation.* 2009;119(10):1386-1397.

35. Pchejetski D, Kunduzova O, Dayon A, Calise D, Seguelas MH, Leducq N, Seif I, Parini A, Cuvillier O. Oxidative stress-dependent sphingosine kinase-1 inhibition mediates monoamine oxidase A-associated cardiac cell apoptosis. *Circ Res.*

المنارة للاستشارات
www.manaraa.com

2007;100(1):41-49.

36. Kaneda R, Ueno S, Yamashita Y, Choi YL, Koinuma K, Takada S, Wada T, Shimada K, Mano H. Genome-wide screening for target regions of histone deacetylases in cardiomyocytes. *Circ Res.* 2005;97(3):210-218.

37. Tang Y, Jackson M, Qian K, Phillips MI. Hypoxia inducible double plasmid system for myocardial ischemia gene therapy. *Hypertension.* 2002;39(2 Pt 2):695-698.

38. Mizukami Y, Ono K, Du CK, Aki T, Hatano N, Okamoto Y, Ikeda Y, Ito H, Hamano K, Morimoto S. Identification and physiological activity of survival factor released from cardiomyocytes during ischaemia and reperfusion. *Cardiovasc Res.* 2008;79(4):589-599.

39. Kimes BW, Brandt BL. Properties of a clonal muscle cell line from rat heart. *Exp Cell Res.* 1976;98(2):367-381.

40. Kim MH, Jung YS, Moon CH, Jeong EM, Lee SH, Baik EJ, Moon CK. Isoform-specific induction of PKC-epsilon by high glucose protects heart-derived H9c2 cells against hypoxic injury. *Biochem Biophys Res Commun.* 2003;309(1):1-6.

41. Kim MJ, Moon CH, Kim MY, Kim MH, Lee SH, Baik EJ, Jung YS. Role of PKC-delta during hypoxia in heart-derived H9c2 cells. *Jpn J Physiol.* 2004;54(4):405-414.

42. Bishai JM, Blood AB, Hunter CJ, Longo LD, Power GG. Fetal lamb cerebral blood flow (CBF) and oxygen tensions during hypoxia: a comparison of laser Doppler and microsphere measurements of CBF. *J Physiol.* 2003;546(Pt 3):869-878.

43. Uetani T, Nakayama H, Okayama H, Okura T, Higaki J, Inoue H, Higashiyama S. Insufficiency of pro-heparin-binding epidermal growth factor-like growth factor shedding enhances hypoxic cell death in H9c2 cardiomyoblasts via the activation of caspase-3 and c-Jun N-terminal kinase. *J Biol Chem.* 2009;284(18):12399-12409.

44. Shin EJ, Schram K, Zheng XL, Sweeney G. Leptin attenuates hypoxia/reoxygenation-induced activation of the intrinsic pathway of apoptosis in rat H9c2 cells. *J Cell Physiol.* 2009;221(2):490-497.

45. Compere SJ, Palmiter RD. DNA methylation controls the inducibility of the mouse metallothionein-I gene in lymphoid cells. *Cancer Cell*. 1980; 25: 233-40.

46. Chuang JC, Yoo CB, Kwan JM, Li TW, Liang G, Yang AS, Jones PA. Comparison of biological effects of non-nucleoside DNA methylation inhibitors versus 5-aza-2'-deoxycytidine. *Mol Cancer Ther.* 2005;4(10):1515-1520.

المنارة للاستشارات
www.manaraa.com

47. Nguyen CT WD, Velicescu M, Gonzales FA, Lin JC, Liang G, Jones PA. Histone H3-lysine 9 methylation is associated with aberrant gene silencing in cancer cells and is rapidly reversed by 5-aza-2'-deoxycytidine. *Cancer Res.* 2002;62(22):6456-6461.

48. Bender CM, Pao MM, Jones PA. Inhibition of DNA methylation by 5-aza-2'-deoxycytidine suppresses the growth of human tumor cell lines. *Cancer Res.* 1998;58(1):95-101.

49. Lee BH YS, Lin X, Nelson WG. Procainamide is a specific inhibitor of DNA methyltransferase 1. *J Biol Chem.* 2005;280(49):40749-40756.

50. Krishnan M, Park JM, Cao F, Wang D, Paulmurugan R, Tseng JR, Gonzalgo ML, Gambhir SS, Wu JC. Effects of epigenetic modulation on reporter gene expression: implications for stem cell imaging. *FASEB J.* 2006;20(1):106-108.

51. Bae S, Zhang L. Gender differences in cardioprotection against ischemia/reperfusion injury in adult rat hearts: focus on Akt and protein kinase C signaling. *J Pharmacol Exp Ther.* 2005;315(3):1125-1135.

52. Nilsson S, Mäkelä S, Treuter E, Tujague M, Thomsen J, Andersson G, Enmark E, Pettersson K, Warner M, Gustafsson JA. Mechanisms of estrogen action. *Physiol Rev*. 2001;81(4):1535-1565.

53. Lim SO, Gu JM, Kim MS, Kim HS, Park YN, Park CK, Cho JW, Park YM, Jung G. Epigenetic changes induced by reactive oxygen species in hepatocellular carcinoma: methylation of the E-cadherin promoter. *Gastroenterology.* 2008;135(6):2128-2140, 2140 e2121-2128.

54. Cannito S, Novo E, Compagnone A, Valfre di Bonzo L, Busletta C, Zamara E, Paternostro C, Povero D, Bandino A, Bozzo F, Cravanzola C, Bravoco V, Colombatto S, Parola M. Redox mechanisms switch on hypoxia-dependent epithelial-mesenchymal transition in cancer cells. *Carcinogenesis.* 2008;29(12):2267-2278.

55. Chandel NS, Maltepe E, Goldwasser E, Mathieu CE, Simon MC, Schumacker PT. Mitochondrial reactive oxygen species trigger hypoxia-induced transcription. *Proc Natl Acad Sci U S A.* 1998;95(20):11715-11720.

56. Mansfield KD, Simon MC, Keith B. Hypoxic reduction in cellular glutathione levels requires mitochondrial reactive oxygen species. *J Appl Physiol.* 2004;97(4):1358-1366.

57. Fan L, Sawbridge D, George V, Teng L, Bailey A, Kitchen I, Li JM. Chronic cocaine-induced cardiac oxidative stress and mitogen-activated protein kinase activation: the role of Nox2 oxidase. *J Pharmacol Exp Ther.* 2009;328(1):99-106.

المنارة للاستشارات
www.manaraa.com

58. Isabelle M, Vergeade A, Moritz F, Dautreaux B, Henry JP, Lallemand F, Richard V, Mulder P, Thuillez C, Monteil C. NADPH oxidase inhibition prevents cocaine-induced up-regulation of xanthine oxidoreductase and cardiac dysfunction. *J Mol Cell Cardiol.* 2007;42(2):326-332.

59. Moritz F, Monteil C, Isabelle M, Bauer F, Renet S, Mulder P, Richard V, Thuillez C. Role of reactive oxygen species in cocaine-induced cardiac dysfunction. *Cardiovasc Res.* 2003;59(4):834-843.

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CHAPTER 3

HYPOXIA-DERIVED OXIDATIVE STRESS MEDIATES EPIGENETIC REPRESSION OF PKCE GENE IN FETAL RAT HEARTS

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Abstract

Aims: Fetal hypoxia causes promoter methylation and gene repression of protein kinase C epsilon (PKCε) in the developing heart resulting in the heightened cardiac susceptibility to ischemia and reperfusion injury in offspring. The present study tested the hypothesis that HIF-1 and/or hypoxia-derived reactive oxygen species (ROS) mediate the hypoxia-induced PKCε gene repression.

Methods and Results: Hypoxic treatment of pregnant rats from day 15 to 21 of gestation resulted in significant decreases in PKCε protein and mRNA in fetal hearts. Similar results were obtained in *ex vivo* hypoxic treatment of isolated fetal hearts and rat embryonic ventricular myocyte cell line H9c2. Increased methylation of PKCε promoter at the SP1 binding sites, -346 and -268, were demonstrated in both fetal hearts of maternal hypoxia and H9c2 cells treated with 1% O_2 for 24 h. In H9c2 cells, hypoxia caused a significant nuclear accumulation of HIF-1α. HIF-1α inhibitors, YC-1 and 2-methoxy estradiol, decreased the hypoxia-mediated HIF-1α nuclear accumulation, but had no effect on the hypoxia-induced PKCε mRNA repression. Hypoxia produced a time-dependent increase in the ROS production in H9c2 cells, which was blocked by ROS scavengers N-acetylcysteine or 4-hydoxy tempo. In accordance, N-acetylcysteine and 4-hydoxy tempo, but not apocynin, inhibited the hypoxic effect and restored PKCε protein and mRNA expressions to the control values in the fetal heart and H9c2 cells. The ROS scavengers blocked hypoxia-induced CpG methylation of the SP1 binding sites and restored the SP1 binding to the PKCε promoter to the control levels.

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Conclusion: The results demonstrate that hypoxia induces epigenetic repression of the PKCε gene through a NADPH oxidase-independent ROS-mediated pathway in the fetal heart.

Keywords: PKCε, HIF-1α, ROS, DNA methylation, H9c2

Introduction

Epidemiological studies have demonstrated a clear association between an adverse intrauterine environment and an increased risk of heart disease later in life [1-4]. Hypoxia is a common form of intrauterine stress. Animal studies have suggested a possible link between chronic maternal hypoxia and increased risk of cardiovascular disease in offspring [3-6]. Recent studies in rats have demonstrated that maternal hypoxia results in the heightened cardiac vulnerability to ischemia and reperfusion injury in offspring in a sex-dependent manner [6]. Additionally, it has been demonstrated that the downregulation of protein kinase C epsilon (PKCε) gene expression in the hearts of adult offspring is a mechanism for the increased heart susceptibility to ischemia and reperfusion injury in the animals exposed to hypoxia before birth [6,7]. PKCε plays an important role in cardioprotection against ischemic injury [8-10]. Further investigation has revealed that hypoxia has a direct effect on epigenetic repression of the PKCε gene in fetal hearts through DNA methylation of the PKCε promoter at the SP1 binding sites [7]. However, the mechanisms underlying hypoxia-mediated methylation of the PKCε promoter has yet to be elucidated.

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One of the most important mediators for the hypoxic effect is hypoxia inducible factor 1 (HIF-1) that regulates many genes involved in external and internal adaptation to hypoxic stress. HIF-1 is composed of two subunits, HIF-1β (constitutively active) and HIF-1α (regulatory subunit). Although recent studies have implicated several mechanisms for HIF-1α regulation, oxygen availability is classically described [11]. Under normoxic conditions, proline hydroxylases (PHDs) add hydoxyl groups to proline residues 402 and 564, which afford the recruitment and binding of von Hippel-Lindau protein (E3 Ligase). This event then primes HIF-1α for proteasomal degradation. Additionally, factor inhibiting HIF (FIH) regulates the transcriptional activity of HIF-1 [12]. In normoxia, FIH hydroxylates arginine 803, preventing the association of HIF-1 and its binding partner CREB-binding protein (CBP)/p300 and thereby inhibits transcriptional activity. Under hypoxic conditions, HIF-1α subunit stabilizes and translocates to the nucleus where it dimerizes with HIF-1β. HIF-1 then interacts with CBP/p300 and influences gene transcription through either direct binding to hypoxic response elements (HRE), or indirect modulation of the activity or expression of other transcription factors [13-15].

Another key mediator in the hypoxic effect is reactive oxygen species (ROS). Under hypoxic conditions, intracellular ROS paradoxically increases [16]. The main site for ROS production is the electron transport system (ETS) located in the inner membrane of the mitochondria. Uncoupling of ETS caused by hypoxia slows the electron flow through the ETS thereby increasing the probability of molecular oxygen interacting with free radicals (*i.e.*, semiubiquione) to produce superoxide ion [16,17]. Cardiomyocytes are major producers of ROS due to their high metabolic demand. Increased ROS can

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significantly alter gene expression patterns through the induction of the integrated stress response that involves PERK activation, eIFα phosphorylation and ATF4-mediated stress gene induction [18]. Recent studies have suggested a link between prolonged oxidative stress and aberrant DNA methylation patterns [19-21].

Since HIF-1 activity is central to cellular adaptation to hypoxic stress and hypoxia-derived ROS is known to influence gene expression, it is plausible that HIF-1 and/or ROS may mediate the hypoxia-induced epigenetic repression of PKCε gene expression in fetal hearts. The present study tests this hypothesis in fetal rat hearts and rat embryonic ventricular cell line H9c2 that has been widely used in a variety of myocardiocyte studies. Our recent study has demonstrated a congruent underlying mechanism in fetal hearts and H9c2 cells in the epigenetic regulation of PKCε gene repression [7]. Herein, we present evidence that blockade of hypoxia-derived ROS, but not HIF-1, inhibits the hypoxia-induced increase in methylation of the SP1 binding sites, reverses the decreased SP1 binding to the PKCε promoter, and restores PKCε mRNA and protein abundance to the control levels.

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% oxygen from gestational day 15 to day 21, as described previously (7). To examine the effect of antioxidant, the rats were treated in the absence or presence of N-acetyl-cysteine (NAC, Sigma) in the drinking water (500

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mg/kg/day). Hearts were isolated from near-term (21 d) fetuses. 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 (7). Hearts were given 24 h of recovery time before being placed in a hypoxic chamber with 1% O_2 for 48 h in the absence or presence of NAC (1 mM). All procedures and protocols were approved by the Institutional Animal Care and Use Committee guidelines, and followed the guidelines by US National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Cell Culture

H9c2 cells were obtained from ATCC (Rockville, MD, USA). Cells were maintained in DMEM and supplemented with 10% FBS and 1% penicillin/streptomycin at 37 °C in 95% air/5% CO_2. Cells were grown and sub-cultured in 6-well plates with experiments performed between 70-80% confluent. For the hypoxic treatment, cells were transferred to the hypoxic chamber and maintained at 1% O_2 for 24 h, as previous described (7).

Western Blot Analysis

Western blots were performed as previously described [7,14,22]. Briefly, hearts or H9c2 cells were homogenized in a lysis buffer containing 150 mM NaCl, 50 mM Tris.HCl, 10 mM EDTA, 0.1% Tween-20, 0.1% β-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, and 5 μg/ml aprotinin, pH 7.4 and allowed to incubate for 1 h on ice. Homogenates were then centrifuged at 4°C for 20 min at 10,000 g, and supernatants collected. Nuclear extracts were prepared using the

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NXTRACT CelLytic Nuclear Extraction Kit (Sigma) with few modifications. Hypotonic buffers were supplemented with 1 μM lactacystin and 1 mM EDTA. 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 min. Proteins were then transferred onto nitrocellulose membranes. Nonspecific binding was blocked for 1 h at room temperature in the blocking buffer. The membranes were then probed overnight with primary antibodies against PKCε (Santa Cruze Biotechnology; Santa Cruz, CA) and HIF-1α (BD Biosciences), as described previously [23]. β-actin antibody (Sigma) was used to normalize loading. After washing, membranes were incubated with IRDye® secondary antibodies (LI-COR biosciences). Proteins were visualized and analyzed with the Odyssey imagine system.

Real-Time RT-PCR

RNA was extracted from hearts or H9c2 cells using TRIzol protocol (Invitrogen, Carlsbad, USA). PKCε mRNA abundance was determined by real-time RT-PCR using Icycler Thermal cycler (Bio-Rad), as described previously [7]. The primers for PKCε are 5'-GCGAAGCCCCTAAGACAAT-3' (forward) and 5'-CACCCCAGATGAAATCCCTAC-3' (reverse). Real-time RT-PCR was performed in a final volume of 25 μl. Each PCR reaction mixture consisted of 600 nM of primers, 33 units of M-MLV reverse transcriptase (Promega, Madison, WI), and iQ SYBR Green Supermix (Bio-Rad) containing 0.625 unit Taq polymerase, 400 μM each of dATP, dCTP, dGTP, and dTTP, 100 mM KCl, 16.6 mM ammonium sulfate, 40 mM Tris-HCl, 6 mM $MgSO_4$, SYBR Green I, 20 nM fluorescing and stabilizers. The following RT-PCR

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protocol was used: 42 °C for 30 min, 95 °C for 15 min, followed by 40 cycles of 95 °C for 15 s, 59 °C for 20 s, 72 °C for 10 s. 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.

Quantitative Methylation-Specific PCR

DNA was isolated from hearts or H9c2 cells using a GenElute Mammalian Genomic DNA Mini-Prep kit (Sigma), denatured with 2 N NaOH at 42 °C for 15 min, and treated with sodium bisulfite at 55 °C for 16 h, as previously described [7,27]. DNA was purified with a Wizard DNA clean up system (Promega) and resuspended in 100 µl of TE buffer. Bisulfite-treated DNA was used as a template for real-time fluorogenic methylation-specific PCR (MSP) using primers created to amplify promoter binding sites containing methylation sites based on the previous sequencing of rat PKCε promoter [23]. GAPDH was used as an internal reference gene. Real-time MSP was performed using the iQ SYBR Green Supermix with iCycler real-time PCR system (Bio-Rad).

Measurement of Intracellular ROS

The fluorescent indicator 2’7’-dichlorofluorescein diacetate was used to measure intracellular ROS in H9c2 cells. 2’7’-Dichlorofluorescein diacetate enters cells where it is de-esterified and converted to the highly fluorescent 2′,7′-dichlorofluorescein (DCF) upon oxidation. Cells were subcultured in black-wall, clear-bottom 96 well plates and allowed to grow to 50-70% confluence. Cells were then exposed to hypoxic treatment at various time points. After the treatment, cells were washed twice with PBS and then

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loaded with 5 μM of 2'7'-dichlorofluorescein diacetate in serum free DMEM and placed in a 37 °C incubator for 30 min. Cells were removed from the incubator and washed twice with PBS. Fluorescence (excitation wavelength at 495 nm and emission wavelength at 515 nm) was measured using a Synergy HT Multi-Mode Microplate Reader (Winooski, VT). All experiments were done with minimal exposure to light and fluorescence was normalized to cell count.

Chromatin Immunoprecipitation (ChIP)

Chromatin extracts were prepared from H9c2 cells. ChIP assays were performed using the ChIP-IT™ Express Chromatin Immunoprecipitation Kit (Active Motif, Carlsbad, CA), as previously described [7,22]. Briefly, cells were exposed to 1% formaldehyde for 10 min to crosslink and maintain DNA/protein interactions. After the reactions were stopped with glycine, cells were washed, chromatin isolated and the DNA sheared into medium fragments (200–1000 base pairs) with 7 pulses at ¼ power using a sonicator. ChIP reactions were performed using a SP1 antibody (Active Motif) to precipitate the transcription factor/DNA complex. Crosslinking was then reversed using a salt solution and proteins digested with proteinase K. Two sets of primers flanking the two SP1 binding sites at -346 and -268 were used: 5′-accatttcctctcgacatgc-3′ (forward) and 5′-agatttcaacccggatcctc-3′ (reverse); 5′-agaggatccgggttgaaatc-3′ (forward) and 5′-ctcacctacctttccgaaaca-3′ (reverse). PCR amplification products were visualized on 1.5% agarose gel stained with ethidium bromide. To quantify PCR amplification, 45 cycles of real-time PCR were carried out with 3 min initial denaturation followed by 95 °C for 30 s, 51 °C for 30 s, and 72 °C for 30 s, using the iQ SYBR Green Supermix with iCycler real-time PCR system (Bio-Rad).

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Statistical Analysis

Data are expressed as mean ± SEM. Experimental number (n) represents the hearts of fetuses from different dams. 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

The Effect of HIF-1α Inhibitors on Hypoxia-Induced Decrease of PKCε Expression

To assess the role of HIF-1α in hypoxia-induced decrease of PKCε expression, YC-1 and 2-methoxyestradiol (2ME) were used to block HIF-1α nuclear accumulation. YC-1 blocks HIF-1α protein by enhancing degradation [24], and 2ME blocks HIF-1α through an oxygen and proteasome independent pathway that involves disruption of mircotubules [25]. H9c2 cells were treated with 1% O_2 for 24 h in the absence or presences of YC-1 (10 or 100 μM) or 2ME (10 or 100 μM). Nuclear extracts were collected for determining HIF-1α nuclear accumulation. Figure 1A shows that HIF-1α protein accumulated in the nuclear compartment under the hypoxic treatment. The addition of YC-1 or 2ME significantly reduced HIF-1α nuclear accumulation (Figure 1A). However, neither YC-1 nor 2ME had significant effects on the hypoxia-induced decrease in PKCε mRNA expression (Figure 1B), suggesting the minimum role of HIF-1α in regulating PKCε gene transcription under hypoxic conditions.

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The Effect of ROS Scavengers on Hypoxia-Induced Decrease of PKCε Expression

To determine the role of ROS in hypoxia-induced decrease of PKCε expression, the ROS scavengers, N-acetyl-cysteine (NAC) and tempol were used to reduce intracellular ROS. H9c2 cells were treated with 1% O_2 for 24 h in the absence or presence of NAC (0.5, 0.75, 1 mM), tempol (1, 2.5, 5 mM). Additionally, apocynin (0.5 mM) was used to determine the role of NADPH oxidase in the hypoxic effect. Hypoxia significantly decreased PKCε protein and mRNA abundance (Figure 2A, B). NAC produced a dose-dependent inhibition of the hypoxic effect on PKCε mRNA repression (Figure 2A). Similar findings of blockade of the hypoxic effect were obtained with tempol (Figure 2A). In contrast, apocynin had no significant effect on the hypoxia-mediated downregulation of PKCε expression (Figure 2A). Consistent with the results of mRNA, both NAC and tempol blocked the hypoxia-induced reduction of PKCε protein expression in H9c2 cells (Figure 2B). In agreement with the findings in H9c2 cells, maternal hypoxia-mediated downregulation of PKCε mRNA and protein expressions in the fetal heart were blocked by NAC (Figure 3A). The similar results were obtained in isolated fetal hearts treated *ex vivo* with 1% O_2, showing the reversal of hypoxia-induced downregulation of PKCε gene expression by NAC (Figure 3B).

Hypoxia Increased ROS Production in H9c2 cells

To determine whether hypoxia significantly alters ROS production in H9c2 cells, we performed a time course experiment using 2’7’-dichlorofluorescein diacetate to measure intracellular ROS production. H9c2 cells were treated with 1% O_2 for 2, 4, 8, 16, and 24 h. Fluorescence of DCF was measured using a microplate reader and normalized

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to cell count. As shown in Figure 4A, ROS levels were significantly elevated at the 2 h treatment. At the 4 h time point, ROS levels peaked and gradually declined afterward until the 16 h mark when it continued to increase again (Figure 4A). We further assessed the effect of NAC or tempol on hypoxia-induced ROS production at the 4 h time point. As shown in Figure 4B, in the presence of NAC or tempol, the hypoxia-induced ROS production was blocked.

ROS Scavengers Abolished Hypoxia-Induced Methylation at the PKCε Promoter

Previous studies have demonstrated prolonged hypoxia treatment significantly increases methylation of the SP1 binding sites -346 and -268 at the PKCε promoter in H9c2 cells [7]. We therefore determined whether the inhibition of ROS significantly altered the methylation status of the SP1 binding sites at the PKCε promoter. H9c2 cells were treated with 1% O_2 for 24 h in the absence or presence of NAC or tempol. Genomic DNA was isolated and methylation-specific PCR was performed using primers that had been previously designed for the SP1 sites -346 and -268 [7,23]. Consistent with the previous findings, hypoxia significantly increased the methylation status of the both SP1 binding sites in the absence of ROS scavengers (Figure 5A). NAC and tempol blocked the hypoxia-induced increase in CpG methylation of the both SP1 binding sites -346 and -268 at the PKCε promoter (Figure 5A). In agreement with the findings in H9c2 cells, the maternal hypoxic treatment revealed a similar pattern of increased methylation of the two SP1 binding sites, -346 and -268, at the PKCε promoter in the fetal hearts, which was inhibited by NAC (Figure 5B).

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ROS Scavengers Restored SP1 Binding to the PKCε Promoter

Previous studies have demonstrated that methylation of the SP1 binding sites -346 and -268 decreases SP1 binding to the PKCε promoter resulting in the reduced transcription activity [7,22]. To evaluate further whether the inhibition of ROS restored the binding of SP1 protein to the SP1 binding sites at the proximal PKCε promoter in the context of intact chromatin, ChIP assays were performed using the SP1 antibody. Figure 6 shows the binding of SP1 to both SP1 elements at −346 and −268 at the PKCε promoter in intact chromatin in H9c2 cells. Hypoxia significantly decreased SP1 binding to the both SP1 binding sites (Figure 6). In the presence of NAC or tempol, the SP1 binding was restored to the control values for the both SP1 binding sites (Figure 6).

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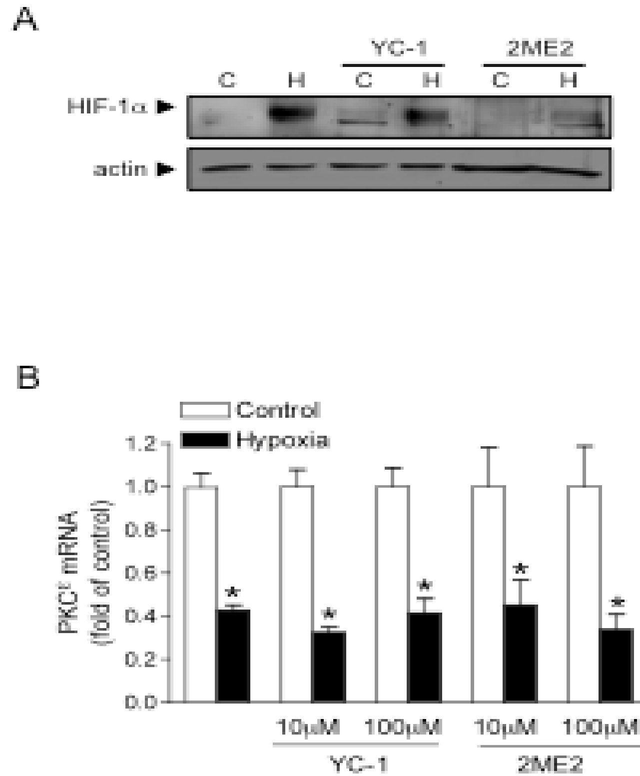

Figure 10. The effect of HIF-1α inhibitors on PKCε expression. **A,** H9c2 cells were treated with 21% O_2 (control, C) or 1% O_2 (hypoxia, H) in the absence or presence of YC-1 (100 μM) or 2-ME2 (100 μM) for 24 h. HIF-1α protein in the nuclear extracts was measured by Western blots. **B,** H9c2 cells were treated with 21% O_2 (Control) or 1% O_2 (Hypoxia) in the absence or presence of YC-1 (10, 100 μM) or 2-ME2 (10, 100μM) for 24 h. PKCε mRNA abundance was determined by real-time RT-PCR. Data are means ± SEM. * $P < 0.05$, hypoxia *vs*. control. n = 10-

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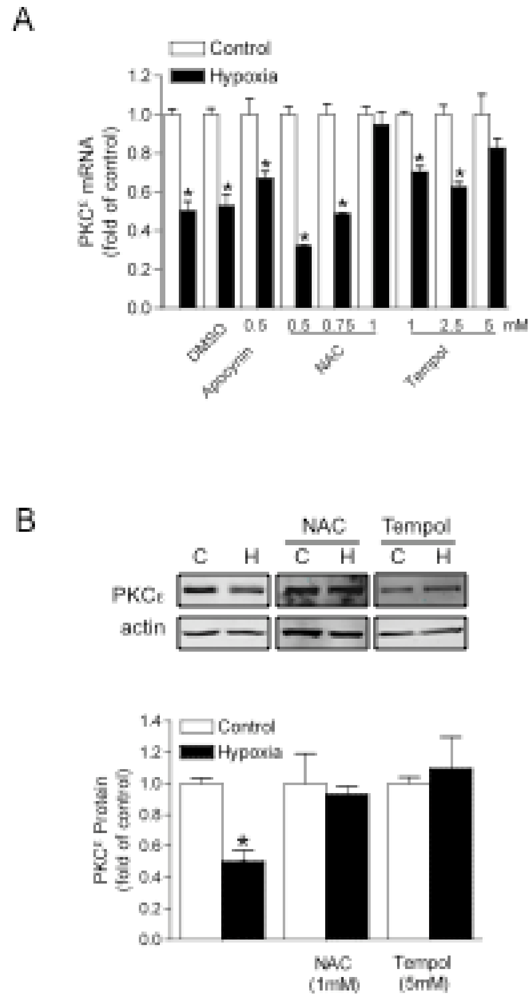

Figure 11. The effect of ROS scavengers on PKCε expression. **A,** H9c2 cells were treated with 21% O_2 (control) or 1% O_2 (hypoxia) in the absence or presence of apocynin, NAC or tempol for 24 h. PKCε mRNA abundance was measured by real-time RT-PCR. Data are means ± SEM. * $P < 0.05$, hypoxia *vs*. control. n = 5 **B,** H9c2 cells were treated with 21% O_2 (control, C) or 1% O_2 (hypoxia, H) in the absence or presence of NAC or tempol for 24 h. PKCε protein abundance was determined by Western blots. Data are means ± SEM. * $P < 0.05$, hypoxia *vs*. control. n = 4-9

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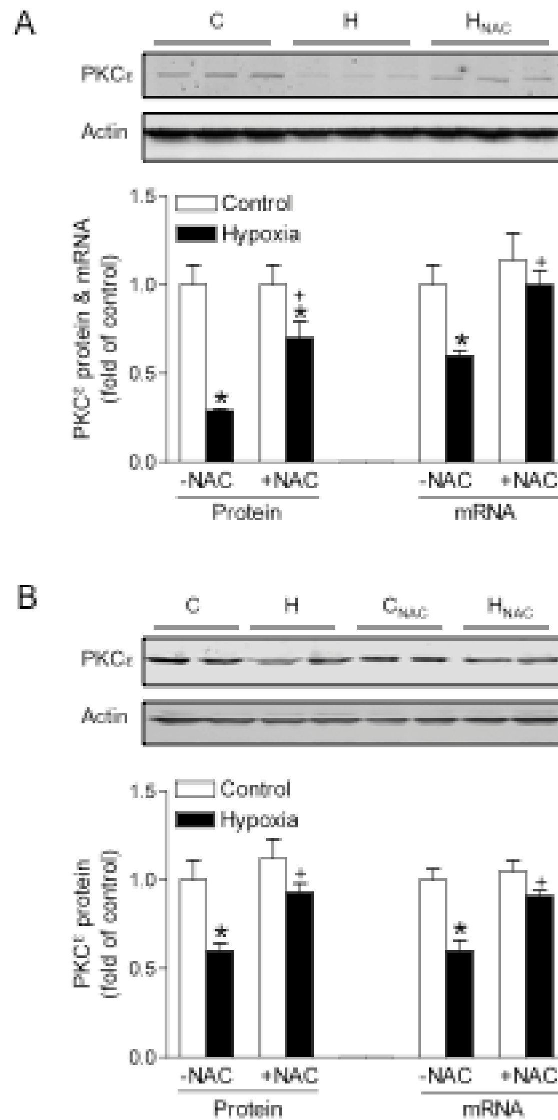

Figure 12. The effect of NAC on PKCε expression in fetal hearts. A, Hearts were isolated from near-term fetuses of pregnant rats treated with control (C) and hypoxia (H) in the absence or presence of NAC. **B**, Isolated fetal hearts were treated *ex vivo* with control (C) and hypoxia (H) in the absence or presence of NAC. PKCε protein abundance was determined by Western blots, and mRNA abundance was determined by real-time RT-PCR. Data are means ± SEM. * $P < 0.05$, hypoxia *vs*. control; † $P < 0.05$, +NAC *vs*. –NAC. n =

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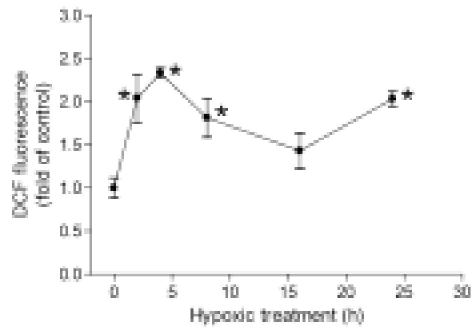

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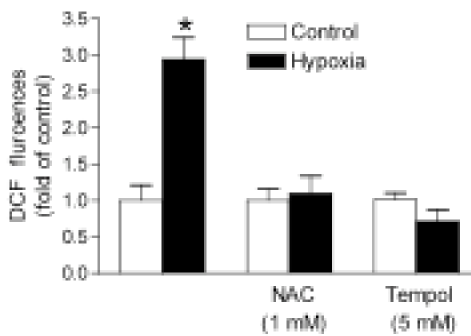

Figure 13. **Measurement of intracellular ROS in H9c2 cells**. **A,** H9c2 cells were treated with 21% O_2 (control) or 1% O_2 (hypoxia) for 0, 2, 4, 8, 16, 24 h. Cells were then loaded with 5 μM of 2'7' dichlorofluorescein diacetate for 30 min and fluorescence measured with a microplate reader. Data are means ± SEM. * $P < 0.05$, hypoxia *vs*. control. n = 4-8 **B.** H9c2 cells were treated with 21% O_2 (control) and 1% O_2 (hypoxia) in the absence or presence of NAC or tempol for 4 h. Cells were then loaded with 5 μM of 2'7' dichlorofluorescein diacetate for 30 min and fluorescence measured with a microplate reader. Data are means ± SEM. * $P < 0.05$, hypoxia *vs*. control. n = 5-6

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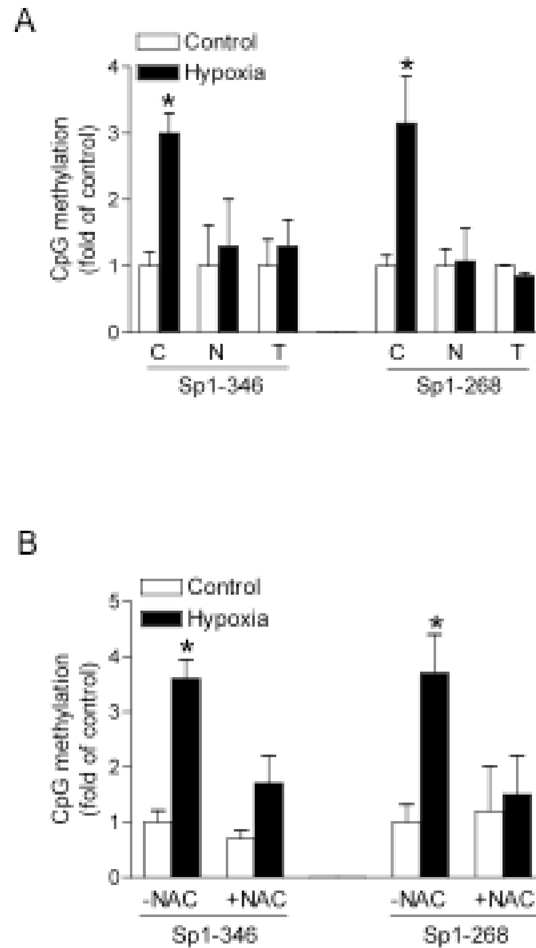

Figure 14. The effect of ROS scavengers on PKCε promoter methylation. **A**, H9c2 cells were treated with 21% O_2 (control) or 1% O_2 (hypoxia) in the absence (C) or presence of NAC (N) or tempol (T) for 24 h. **B**, Hearts were isolated from near-term fetuses of pregnant rats treated with control and hypoxia in the absence or presence of NAC. Methylation of the SP1 binding sites at -346 and -268 was determined by methylation specific PCR. Data are means ± SEM. * $P < 0.05$, hypoxia *vs*. control. n = 4-

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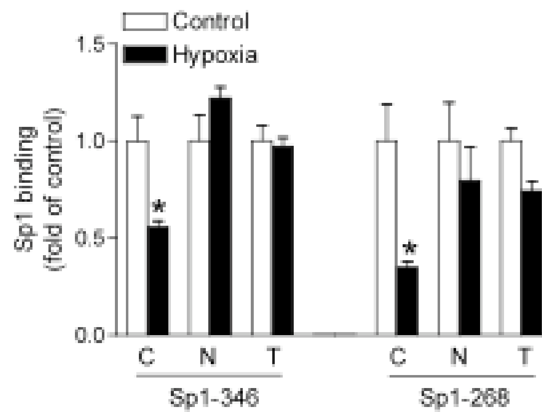

Figure 15. The effect of ROS scavengers on SP1 binding to the PKCε promoter. H9c2 cells were treated with 21% O_2 (control) or 1% O_2 (hypoxia) in the absence (C) or presence of NAC (N) or tempol (T) for 24 h. SP1 binding to the PKCε promoter at -346 and -268 in the context of intact chromatin was determined by ChIP assays. Data are means ± SEM. * $P < 0.05$, hypoxia *vs*. control. n = 4-9

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Discussion

The present study presents evidence for the first time that prolonged hypoxia mediates epigenetic repression of the PKCε gene in the fetal heart through a ROS, but not HIF-1α, dependent pathway. The studies demonstrate that the attenuation of hypoxia-derived ROS restores PKCε protein and mRNA expressions by inhibiting CpG methylation of the SP1 binding sites and restoring SP1 binding to the PKCε promoter. In contrast, the inhibition of HIF-1α did not affect the hypoxic effect on repression of the PKCε gene.

The present study builds upon the recent work that demonstrated the heightened cardiac susceptibility to ischemia and reperfusion injury in offspring that had experienced prolonged hypoxia before birth [5,6]. Interestingly, the ventricles of these offspring showed significantly less PKCε abundance compared to the control animals [6]. Previous studies have shown that acute exposure to hypoxia increases the activity of PKCε in the adult heart [26]. We have demonstrated that chronic gestational hypoxia decreases the expression of PKCε in the fetal heart, suggesting that prolonged hypoxia *in utero* suppresses PKCε gene activity [7]. Further investigation has revealed that chronic hypoxia directly regulates PKCε gene expression through increased methylation of two SP1 binding sites at the PKCε promoter [7]. This pattern of increased promoter methylation was present from the fetal heart and persists into the adulthood [6,7]. Consistent with the increased methylation, SP1 binding to the PKCε promoter in the context of intact chromatin was significantly decreased [7,22,23]. Site-directed methylation of PKCε promoter-luciferase constructs for both SP1 sites, but not either site alone, caused a significant decrease in the promoter activity in H9c2 cells, demonstrating

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an important epigenetic mechanism involving the two SP1 binding sites in regulating PKCε gene transcription activity [7,22]. Furthermore, the causal role of DNA methylation in the hypoxia-induced PKCε gene repression was demonstrated using a methylation inhibitor, 5-aza-2-deoxycytdine that blocked the hypoxic effect on the downregulation of PKCε gene expression, thereby restoring PKCε protein and mRNA to the control values [7].

The present findings add new insights into the hypoxia-mediated regulation of PKCε expression in cardiomyocytes and demonstrate that hypoxia-derived ROS mediates the epigenetic repression of PKCε gene in the fetal heart. The finding that NAC and tempol, but not apocynin, blocked the hypoxic effect on the PKCε repression is intriguing and suggests a role of NADPH oxidase-independent ROS in the hypoxia-mediated effect in the fetal heart. While NADPH oxidase has been shown to play a role in regulating the ROS production under chronic hypoxic conditions in some cell types, particularly in the pulmonary vasculature and carotid body, its involvement in hypoxia-mediated ROS production and hypoxia-related gene regulation appears to be tissue and organ selective. Consistent with the finding of minimum role of NADPH oxidase in the hypoxia-mediated effect in the present study, previous studies in guinea pig ventricular myocytes demonstrated that NADPH oxidase did not appear to contribute substantially in the hypoxia-induced ROS production and myocyte dysfunction [27]. The present findings that the ROS scavengers NAC and tempol inhibited hypoxia-mediated methylation of the SP1 binding sites and restored SP1 binding to the PKCε promoter indicates that hypoxia-derived ROS plays a vital role in causing DNA methylation of the PKCε promoter in the fetal heart. Similar findings showed that NAC significantly reduced global DNA

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methylation during anchorage blockade in murine nontumorigenic melanocyte, supporting the notion that ROS plays an important role in regulating DNA methylation [28]. Consistent with these findings, previous studies demonstrated that prolonged exposure to ROS caused significant hypermethylation of the E-cadherin promoter [21]. ROS-mediated methylation of E-cadherin promoter involved up-regulation of Snail, which recruited epigenetic effectors (*i.e.* DNA methyltransferase 1) to suppress gene transcription. Interestingly, Snail overexpression alone was sufficient to induce hypermethylation of E-cadherin promoter, suggesting Snail regulation was a key factor in mediating epigenetic modification of gene promoters [21]. Determining whether Snail activity is important in hypoxia-induced heightened methylation of CpG dinucleotides at transcription factor binding sites for the PKCε promoter deserves further investigation. Furthermore, understanding whether the mechanism by which hypoxia through ROS mediates methylation of the SP1 binding sites is a broad event (occurring in many genes) or selective (occurring in a few genes) warrants future research.

Previous studies in myocardial, non-myocardial tissues and H9c2 cell line have found that hypoxia increases ROS [18,29,30]. Consistent with these findings, we found that hypoxia significantly increased ROS in H9c2 cells using 2'7'-dichlorofluorescein diacetate. Time course studies revealed a biphasic production of ROS in H9c2 cells with an initial peak at 4 h treatment, which declined to the 16 h mark, then was significantly increased by 24 h. Time course studies by Chen et al. [31] showed a similar biphasic elevation of ROS in human embryonic kidney and glioma cell lines treated with mitochondria complex inhibitor I rotenone or mitochondraia complex II inhibitor TTFA. As shown in the present study, the prolonged hypoxia treatment for 24 h maintained

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significantly higher levels of ROS and produced heightened and prolonged oxidative stress. Although acute exposure to ROS increases the activity of PKCε and promotes a cardioprotective phenotype often observed in acute ischemia and reperfusion setting in the adult heart [26], the present study demonstrates that prolonged hypoxia cause a sustained increase in ROS that results in the downregulation of PKCε gene expression in H9c2 cells. These findings suggest differential regulations of the PKCε activity and gene expression in response to acute or chronic hypoxia. This difference may represent a negative feedback loop where short-term hypoxia significantly enhances the PKCε activity to promote a cardioprotective phenotype, while long-term exposure to hypoxia-derived ROS promotes adaptive changes that include the downregulation of PKCε gene expression.

The present study found that H9c2 cells exposed to 1% O_2 for 24 h resulted in significant nuclear accumulation of HIF-1α that is a marker of hypoxia. This is consistent with the previous study showing that maternal hypoxia increased HIF-1α protein levels in the fetal heart [32], indicating tissue hypoxia of the fetal heart in response to maternal hypoxia. Other studies have found that similar oxygen levels and exposure are sufficient to induce HIF-1α stabilization and nuclear accumulation [33]. YC-1 and 2-ME have been widely used to inhibit HIF-1α nuclear accumulation. Previous studies suggested that YC-1 inhibited HIF-1α protein by enhancing its degradation through FIH-dependent COOH-terminal transactivation domain (CAD) inactivation [24]. 2-ME inhibits HIF-1α independent of oxygen and proteasome pathways by disrupting microtubules preventing the translocation of HIF-1α into the nuclear compartment, thus preventing the HIF-1 activity [25]. Interestingly, both YC-1 and 2-ME have been shown to block HIF-2α

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nuclear accumulation as well, thereby inhibiting the HIF-2 activity. Although HIF-2α is not the focus of this study, the use of YC-1 and 2-ME may also provide clues as to the role of HIF-2α in hypoxia-induced PKCε gene repression.

Little is known concerning the role of HIF-1α in the methylation of specific gene promoters [34]. HIF-1α regulates the expression of epigenetic effectors, namely histone deacetylases and demethylase (JMJD1A) [35], but it is unclear whether HIF-1α directly or indirectly regulates DNA methyltransferase (DNMT). Previous studies have shown that HIF-1 regulates the c-myc activity [14]. C-myc has been shown to recruit DNA methyltransferase resulting in promoter hypermethylation for some genes [36], suggesting a possible mechanism whereby HIF-1 may influence methylation of promoter regions. Interestingly, Watson et al. reported that chronic hypoxia increased global methylation patterns and expression of DNA methyltransferase 3b in prostate cell lines absent of HIF-1α protein expression [37], suggesting chronic hypoxia can influence DNA methylation independent of HIF-1. In the present study, we found that the inhibition of HIF-1α with YC-1 or 2-ME had no significant effect on hypoxia-induced repression of PKCε mRNA, suggesting that HIF-1α does not play a significant role in altering PKCε promoter methylation. Importantly, it has been demonstrated that hypoxia induces the stabilization of HIF-1α protein through alterations in the redox state. The mechanism is thought to be through mitochondrial derived ROS from complex III that regulate the prolyl hydroxylases activity [20,38]. Other studies contend that oxygen availability instead of ROS production is the main stimulus altering prolyl hydroxylase activity and therefore HIF-1α stabilization [39]. In the present study, we demonstrate that attenuation

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of ROS, but not HIF-1α, plays a major role in hypoxia-induced reduction of PKCε expression.

A model used in the present study was the embryonic rat ventricular myoctye cell line H9c2. The H9c2 cell is a widely used system for studying cardiomyocytes, including cell death, differentiation and fetal programming [7,29,32]. Electrophysically, H9c2 cells are similar to primary cardiomyocytes, but differ phenotypically [40,41]. Although differences exist, recent studies using the H9c2 cell line to study the effects of hypoxia on PKCε abundance have found consistent results with freshly isolated fetal cardiomyocytes and intact hearts [7]. Thus, H9c2 cells exposed to 1% O_2 for 24 hours displayed a similar pattern of decreased PKCε protein and mRNA as those seen in freshly isolated fetal rat cardiomyocytes and intact hearts exposed to 1% O_2 [7]. Furthermore, both models found increased methylation of CpG dinucleotides at SP1 binding sites at the PKCε promoter. This suggests the underlying mechanism for hypoxia-induced decrease in PKCε gene expression is similar in both the freshly isolated fetal rat hearts and H9c2 cell line. Other studies also demonstrated that prolonged hypoxia in the presence of low or high glucose significantly decreased PKCε protein abundance in H9c2 cells [42,43]. Consistent with these findings, the present study demonstrates a congruent underlying mechanism of the heightened ROS in hypoxia-mediated PKCε gene repression in fetal hearts and H9c2 cells, supporting the use of the H9c2 cells in investigating the epigenetic mechanisms of PKCε gene expression patterns.

In summary, the present study identifies a novel mechanism of hypoxia-derived ROS in inducing CpG methylation of sequence specific transcription factor binding sites at the PKCε promoter and its gene repression in the fetal heart. Although it is difficult to

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translate directly these findings into humans, linking chronic exposure to hypoxia-derived ROS with the downregulation of a cardioprotective gene has significance clinical implications. Elevated levels of ROS have been implicated in numerous disease models and thus, may initiate epigenetic modification of cardioprotective genes in long-term leading to an increased susceptibility to ischemic heart disease. Potentially, this knowledge may lead to interventions involving antioxidants defense during gestation that may prevent the long-term adverse effects of chronic intrauterine hypoxia.

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References

1. Barker DJ, Osmond C. Infant mortality, childhood nutrition, and ischaemic heart disease in England and Wales. Lancet 1986;1:1077-81.

2. Bateson P, Barker D, Clutton-Brock T, Deb D, D'Udine B, Foley RA, Gluckman P, Godfrey K, Kirkwood T, Lahr MM, McNamara J, Metcalfe NB, Monaghan P, Spencer HG, Sultan SE. Developmental plasticity and human health. Nature 2004;430:419-21.

3. Gluckman PD, Hanson MA, Cooper C, Thornburg KL. Effect of in utero and early-life conditions on adult health and disease. N Engl J Med 2008;359:61-73.

4. McMillen IC, Robinson JS. Developmental origins of the metabolic syndrome: prediction, plasticity, and programming. Physiol Rev 2005;85:571-633.

5. Li G XY, Estrella JL, Ducsay CA, Gilbert RD, Zhang L. Effect of fetal hypoxia on heart susceptibility to ischemia and reperfusion injury in the adult rat. J Soc Gynecol Investig 2003;10:265-74.

6. Xue Q, Zhang L. Prenatal hypoxia causes a sex-dependent increase in heart susceptibility to ischemia and reperfusion injury in adult male offspring: Role of PKCε. J Pharmacol Exp Ther 2009;330:624-32.

7. Patterson AJ, Chen M, Xue Q, Xiao D, Zhang L. Chronic Prenatal Hypoxia Induces Epigenetic Programming of PKCε Gene Repression in Rat Hearts. Circ Res 2010;107:365-73.

8. Ardehali H. Signaling mechanisms in ischemic preconditioning: interaction of PKCε and MitoK$_{ATP}$ in the inner membrane of mitochondria. Circ Res 2006;99:798-800.

9. Inagaki K, Begley R, Ikeno F, Mochly-Rosen D. Cardioprotection by epsilon-protein kinase C activation from ischemia: continuous delivery and antiarrhythmic effect of an epsilon-protein kinase C-activating peptide. Circulation 2005;111:44-50.

10. Bertolotto C, Maulon L, Filippa N, Baier G, Auberger P. Protein kinase C theta and epsilon promote T-cell survival by a rsk-dependent phosphorylation and inactivation of BAD. J Biol Chem 2000;275:37246-50.

11. Semenza GL. Hypoxia-inducible factor 1: master regulator of O2 homeostasis. Curr Opin Genet Dev 1998;8:588-94.

12. Mahon PC HK, Semenza GL. FIH-1: a novel protein that interacts with HIF-1alpha and VHL to mediate repression of HIF-1 transcriptional activity. Genes Dev 2001;15:2675-86.

المنارة للاستشارات
www.manaraa.com

13. Wang GL JB, Rue EA, Semenza GL. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. Proc Natl Acad Sci USA 1995;92:5510-4.

14. Huang LE. Carrot and stick: HIF-alpha engages c-Myc in hypoxic adaptation. Cell Death Differ 2008;15:672-7.

15. Scheuermann TH, Yang J, Zhang L, Gardner KH, Bruick RK. Hypoxia-inducible factors Per/ARNT/Sim domains: structure and function. Methods Enzymol 2007;435:3-24.

16. Poyton RO, Ball KA, Castello PR. Mitochondrial generation of free radicals and hypoxic signaling. Trends Endocrinol Metab 2009;20:332-40.

17. Hervouet E, Simonnet H, Godinot C. Mitochondria and reactive oxygen species in renal cancer. Biochimie 2007;89:1080-8.

18. Duranteau J, Chandel NS, Kulisz A, Shao Z, Schumacker PT. Intracellular signaling by reactive oxygen species during hypoxia in cardiomyocytes. J Biol Chem 1998;273:11619-24.

19. Liu L, Wise DR, Diehl JA, Simon MC. Hypoxic reactive oxygen species regulate the integrated stress response and cell survival. J Biol Chem 2008;283:31153-62.

20. Galanis A, Pappa A, Giannakakis A, Lanitis E, Dangaj D, Sandaltzopoulos R. Reactive oxygen species and HIF-1 signalling in cancer. Cancer Lett 2008;266:12-20.

21. Lim SO, Gu JM, Kim MS, Kim HS, Park YN, Park CK, et al. Epigenetic changes induced by reactive oxygen species in hepatocellular carcinoma: methylation of the E-cadherin promoter. Gastroenterology 2008;135:2128-40, 40 e1-8.

22. Meyer K, Zhang H, Zhang L. Direct effect of cocaine on epigenetic regulation of PKCepsilon gene repression in the fetal rat heart. J Mol Cell Cardiol 2009;47:504-11.

23. Zhang H, Meyer KD, Zhang L. Fetal exposure to cocaine causes programming of Prkce gene repression in the left ventricle of adult rat offspring. Biol Reprod 2009;80:440-8.

24. Li SH, Shin DH, Chun YS, Lee MK, Kim MS, Park JW. A novel mode of action of YC-1 in HIF inhibition: stimulation of FIH-dependent p300 dissociation from HIF-1α. Mol Cancer Ther 2008;7:3729-38.

25. Mabjeesh NJ, Escuin D, LaVallee TM, Pribluda VS, Swartz GM, Johnson MS, et al. 2ME2 inhibits tumor growth and angiogenesis by disrupting microtubules and dysregulating HIF. Cancer Cell 2003;3:363-75.

المنارة للاستشارات
www.manaraa.com

26. Kabir AM CJ, Tanno M, Cao X, Hothersall JS, Dashnyam S, Gorog DA, Bellahcene M, Shattock MJ, Marber MS. Cardioprotection initiated by reactive oxygen species is dependent on activation of PKCε. Am J Physiol Heart Circ Physiol 2006;291:H1893-9.

27. Hool LC, Di Maria CA, Viola HM, Arthur PG. Role of NAD(P)H oxidase in the regulation of cardiac L-type Ca2+ channel function during acute hypoxia. Cardiovasc Res 2005;67:624-35.

28. Campos AC, Molognoni F, Melo FH, Galdieri LC, Carneiro CR, D'Almeida V, et al. Oxidative stress modulates DNA methylation during melanocyte anchorage blockade associated with malignant transformation. Neoplasia 2007;9:1111-21.

29. Kumar S, Sitasawad SL. N-acetylcysteine prevents glucose/glucose oxidase-induced oxidative stress, mitochondrial damage and apoptosis in H9c2 cells. Life Sci 2009;84:328-36.

30. Ishida I KH, Suzuki S, Suzuki T, Akashi S, Inoue K, Maeda S, Kikuchi H, Sasaki H, Kondo T. Hypoxia diminishes toll-like receptor 4 expression through reactive oxygen species generated by mitochondria in endothelial cells. J Immunol 2002;169:2069-75.

31. Chen Y, McMillan-Ward E, Kong J, Israels SJ, Gibson SB. Mitochondrial electron-transport-chain inhibitors of complexes I and II induce autophagic cell death mediated by reactive oxygen species. J Cell Sci 2007;120:4155-66.

32. Hwang JM WY, Lin JA, Bau DT, Ko FY, Tsai FJ, Tsai CH, Wu CH, Lin PC, Huang CY, Kuo WW. Hypoxia-induced compensatory effect as related to Shh and HIF-1alpha in ischemia embryo rat heart. Mol Cell Biochem 2008;311:179-87.

33. Shaw RJ OM, Rokadiya S, Kogera FA, Lowe D, Hall GL, Woolgar JA, Homer J, Liloglou T, Field JK, Risk JM. Cytoglobin is upregulated by tumour hypoxia and silenced by promoter hypermethylation in head and neck cancer. Br J Cancer 2009;101:139-44.

34. Beyer S, Kristensen MM, Jensen KS, Johansen JV, Staller P. The histone demethylases JMJD1A and JMJD2B are transcriptional targets of hypoxia-inducible factor HIF. J Biol Chem 2008;283:36542-52.

35. Benanti JA WM, Myers HE, Robinson KL, Grandori C, Galloway DA. Epigenetic down-regulation of ARF expression is a selection step in immortalization of human fibroblasts by c-Myc. Mol Cancer Res 2007;5:1181-9.

36. Watson JA, Watson CJ, McCrohan AM, Woodfine K, Tosetto M, McDaid J, et al. Generation of an epigenetic signature by chronic hypoxia in prostate cells. Hum Mol Genet 2009;18:3594-604.

المنارة للاستشارات
www.manaraa.com

37. Chandel NS MD, Feliciano CE, Wood TM, Melendez JA, Rodriguez AM, Schumacker PT. Reactive oxygen species generated at mitochondrial complex III stabilize hypoxia-inducible factor-1alpha during hypoxia: a mechanism of O2 sensing. J Biol Chem 2000;275:25130-8.

38. Chua YL, Dufour E, Dassa EP, Rustin P, Jacobs HT, Taylor CT, et al. Stabilization of hypoxia-inducible factor-1alpha protein in hypoxia occurs independently of mitochondrial reactive oxygen species production. J Biol Chem 2010;285:31277-84.

39. Hescheler J, Meyer R, Plant S, Krautwurst D, Rosenthal W, Schultz G. Morphological, biochemical, and electrophysiological characterization of a clonal cell (H9c2) line from rat heart. Circ Res 1991;69:1476-86.

40. Kimes BW, Brandt BL. Properties of a clonal muscle cell line from rat heart. Exp Cell Res 1976;98:367-81.

41. Kim MH, Jung YS, Moon CH, Jeong EM, Lee SH, Baik EJ, et al. Isoform-specific induction of PKCε by high glucose protects heart-derived H9c2 cells against hypoxic injury. Biochem Biophys Res Commun 2003;309:1-6.

42. Kim MJ, Moon CH, Kim MY, Kim MH, Lee SH, Baik EJ, et al. Role of PKCδ during hypoxia in heart-derived H9c2 cells. Jpn J Physiol 2004;54:405-14.

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CHAPTER 4

GENERAL DISCUSSION

Hypoxic stress is one of the most common insults to the developing fetus. Acute exposure to hypoxia has beneficial effects on heart development. Short-term hypoxia causes outflow tract remodeling and coronary vasculargenesis. However, chronic pathophysiological hypoxia is associated with significant harmful effects that may produce structural, functional, and gene expression changes in fetal heart that may persist throughout life (Patterson and Zhang 2010). In rodents, chronic maternal hypoxia induces hypertropic growth, and reduces proliferation of fetal cardiomyocytes. In adult male offspring, chronic maternal hypoxia causes a significant increase in susceptibility to ischemia reperfusion injury and considerably reduces the expression of the cardioprotective gene PKCε (Xue and Zhang 2009). PKCε translocation inhibitors reduce postischemic recovery in control animals, linking PKCε gene regulation to protection against ischemia reperfusion injury (Xue and Zhang 2009). Previous work in the cocaine model of intrauterine stress has established DNA methylation of CpG dinucleotides at the PKCε promoter as the major mechanism for long-term repression of PKCε. This study establishes epigenetic repression through DNA methylation of PKCε gene as the principle process underlying long-term repression of PKCε expression in fetal hearts exposed to chronic maternal hypoxia. This study presents additional evidence that oxidative stress independent of HIF-1 initiates events responsible for epigenetic modification of PKCε gene expression.

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The Direct effects of Hypoxia on Fetal Heart

In chapter 2, we demonstrated hypoxia significantly alters PKCε protein and mRNA in fetuses exposed to maternal hypoxia (10.5% O_2) from day 15 through 21. This finding builds on previous work that found maternal hypoxia significantly reduced PKCε expression in adult male offspring (Li et al, 2003, Xue and Zhang 2009). Our finding supports studies done by Hlaváčková et al. (2010) who found chronic intermittent hypoxia (CIH) significantly decreased PKCε protein but did not alter phospho- PKCε levels in adult wistar rats. In addition, Hlaváčková et al. (2010) reported CIH increased the total expression of total PKCδ and phospho-PKCδ. PKCδ expression has been linked with increased ischemia reperfusion injury (Budas et al., 2007). The effect of chronic maternal hypoxia on PKCδ expression in fetal hearts is not clear and deserves future investigation.

Chronic maternal hypoxia effects fetal rat heart architecture by inducing hypertropic growth, reducing cellular proliferation of fetal cardiomyocytes, reducing metalloproteinase-2 activity, increasing collagen I and III expression and increasing the heart to body ratio (Bae et al 2003, Xu et al, 2006). These findings suggest hypoxic insult significantly alters fetal cardiomyocyte homeostasis, producing cellular and organ specific changes that in the short term may be compensatory, but in the long term increases the hearts susceptibility to cardiomyopathies in adulthood. In chapter 2, our data confirmed that prenatal hypoxia causes intrauterine programming of rat offspring. We found that selective PKCε activation protects hearts exposed to maternal hypoxia, thus supporting studies done by Inagaki et al. (2004), who demonstrated selective PKCε activation conferred *in vivo* protection against ischemic reperfusion injury in mice and

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porcine hearts. These findings place PKCε regulation as central in conferring cardioprotection in adult rat hearts.

Since maternal hypoxia can cause significant maternal stress that may result in dramatic systemic adaptation, which may affect the fetus and account for the downregulation of PKCε and increased sensitivity to ischemia reperfusion injury, it was necessary to determine the direct effect of hypoxia on fetal hearts. Therefore, we demonstrated in chapter 2 by using a whole organ culture model that chronic hypoxia is sufficient to induce a direct effect on PKCε expression in fetal hearts. We exposed fetal hearts to 1% O_2 in cultured media for 48 hours and found significant reduction in PKCε protein and mRNA as compared to control. Similarly, cocaine exposure of culture intact fetal hearts also resulted in significant decrease in PKCε protein and mRNA (Meyer 2009B). These findings demonstrate hypoxia directly regulates PKCε expression in fetal rat hearts. The extent to which other maternal derived stressors induced by chronic hypoxia such as increased circulating catecholamines or glucocortoids affects PKCε expression is not clear. In fetal sheep, chronic hypoxia modulates the fetal hypothalamic-pituitary-axis (HPA) causing the elevation of in plasma precursor ACTH that allow for heighten cortisol secretion in response to secondary stressor (Carmicheal et al., 1997; Duscay, 1998;). Chronic hypoxia also induces arterial sympathetic hyperinnervation, elevation in epinephrine and noreprinephrine content in the heart (Jelinek and Jensen, 1991; Ruijtenbeek et al., 2000). These finding suggest additional factors produced by in response to hypoxia may potentiate the effect of hypoxia on PKCε expression. The combined effect of hypoxia and stress hormones on cardioprotective genes such as PKCε is intriguing and warrants further study.

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The effect of Hypoxia *In vitro*

Although the use of maternal hypoxia and intact fetal heart models revealed significant information pertaining the effects of hypoxia on rat heart, we determined to use the cell culture model to study more closely the mechanisms responsible for PKCε repression. We selected the embryonic rat ventricular myoctye cell line H9c2. The advantage in selecting the H9c2 cell line pertains to its unique fetal heart origin, which afforded us a glimpse at fetal cardiomyocyte behavior from the controlled environment of cell culturing. H9c2 cells retain many properties consistent with normal cardiomyocyte physiology, including electrophysical characteristics (Hescheler, 1991). In addition, the H9c2 cell line is used to study a variety of areas including cell death, differentiation and ischemia reperfusion injury (Chong, 1998; Hwang, 2008; Graf, 2006).

There are obvious drawbacks in using a cell model. The cell model cannot reproduce the microenvironment of the fetal heart. The fetal heart alone includes a variety of cell populations such as fibroblast, endothelial cells, various types of cardiomyocytes and neuronal cell types. Although paracrine and endocrine signaling cannot be reproduced in this model, the use of the whole animal and intact heart model gives insight into this process. The cell line selected does differ from normal freshly isolated fetal cardiomyocytes. Freshly isolated cardiomyocytes are contractile and display a restricted level of proliferation, whereas H9c2 are not contractile and under the right conditions are capable of continuous proliferation. The H9c2 cell line may also differentiate into a different phenotype over time with numerous passages, and therefore must be constantly monitored. We were careful to confirm results obtained in the H9c2 cell line intact whole hearts and hearts exposed to maternal hypoxia.

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In chapter 2 & 3, we demonstrated that PKCε protein and mRNA was decreased in H9c2 cells exposed to 1% O_2 for 24 hours. This finding is consistent with the maternal hypoxia and intact fetal heart models, which suggest that the underlying mechanism for PKCε repression is consistent across each model. Our findings are consistent with studies that demonstrated prolonged hypoxia in the presence of low or high glucose significantly decreased PKCε protein abundance in H9c2 cells (Kim 2003, 2004). In tumor cells, chronic hypoxia significantly reduced PKCε protein causing increased sensitivity to TNF-related apoptosis ligand (TRAIL) (Gobbi, 2010). Moreover, in chapter 3, we confirmed hypoxia-induced nuclear accumulation of HIF-1α in H9c2 cells, which is consistent with studies that have demonstrated HIF-1α protein accumulation in fetal hearts, H9c2 and freshly isolated cardiomyocytes cells (Bae et al, 2003; Hwang 2008; Gobbi, 2010). Given these results, it seems H9c2 cells responds to hypoxia in a manner consistent with normal cardiomyocytes and therefore supports the use of this model in examining the underlying mechanism of hypoxia-induced gene repression.

An area of considerable discussion pertains to the use of 21% O_2 as control for cell culturing experiments. Since we chose to study fetal cardiomyocytes, the question of what is normal physiological oxygen tension is critical. Fetal cardiomyocytes develop at low pO_2 values as compared to normal adult cardiomyocytes (Patterson and Zhang, 2010). Some studies have found fetal oxygen tension to be as low as ~20 mm Hg (Bishai, 2003; Webster 2007). In addition, fetal cardiomyocytes utilize large amounts of oxygen due to high metabolic activity (Ascuitto and Ross-Ascuitto, 1996). This suggests that normal physiological oxygen tension for fetal cardiomyocytes is significantly lower than the 21% O_2 (~147 mm Hg) used in standard culturing techniques, which could

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significantly distort results. These concerns were also highlighted in a review by Dr. Ivanovic (2009), who argued that standard culture condition do not reflect normal oxygen tension levels in a variety of tissues (i.e. bone marrow) in the mature organ system. He argued the term normoxia, which represented 21% O_2 in classical physiology was also adopted in cell biology and culturing but is not an accurate representation of normal for tissue and cell environments (Ivanovic 2009). We also recognized these concerns when considering the H9c2 cell model. In chapter 2, we designed experiments using various levels of oxygen tension and demonstrated that there was no difference in PKCε protein or mRNA when we used 21% (~147 mm Hg), 10.5% (~73.5 mm Hg) or 3% (~21 mm Hg) O_2, but found significant differences in PKCε protein and mRNA at 1% (~7 mm Hg) O_2. Furthermore, in chapter 3 we demonstrated nuclear accumulation of HIF-1α protein at 1% O_2, while no HIF-1α protein could be detected at 21% O_2. These findings support our hypothesis that the H9c2 model system using 21% O_2 as control and 1% O_2 as hypoxia is consistent with the maternal hypoxia model and is ideal for studying the molecular mechanisms regulating PKCε expression.

PKCε Regulation

The regulation of PKCε expression under hypoxic condition is the focus of this project. Normally, PKCε expression increases 10-fold in the rat heart from neonatal to adult periods (Clark et al., 1995). Our lab has shown maternal hypoxia from day 15 to 21 of gestation resulted in increase sensitivity to ischemia reperfusion and injury and decrease PKCε abundance in the ventricles of adult male rats (Li, 2003; Xue and Zhang 2009). Interestingly, researchers have demonstrated no significant change in the ratio of

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phospho-PKCε/ PKCε between control and maternal hypoxia groups, suggesting PKCε activity is not affected by chronic gestational hypoxia (Xue and Zhang 2009). In chapter 2, we showed that hypoxia directly regulates PKCε abundance in isolated intact fetal heart and H9c2 cells. Studies examining the effect of cocaine and nicotine through catecholamines on PKCε expression have found concurrent findings with the hypoxia model, suggesting a similar pattern of PKCε regulation (Meyer 2009B; Lawrence 2008). In fact, cocaine and nicotine exert vascular altering properties that may affect fetal oxygen supply (Wood, 1987; Lawrence, 2008). Indeed, these findings may shed clues as to a general mechanism for adaptation to intrauterine stress and the consequences in terms of PKCε gene regulation in fetal heart.

Our lab has found cocaine directly regulates PKCε gene repression through increased DNA methylation for transcription factor binding sites of PKCε promoter (Meyer 2009B). Specifically, cocaine treatment significantly increased methylation at Sp1 binding sites -346 and -268 and this significantly reduced binding of SP1 to those sites (Meyer 2009B). In chapter 2 & 3, we found chronic hypoxia also significantly increased methylation of SP1 binding sites -346 and -268. We confirmed with EMSA assays that methylation at the core of SP1 binding sites -346 and -268 abolished SP1 binding and that hypoxia does not significantly alter the binding affinity of SP1 protein to SP1 binding sites -346 and -268. Reporter gene assay indicated the regions encompassing SP1 binding sites -346 and -268 play a significant role in promoter activity (Zhang 2009). While methylation of a single SP1 site does not substantially alter PKCε promoter activity, methylation of both SP1 sites (-346 and -268) significantly decreases PKCε promoter activity (Meyers, 2009B). In H9c2 cells, we demonstrated with CHIP

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assay that chronic hypoxia decreases the binding of SP1 protein to Sp1 binding sites -346 and -268 *in vivo* and that SP1 binding is restored in the presence of DNA methylation inhibitor 5-aza-2-deoxycytidine. This finding supports previous work that found cocaine-induced repression of SP1 binding is restored in the presence of DNA methylation inhibitors 5-aza-2-deoxycytidine and procainamide (Meyer, 2009). We further demonstrated that 5-aza-2-deoxycytidine blocked the hypoxic effect on PKCε protein and mRNA, definitively linking epigenetic repression through CpG dinucleotide methylation as chief mechanism of hypoxia-induced PKCε repression (See Figure 16). The observed heightened sensitivity of the PKCε promoter to methylation in response to cocaine, nicotine and chronic hypoxia in fetal hearts is intriguing and suggest a common pathway of regulation in response to intrauterine stress.

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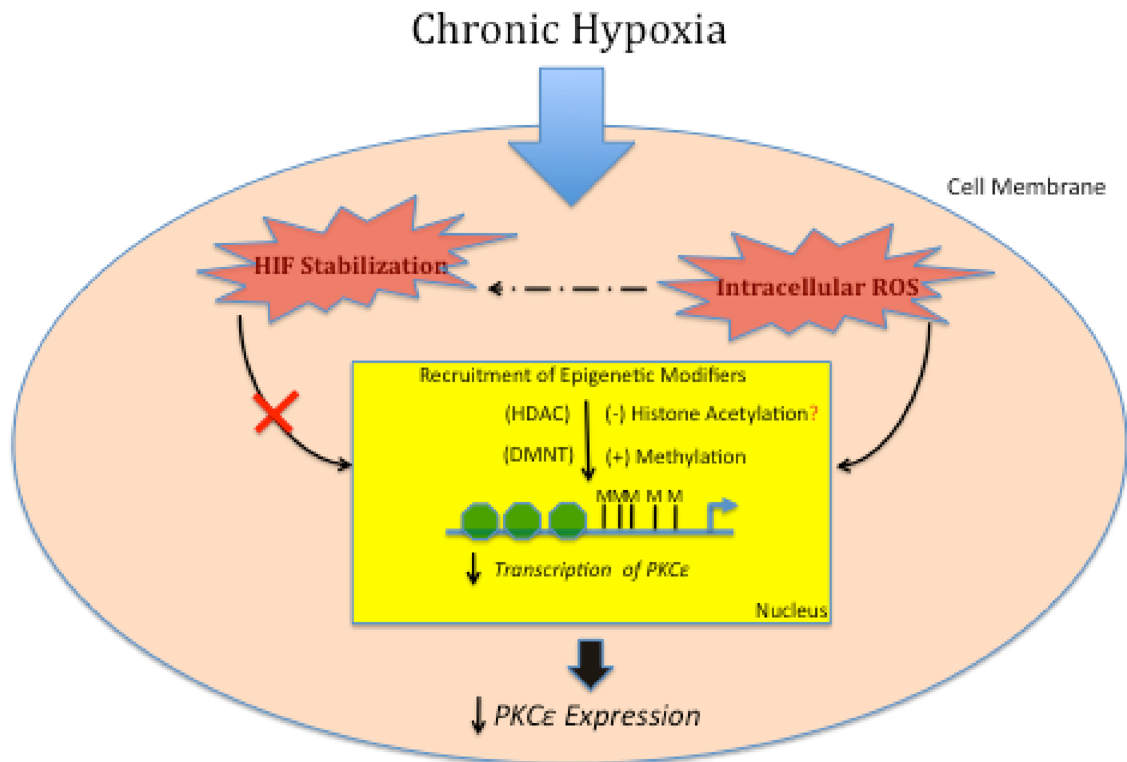

Figure 16: Plausible mechanisms for hypoxia-induce downregulation of PKCε in hearts. Hypoxia causes the stabilization of HIF-1α and increases ROS production in fetal hearts. Increase ROS causes the recruitment of epigenetic modifiers, *i.e.* DNA methyltransferase (DNMT) and possibly histone deacetylases (HDAC). These modifiers increase methylation of promoter at transcription factor binding sites and deacetylate histone residues resulting in the decreased transcription of PKCε.

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We have presented data showing PKCε expression is altered *in utero* in the hearts of fetus exposed to chronic maternal hypoxia. It is not clear, however, the affect of chronic hypoxia on PKCε gene expression in other organ systems such as in brain or liver and the long-term implication. Studies in brain have found PKCε is important in preconditioning of ischemia reperfusion injury (Raval AP, 2003; Kim et al., 2007). PKCε also plays an important role in hyperalgesic priming, a process whereby one or several acute inflammatory events involving pain becomes a chronic condition with heightened pain sensation (Reichling D, 2009). PKCε plays an important role in nociceptors progression from acute to chronic pain sensitization (Reichling D, 2009). Interestingly, the selective activation of PKCε with bryostatin1 or DCP-LA has been shown to reverse or prevent synaptic lost in the hippocampus, reduced Aβ protein accumulation and improve cogitative memory in Alzheimer's disease models (Hongpaisan et al., 2011). In each model, oxidative stress has been shown to contribute to disease progression. Perhaps, ROS-signaling may be the common pathway modulating PKCε expression. The apparent involvement of PKCε in multiple organ systems suggests that perinatal regulation of PKCε expression has important implications for a variety of diseased and non-diseased states later in life.

DNA Methylation

DNA methylation is the chief mechanism for transcriptional repression. Methylation of cytosine plays an important role in normal physiology. Among other examples, genomic imprinting during early stages of development utilizes DNA methylation (Okano et al., 1999; Miranda and Jones 2007). Differentiation of stem cells

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into specialized tissue such as cardiomyocytes, neurons, and hepatocytes involves selective repression of genes through DNA methylation. The role of DNA methylation in fetal programming of disease is intriguing. A classic example of the importance of DNA methylation in gestational epigenetics is found in studies done in the Agouti mouse model (Waterland and Jirtle, 2004). A^{vy}/a mice display a range of fur colors from yellow to mottle to brown (Waterland and Jirtle, 2004). The yellow agouti (A^{vy}) mouse has a mutation that causes yellow pigmentation. When pregnant yellow agouti (A^{vy}) mice were supplement with methyl rich or high soy diets, the fur of the offspring shifted towards brown fur color, and in the case of the high soy diets significantly reduced the weight of brown offspring in comparison to yellow (Dolinoy, 2006). In comparison, reduced folate intake during pregnancy can lead to genetic instability with abnormal chromosomal re-arrangement as result of poor gene regulation due to hypomethylation (Okano et al., 1999). Methyltetrahydrofolate (folate) is an important methyl donor to S- adensonyl Methionine (SAM). DNA methylation is mediate by a family of enzymes known as DNA methyltransferases (DNMT). DNMT uses methyl groups from SAM to methylate CpG dinucleotides of target genes. It is not clear whether DNMT expression or activity is altered by hypoxic insult. Studies in prostate cell line PwR-1E have shown increased DNMT3b (a isoform responsible for de novo methylation) activity in response to chronic hypoxia (Watson, 2009). The effect of hypoxia on the activity of DNMT's and global methylation patterns in fetal cardiomyocytes is an area yet to be elucidated. We have found that in response to chronic hypoxia, fetal cardiomyocytes adjust gene expression patterns through targeted methylation of SP1 binding sites at the PKCε promoter. The term "targeted" is used since the methylation levels for most transcriptional factor

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binding sites with CpG dinucleotides in the proximal PKCε promoter (except MTF1-2 -168) were not significantly changed as a result of chronic hypoxia (See chapter 2, figure 3). Only Sp1 binding sites (-346, and -268) in both H9c2 and fetal hearts and Egr1 (-1008) in fetal hearts were significantly increased by chronic hypoxia. Methylation of Sp1 binding sites does not completely abolish gene transcription but significantly reduces basal levels (Zhang, 2009), which leads to heighten sensitivity to stress in adult life. Interestingly, our lab has reported similar patterns of methylation for PKCε promoter in cocaine (Sp1) and nicotine (Egr1) models of intrauterine stress (Zhang, 2009; Lawrence 2011). It is unknown whether the process of methylation of PKCε in cardiomyocytes is present in other forms of intrauterine stress such as undernutrition, alcohol exposure, or elevated stress hormones. Moreover, the effect of chronic intrauterine hypoxia on the methylation status of the PKCε promoter in other tissues such as brain is not known and warrants future study.

Hypoxia and Sex Dimorphism of Cardiac Vulnerability

Previous studies have reported significant sex differences in the effect of maternal hypoxia on cardiac vulnerability in adulthood. Maternal hypoxia causes increase susceptibility to ischemia reperfusion injury in male rat offspring (Xue and Zhang 2009). Further investigation revealed reduced expression of PKCε protein in male rats compared to female rats. In chapter 2, we demonstrated maternal hypoxia caused significant decrease in PKCε mRNA and increase methylation of both SP1 bindings sites (-346 and -268) for fetuses and adult offspring. The degree to which methylation was increased in female hearts was considerably lower than in male hearts. Fetal hearts exposed to

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maternal cocaine responded in a similar sex dependent manner (Zhang, 2009). Maternal cocaine exposure caused increase CpG methylation for SP1 binding sites -346 and -268 in male and -268 only in female offspring (Zhang, 2009). Likewise, maternal nicotine exposure caused increased susceptibility to ischemia reperfusion injury in both male and female offspring, with poorer recovery in female offspring (Lawrence 2008). PKCε protein was significantly reduced in both male and female hearts exposed to prenatal nicotine, which suggest nicotine induces a different pattern of regulation in fetal hearts (Lawrence, 2008). Furthermore, Netuka et al. (2006) found maternal hypoxia significantly improved cardiac tolerance to ischemic arrhythmias in female offspring, but had the opposite effect in male offspring. These findings indicate sex dependent mechanisms for *in utero* programming of the myocardium.

Studies have found a general tendency for female offspring to be more resistant to cardiovascular diseases induced by prenatal stressors (Zhang 2009, Xue 2009). Indeed, cardiomyocytes isolated from female hearts are more resistant to ischemia reperfusion injury (Rank et al. 2001). Sex specific differences in gene expression become evident by midgestation in rodents (Dewing 2003). The contribution of (XX) and (XY) dependent genes in reduced or enhanced cardioprotection of male or female offspring respectively, is not clear. In neonatal brains, a PARP1 knockout potentiate differences in relative NAD+ utilization and expression between male and females, and has therefore been proposed as a contributing factor in increased sensitivity of male neonates to ischemia reperfusion injury (Hurn, 2005). The increase cardioprotection observed in female offspring may inpart be explained by the presence of estrogens sex hormone. Removal of the ovaries and estrogen replacement in rats has implicated estrogens in cardioprotection

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against ischemia reperfusion injury in female rat hearts (Zhai et al. 2000). The exact mechanism is not clear, but studies in female rat hearts have found estrogens replacement reduced the expression of tumor necrosis factor-alpha (Xu 2006). Further studies have found estrogen receptor alpha agonist (4,4',4''-[4-propyl-(1H)-pyrazole-1,3,5-triyl]tris-phenol [PPT]) or selective beta agonist (2,3-bis(4-hydroxyphenyl)-propionitrile [DPN]) improve myocardial recovery from acute ischemia reperfusion insult (Vornehm 2009). These findings are interesting given that male and female fetuses are likely exposed to similar concentration of steroid hormones. In chapter 2, we demonstrated significant higher expression of ERα and ERβ in female fetal hearts compared to male, which may in part explain the enhanced protection afforded in female hearts. Furthermore, we demonstrated using ChIP assay that ERα and ERβ interact with SP1 binding sites -346 and -268 at PKCε promoter. The functional significance of this interaction is yet to be understood, however, several studies examining multiple genes have demonstrated ERα and ERβ are capable of binding GC rich regions and forming ER-SP1 complexes that are important in gene transactivation (Safe, 2008; Sisci 2010; Nilsson 2001). The relative gender differences in PKCε promoter methylation and ERα and ERβ expression *in utero* are fascinating and deserve future study.

HIF-1α and Epigenetic Repression of PKCε Gene

HIF-1α plays an important regulatory role in cellular and tissue adaptation to hypoxic stress. HIF-1α influences the expression of genes involved in apoptosis, cellular differentiation, metabolism and cell signaling (Semenza, 2007). Previous work in our lab and has demonstrated increased HIF-1α expression in the fetal rat hearts exposed to

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maternal hypoxia in late gestation (Bae et al., 2003). Studies in the developing chick embryo have also demonstrated HIF-1α stabilization (Sugishita et al., 2004A). In fetal heart development, HIF-1α plays a critical role in modulating cardiomyocyte survival and death pathways, thereby orchestrating remodeling events during cardiogenesis (Sugishita et al, 2004A; Patterson and Zhang, 2010). The role HIF-1α plays in epigenetic modulation through methylation of CpG dinucleotides is not well understood. To our knowledge, interaction between HIF-1 and DNMT has not been described. HIF-1α has been reported to interact with histone modulators such as histone deacetylases and methylases (Granger 2008; Beyer 2008). In particular, HIF-1 transactivates histone demethylase JMJD1A, JMJD2A, JMJD2B, and JMJD2C (Wellman et al. 2008). JMJD1A and JMJD2A remove dimethyl marks on histone 3 lysine 9 (H3K9me2), while JMJD2B removes trimethyl marks (H3K9me3) and more weakly JMJD2C which converts H3K9me3 to me2 (Xia et al. 2009; Pollard et al. 2008; Maltepe et al. 2005; Charron et al. 2009). H3K9 methylation produces compaction of chromatin thereby decreasing exposure of DNA to damage. H3K9 demethylation is one epigenetic mechanism that is thought to be link to chronic inflammation and HIF-1 activity in carcinogenesis (Brigati 2010). Additionally, chemically induced hypoxia in prostate cancer cell lines induces HIF-1 stabilization with concurrent reduction in miR-101 (Cao, 2010). Although the researchers in this study did not distinguish between the ROS and HIF-1 effects, this finding implies that hypoxia via HIF-1 may modulate micro-RNA expression suggesting an additional role for HIF-1 in epigenetic regulation.

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In chapter 3, we demonstrated inhibition of HIF-1α with YC-1 or 2-me2 did not attenuate the hypoxia-induced repression of PKCε mRNA, suggesting HIF-1 does not play a significant role in epigenetic repression of PKCε gene (See Figure 16). Since YC-1 and 2-me2 have been shown to diminish HIF-2α nuclear accumulation, it is unlikely HIF-2 plays a significant role in epigenetic repression of PKCε. The role of HIF-3 in epigenetics is unknown. However, studies using a dominant negative HIF-3alpha splice variant suggest HIF-3 negatively regulates HIF-1 & 2 activity (Maynard 2007).

Hypoxia, Oxidative Stress and PKCε

The role of oxidative stress during hypoxia is a fascinating area of research. Studies have shown hypoxia increases ROS in cardiomyocytes resulting in reduced contractility (Duranteau et al. 1998). In Chapter 3, we demonstrated chronic hypoxia increases ROS in H9c2 cells. The exact mechanism is not clear, however the consensus in the field suggests complex III of the mitochondrial electron transport chain is the primary sites of ROS generation during hypoxia (Galansis et al., 2008). Interestingly, a recent study found mitochondrial derive ROS plays a critical role in cardiac hypertrophy and failure (Dai et al., 2011). We demonstrated in chapter 3 that N-acetyl cysteine or tempol, but not apocynin (NADPH oxidase inhibitor) blocked hypoxia-induced repression of PKCε protein and mRNA in hearts exposed to hypoxia pregnant rats from day 15 to 21 of gestation, in isolated whole fetal hearts, and H9c2 cells, implicating oxidative stress in the hypoxia-induced decrease PKCε expression. Although NADPH oxidase has been shown to regulate ROS production under chronic hypoxic conditions in some cell types, its role in hypoxia-induced repression of PKCε expression is minimal. Our lab has also

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found cocaine induce repression of PKCε in fetal hearts involves oxidative stress that is independent of NADPH oxidase activity (unpublished data). This finding further supports the concurrent mechanism of *in utero* repression of PKCε expression. Interestingly, acute exposure to ROS increases the activity of PKCε by promoting the translocation from cytosolic to particulate fraction and thus a cardioprotective phenotype often observed in acute ischemia and reperfusion models (Golberg et al., 1996; Kabir et al., 2006). These finding suggests ROS differentially regulates PKCε gene, perhaps through a negative feedback loop that is dependent on the duration of the insult (See Figure 17). This implies that while short bouts of hypoxia are protective by stimulating PKCε activity, long-term hypoxia significantly reduces PKCε expression thereby considerably reducing the threshold necessary for cell death to occur. Interestingly, NAC attenuates apoptosis and ischemia reperfusion injury in H9c2 cells and *ex vivo* neonatal rabbit hearts (Peng et al., 2011). Although PKCε expression was not examined in this study, the notion that antioxidant defense protects against ischemia reperfusion injury supports our findings that PKCε gene activity is maintained at basal levels in the presence of NAC. Previous work in our lab has shown a cause and effect relationship between PKCε expression and postischemic recovery (Li et al., 2003; Xue et al., 2009). Perhaps, the preservation of PKCε expression with antioxidant exposure during chronic maternal hypoxia will improve postischemia recovery in those animals.

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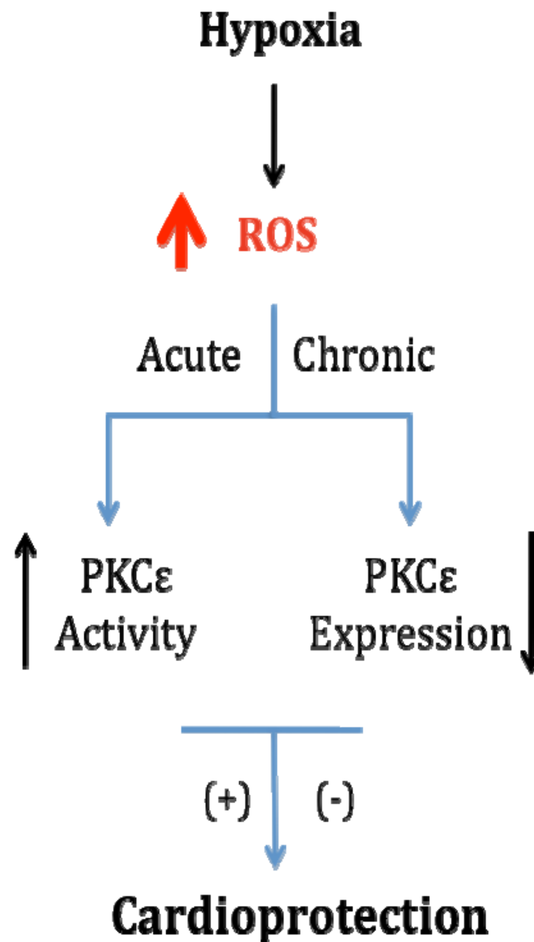

Figure 17: The short and long term effects of ROS on PKCε in cardiomyoctes. Hypoxia causes the increase reactive oxygen species (ROS). Acute exposure to ROS causes PKCε migration from soluble to particulate fraction which is indicative of increase activity and cytoprotection. Chronic exposure to ROS induces reduced PKCε expression and concurrent reduced cardioprotection.

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Recently, the connection between oxidative stress and epigenetics has gained interest. Studies using superoxide dismutase knockout mice have found increased oxidative stress associate with reduce global DNA methylation (Bhusari et al., 2010). Generation of free radicals through X-ray, UV, and γ-rays causes widespread DNA damage that significantly reduces the methyl-accepting ability of DNA (Wilson and Jones, 1983; Becker et al., 1985; Kalinich et al., 1989). The presence of 8-OHdG in CpG dinucleotides has also been shown to significantly impede methylation of adjacent cytosines (Weitzman et al., 1994; Turk et al., 1995). The presence of single stranded DNA can signal *De novo* methylation (Christman et al., 1995). Perhaps the presence of single strand breaks caused by oxidant damage may promote aberrant methylation patterns that can lead to disease progression. Indeed, oxidative damage reduces global methylation but increases regional methylation patterns. Studies in tumoriogenesis have found hypoxia mediates suppression of E-cadherin expression through methylation of E-cadherin promoter in ovarian carcinoma (Imai et al., 2003). The mechanism involves an increase in oxidative stress that promotes epigenetic modification of E-cadherin promoter through CpG methylation that is SNAIL dependent (Imai et al., 2003; Cannito et al., 2008). Furthermore, we found ROS scavengers blocked hypoxia-induced methylation of PKCε promoter suggesting oxidative stress plays an important role in regulating PKCε promoter methylation. Interestingly, studies have found certain fruits and vegetables protect against aberrant methylation in prostate cells, which further supports the use of antioxidants in protection against oxidative damage that leads to mutagenic processes (Ornish et al., 2005; Donkena et al., 2010). Together, these findings reflect a complex

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system of regulation involving cellular adaptation to hypoxia-induced oxidative stress and the *in utero* programming PKCε expression (Figure 16).

Clinical Significance

The clinical significance of this project is immense. Hypoxia is one of the most common gestational insults. Hypoxia during critical periods of development can alter organ and tissue maturation. There is an increased risk of fetal hypoxia in pregnancy where there is cord compression, preexisting maternal illness, smoking, drug use, placenta abnormalities and high altitude (Zhang 2005; Patterson 2010). Our study indicates that chronic hypoxia during the latter stages of fetal rat heart development causes the epigenetic modification of PKCε promoter leading to reduced PKCε expression. We were able to demonstrate that ROS scavenging with NAC was able to block the hypoxia-induced methylation of PKCε promoter and reduced expression. The clinical importance of linking oxidative stress to hypoxia-induced repression of PKCε *in utero* is profound. Currently, clinical studies are examining the role of pregestational diabetes during pregnancy in altering the epigenetic landscape of the fetus (NCT01255384). The rationale for this study is based on the understanding that the diabetic environment increases oxidative stress and causes significant changes in gene expression that may involve epigenetic mechanisms. In addition, a recent clinical study examining the effect of NAC on the prevention of preterm delivery has recently completed phase IV trials (NCT00568113). The results from this study are yet to be reported, however, it raising the issue that oxidative stress is important in the fetal development and disease. The implication of oxidative stress in hypoxia-mediated

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epigenetic repression may influence future clinical studies examining intrauterine stress and fetal outcomes.

PKCε plays a critical role in myocardial protection against ischemia reperfusion injury. In the rat model, chronic maternal hypoxia causes long-term repression of PKCε expression in myocardial tissue leading to enhance vulnerability to ischemia reperfusion later in life. We have demonstrated that chronic hypoxia represses PKCε expression through methylation of CpG dinucleotides at the PKCε promoter. PKCε is involved in a wide array of physiological and pathophysiological processes. PKCε is expressed in tissues throughout the body and has been implicated in precondition against ischemia reperfusion injury, oncogenesis, addiction, hyperanalgesia and thrombosis (Rechling and Levine, 2009; Harper and Poole, 2010; Ardehali, 2006; Barnett, 2007; Lesscher, 2009). The intrauterine regulation of PKCε in cardiomyocyte may reflect broader regulation of PKCε and other protective genes, placing gestational epigenetics as critical to understanding adult disease. Currently, there are several clinical trials examining the effect of selective inhibition of several PKC isoforms. One example involves a double-blinded randomized clinical study examining the efficacy of using KAI-9803 to block PKCδ in reducing infarct size in-patient with myocardial infarction undergoing a percutaneous coronary intervention (NCT00785954). PKCε on the other hand is fairly unstudied clinically. However, clinical trials are examining the efficacy in using selective PKCε blockers (KAI-1678) for the treatment of postoperative pain and PKCε activators (Bryostatin) in treating Alzheimer disease (NCT01015235, NCT00606164). Perhaps, in utero programming of PKCε gene may have tissue specific patterns and therefore influence the function of several organ systems. In myocardial studies, PKCε is

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used as a primary outcome indicator for the use of blood pressure cuff induced preconditioning in patients recovering from heart surgery (NCT00546390). Indeed, chronic oxygen insufficiency *in utero* may suppress or deregulate genes in myocardial and nonmyocardial tissues, reducing the organ systems ability to adapt to insults later in life. With an increased understanding of hypoxia-induced intrauterine modulation of PKCε expression in fetal hearts, preventative and therapeutic measures may be employed to improve patient outcomes.

Future Studies

The findings observed in this project have produced several questions that warrant further investigation. We found hypoxia mediates repression of PKCε expression through CpG dinucleotide methylation of Sp1 binding sites. The mechanism involves oxidative stress dependent pathways. We demonstrated in an *in vivo, ex vivo*, and H9c2 cell line model that ROS scavengers block the hypoxia-induced repression of the PKCε gene. The functional significance of this finding suggests attenuation of oxidative stress *in utero* will improve postischemia reperfusion recovery in adult rat hearts. However, it has not been determined whether attenuation of maternal hypoxia-derived oxidative stress translates into improved postischemia recovery in adult rat offspring. Attenuation of hypoxia-derived oxidative stress maintains basal expression of PKCε in fetal rat hearts and therefore would likely improve cardiac performance in adult offspring, but this has not been proven experimentally. Interestingly, when Wistar rats exposed to undernutrition during fetal development were pretreated with NAC for 48 hours 6 months after delivery, they showed improved recovery from ischemia reperfusion injury (Elmes

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et al., 2007). Furthermore, determining whether the use of antioxidants such as vitamin A, C or E can produce effects similar to NAC is of considerable importance. Previous studies have shown Vitamin A, C, and E given during late gestation reduces hypoplasia associated with congenital diaphragmatic hernia in fetal rat hearts exposed to Nitrofen (Gonzalez-Reyes, 2005). Vitamin A, C and E are found in numerous plant-based foods and are widely consumed in the general population. If the actions of the vitamins mirror those of NAC in the *in vivo, ex vivo*, and H9c2 models, then this may provide a natural therapeutic model to protect fetal hearts from intrauterine stress.

Oxidative Regulation of PKCε

In chapter 3, we demonstrated that maternal hypoxia represses PKCε promoter through an ROS dependent pathway involving methylation of CpG dinucleotides for Sp1 binding sites. The primary source of hypoxia-derived ROS was reported to originate in Complex III of the electron transport chain (Guzy & Schumacker, 2006). The mitochondrial production of ROS via complex III is also reported to regulate HIF-1α stability (Reviewed by Chandel NS, 2010). The role of complex III activity in hypoxia-induced repression of PKCε has not been elucidated. The effect of complex III antagonists (i.e. Stigmatellin, myxothiazol) on hypoxia-induced ROS production and repression of PKCε expression is an interesting question that deserves further study. If the mitochondria were the primary producer of ROS, then the attenuation of mitochondrial derived ROS would answer multiple questions pertaining to the signaling events initiating PKCε gene repression. Xanthine oxidase activity is another possible player in hypoxia-induced PKCε repression. Kayyali et al (2001) demonstrated that

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hypoxia induced the phosphorylation and increased activity of xanthine oxidase. Xanthine oxidase catalyzes the conversion of hypoxanthine to xanthine and xanthine to uric acid (Harris CM et al., 1999). Xanthine oxidase preferentially uses molecular oxygen as the electron acceptor thus producing superoxide radical. The effect of blocking xanthine oxidase with allopurinol, a xanthine oxidase antagonist on hypoxia induced repression of PKCε expression is interesting and deserves further study.

The exact mechanism of hypoxia-induced increase in methylation of the PKCε promoter is yet to be elucidated. Hypoxia-induced hypermethylation of E-cadherin promoter was reported to involve SNAIL transcription factor activity. SNAIL is involved in the repression of epithelial specific genes such as occuludins, E-cadherin and stimulating mesenchymal gene transcription (Reviewed by De Herreros et al., 2010). Whether SNAIL activity is involved in hypoxia-induced repression of PKCε gene is an intriguing question. Since both cocaine and nicotine have been reported to increase oxidative stress, further understanding of SNAIL in hypoxia-induced repression of PKCε may yield insight into a common pathway of intrauterine stress adaptation in fetal heart cells.

Fetal Hypoxia and Cardioprotective Genes

The effect of maternal hypoxia on the expression of cardioprotective genes goes beyond PKCε. Previous work in our lab has demonstrated maternal hypoxia causes a reduction in the expression of endothelial nitric oxide synthase (eNOS) in adult offspring (Li et al., 2003). NO plays an important role in maintaining normal vascular integrity. While the cardioprotective role is controversial, with studies suggesting nitric oxide (NO)

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accumulation is important in ischemia reperfusion injury (Csonka, 1999) and others showing NO to be protective (Boli, 2001), the use of gain of function/ lost of function experiments in transgenic mice seem to suggest a strong correlation of NO in promoting cardioprotection against ischemia reperfusion injury (Reviewed by Jones SP, 2006). Interestingly, SP1 transcription factor is important for critical basal expression of eNOS in endothelial cells (Zhang et al., 1995). Furthermore, mutation of Sp1 concensus sequence at -104 of the eNOS promoter significantly reduced basal eNOS activity (Kumar et al., 2008). The GC rich region at -104 contains two potential CpG dinucleotide sequence at the core the binding motif (Kumar et al., 2008). The sequence is as follows (-119 5′ATT GTG TAT GGG ATA GGG GCG GGG CGA G 3′ -92). Kumar et al. (2008), was able to demonstrate H_2O_2 significantly reduces eNOS promoter activity by decreasing SP1 activity. In addition, methylation of eNOS promoter plays an important role in regulating cell specific expression of eNOS (Chan et al., 2004). Whether chronic hypoxia directly regulates eNOS promoter through increase CpG dinucleotide methylation of Sp1 binding sites in fetal hearts is intriguing and warrants further study.

Heat shock protein 70 (Hsp70) presents another interesting cardioprotective gene that was reported to be sensitive to maternal hypoxia. Studies have shown chronic maternal hypoxia significantly reduces protein and mRNA levels in fetal hearts (Bae et al., 2003). The down regulation on Hsp70 gene persists into adulthood (Li et al, 2003). Hsp70 plays an important role in protection against ischemia reperfusion injury and injury due to heat shock (Snoeckx et al., 2001; Okubo et al., 2001 Jayakumar et al., 2001). The amount of Hsp70 directly correlates with the degree of cardioprotection conferred (Hutter et al., 1994). Our lab has demonstrated that chronic maternal hypoxia

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reduces the expression of Hsp70 and reduces recovery from heat shock in adult rat hearts (Li et al., 2004). Morgan (1989) demonstrated Sp1 binding to Hsp70 promoter promotes transcription activity. Interestingly, Morgan (1989) identified two Sp1 binding sites at the proximal promoter (-172 to -163 and -50 to -41) that possess CpG dinucleotides in their consensus sequence. Whether methylation of those binding sites reduces SP1 binding and Hsp70 promoter activity is an intriguing question. Whether those binding sites are sensitive to chronic hypoxia in fetal hearts is interesting and warrants future investigation.

Previous studies in our lab have shown prenatal hypoxia causes the increased expression of β_2AR and the ratio of $G_s\alpha/G_i\alpha$. The functional significance of this finding is not clear. However, studies in mice have shown overexpression of β_2AR increased contractility and ischemia reperfusion injury in male but not female hearts (Cross et al., 2002). In addition, β_2AR knockout mice showed reduce injury from middle cerebral artery occlusion or wild-type mice pretreated with β_2AR antagonist. Studies looking at epigenetic modifications to the proximal promoter of β_2AR gene are few, but recent work done by McAlees et al. (2011) found that methylation of proximal promoter of β_2AR occurred in CD4+ T cells. Perhaps this region is sensitive to intrauterine stress in fetal cardiomyocytes, resulting in changes in methylation patterns for β_2AR gene leading to enhanced vulnerability to ischemia reperfusion injury.

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Conclusion

The findings from this study demonstrate chronic hypoxia directly regulates PKCε expression in fetal hearts through increase methylation of two Sp1 binding sites located at the proximal promoter. The mechanism initiating the epigenetic modification of PKCε promoter involves the elevation of ROS but does not require HIF-1α nuclear accumulation. Our findings contribute significant understanding to the processes involved in fetal programming. Indeed, the methods used to elucidate PKCε regulation in the context of the developing myocardium may be applied to other tissues. The effect of chronic intrauterine hypoxia on cardioprotective, neuroprotective, and tumorigenic genes may shed new understanding that will allow for future interventions, which may protect against the onset of adult diseases.

المنارة للاستشارات
www.manaraa.com

REFERENCES

Adelman DM, G.M., Nagy A, Simon MC, Maltepe E. (2000) Placental cell fates are regulated in vivo by HIF-mediated hypoxia responses. Genes Dev. 14, 3191-203.

Arcasoy, M.O. (2008) The non-haematopoietic biological effects of erythropoietin. Br J Haematol 141, 14-31.

Ardehali, H. (2006) Signaling mechanisms in ischemic preconditioning: interaction of PKCepsilon and MitoK(ATP) in the inner membrane of mitochondria. Circ Res 99, 798-800.

Ascuitto RJ, R.-A.N. (1996) Substrate metabolism in the developing heart. Semin Perinatol 20, 542-63.

Bae, S., Xiao, Y., Li, G., Casiano, C.A. and Zhang, L. (2003) Effect of maternal chronic hypoxic exposure during gestation on apoptosis in fetal rat heart. Am J Physiol Heart Circ Physiol 285, H983-90.

Barbera, A., Giraud, G.D., Reller, M.D., Maylie, J., Morton, M.J. and Thornburg, K.L. (2000) Right ventricular systolic pressure load alters myocyte maturation in fetal sheep. Am J Physiol Regul Integr Comp Physiol 279, R1157-64.

Barbosky L, L.D., Karunamuni G, Wikenheiser JC, Doughman YQ, Visconti RP, Burch JB, Watanabe M. (2006) Apoptosis in the developing mouse heart. Dev Dyn. 235, 2592-602.

Barker, D.J. and Osmond, C. (1986) Infant mortality, childhood nutrition, and ischaemic heart disease in England and Wales. Lancet 1, 1077-81.

Barker, D.J., Osmond, C., Golding, J., Kuh, D. and Wadsworth, M.E. (1989) Growth in utero, blood pressure in childhood and adult life, and mortality from cardiovascular disease. BMJ 298, 564-7.

Barker, D.J. (1990) The fetal and infant origins of adult disease. BMJ 301, 1111.

Barker, D.J. (1994) Outcome of low birthweight. Horm Res 42, 223-30.

Barker, D.J. (1997) Fetal nutrition and cardiovascular disease in later life. Br Med Bull 53, 96-108.

المنارة للاستشارات
www.manaraa.com

Barnett ME, M.D., Takemoto DJ. (2007) Protein Kinase C as a Stress Sensor. Cell Signal 19, 1820-9.

Becker FF, H.P., Ruchirawat M, Lapeyre JN. (1985) Perturbation of maintenance and de novo DNA methylation in vitro by UVB (280-340 nm)-induced pyrimidine photodimers. Proc Natl Acad Sci U S A 82, 6055-9.

Bettencourt-Dias, M., Mittnacht, S. and Brockes, J.P. (2003) Heterogeneous proliferative potential in regenerative adult newt cardiomyocytes. J Cell Sci 116, 4001-9.

Bertolotto, C., Maulon, L., Filippa, N., Baier, G. and Auberger, P. (2000) Protein kinase C theta and epsilon promote T-cell survival by a rsk-dependent phosphorylation and inactivation of BAD. J Biol Chem 275, 37246-50.

Beyer, S., Kristensen, M.M., Jensen, K.S., Johansen, J.V. and Staller, P. (2008) The histone demethylases JMJD1A and JMJD2B are transcriptional targets of hypoxia-inducible factor HIF. J Biol Chem 283, 36542-52.

Bhusari, S.S., Dobosy, J.R., Fu, V., Almassi, N., Oberley, T. and Jarrard, D.F. (2010) Superoxide dismutase 1 knockdown induces oxidative stress and DNA methylation loss in the prostate. Epigenetics 5, 402-9.

Bishai, J.M., Blood, A.B., Hunter, C.J., Longo, L.D. and Power, G.G. (2003) Fetal lamb cerebral blood flow (CBF) and oxygen tensions during hypoxia: a comparison of laser Doppler and microsphere measurements of CBF. J Physiol 546, 869-78.

Blackburn S. T. (2007) Maternal, Fetal, & Neonatal Physiology: A Clinical Perspective. 3rd ed. Maryland Heights, MO: Elsevier Science.

Bogoyevitch MA, P.P., Sugden PH. (1993) Characterization of protein kinase C isotype expression in adult rat heart. Protein kinase C-epsilon is a major isotype present, and it is activated by phorbol esters, epinephrine, and endothelin. Circ Res 72, 757-67.

Brenner C, D.R., Didelot C, Loriot A, Viré E, De Smet C, Gutierrez A, Danovi D, Bernard D, Boon T, Pelicci PG, Amati B, Kouzarides T, de Launoit Y, Di Croce L, Fuks F. (2005) Myc represses transcription through recruitment of DNA methyltransferase corepressor. EMBO J 24, 336-46.

Breuer, E., Barta, E., Zlatos, L. and Pappova, E. (1968) Developmental changes of myocardial metabolism. II. Myocardial metabolism of fatty acids in the early postnatal period in dogs. Biol Neonat 12, 54-64.

Brigati C, B.B., di Vinci A, Casciano I, Allemanni G, Forlani A, Borzì L, Romani M. (2010) Inflammation, HIF-1, and the epigenetics that follows. Mediators Inflamm. Epub 2010 Dec 19

المنارة للاستشارات
www.manaraa.com

Browne, V.A., Stiffel, V.M., Pearce, W.J., Longo, L.D. and Gilbert, R.D. (1997) Activator calcium and myocardial contractility in fetal sheep exposed to long-term high-altitude hypoxia. Am J Physiol 272, H1196-204.

Budas, G.R., Churchill, E.N. and Mochly-Rosen, D. (2007) Cardioprotective mechanisms of PKC isozyme-selective activators and inhibitors in the treatment of ischemia-reperfusion injury. Pharmacol Res 55, 523-36.

Cannito, S., Novo, E., Compagnone, A., Valfre di Bonzo, L., Busletta, C., Zamara, E., Paternostro, C., Povero, D., Bandino, A., Bozzo, F., Cravanzola, C., Bravoco, V., Colombatto, S. and Parola, M. (2008) Redox mechanisms switch on hypoxia-dependent epithelial-mesenchymal transition in cancer cells. Carcinogenesis 29, 2267-78.

Cao P, D.Z., Wan M, Huang W, Cramer SD, Xu J, Lei M, Sui G. (2010) MicroRNA-101 negatively regulates Ezh2 and its expression is modulated by androgen receptor and HIF-1alpha/HIF-1beta. Mol Cancer 17, 108

Chan, Y., Fish, J.E., D'Abreo, C., Lin, S., Robb, G.B., Teichert, A.M., Karantzoulis-Fegaras, F., Keightley, A., Steer, B.M. and Marsden, P.A. (2004) The cell-specific expression of endothelial nitric-oxide synthase: a role for DNA methylation. J Biol Chem 279, 35087-100.

Chandel, N.S., Maltepe, E., Goldwasser, E., Mathieu, C.E., Simon, M.C. and Schumacker, P.T. (1998) Mitochondrial reactive oxygen species trigger hypoxia-induced transcription. Proc Natl Acad Sci U S A 95, 11715-20.

Chandel NS, M.D., Feliciano CE, Wood TM, Melendez JA, Rodriguez AM, Schumacker PT. (2000) Reactive oxygen species generated at mitochondrial complex III stabilize hypoxia-inducible factor-1alpha during hypoxia: a mechanism of O2 sensing. J Biol Chem 275, 25130-8.

Chandel, N.S. (2010) Mitochondrial complex III: an essential component of universal oxygen sensing machinery? Respir Physiol Neurobiol 174, 175-81.

Chapman, J.D. (1979) Hypoxic sensitizers--implications for radiation therapy. N Engl J Med 301, 1429-32.

Charron, C.E., Chou, P.C., Coutts, D.J., Kumar, V., To, M., Akashi, K., Pinhu, L., Griffiths, M., Adcock, I.M., Barnes, P.J. and Ito, K. (2009) Hypoxia-inducible factor 1alpha induces corticosteroid-insensitive inflammation via reduction of histone deacetylase-2 transcription. J Biol Chem 284, 36047-54.

Chen H, Y.Y., Davidson TL, Shinkai Y, Costa M. (2006) Hypoxic stress induces dimethylated histone H3 lysine 9 through histone methyltransferase G9a in mammalian cells. Cancer Res 66, 9009-16.

المنارة للاستشارات
www.manaraa.com

Chen, W., Ostrowski, R.P., Obenaus, A. and Zhang, J.H. (2009) Prodeath or prosurvival: two facets of hypoxia inducible factor-1 in perinatal brain injury. Exp Neurol 216, 7-15.

Chong, K.Y., Lai, C.C., Lille, S., Chang, C. and Su, C.Y. (1998) Stable overexpression of the constitutive form of heat shock protein 70 confers oxidative protection. J Mol Cell Cardiol 30, 599-608.

Christman JK, S.G., Marasco CJ, Sufrin JR. (1995) 5-Methyl-2'-deoxycytidine in single-stranded DNA can act in cis to signal de novo DNA methylation. Proc Natl Acad Sci U S A 92, 7347-51.

Chua, Y.L., Dufour, E., Dassa, E.P., Rustin, P., Jacobs, H.T., Taylor, C.T. and Hagen, T. (2010) Stabilization of hypoxia-inducible factor-1alpha protein in hypoxia occurs independently of mitochondrial reactive oxygen species production. J Biol Chem 285, 31277-84.

Clerk, A., Bogoyevitch, M.A., Fuller, S.J., Lazou, A., Parker, P.J. and Sugden, P.H. (1995) Expression of protein kinase C isoforms during cardiac ventricular development. Am J Physiol 269, H1087-97.

Compernolle V, B.K., Franco D, Moorman A, Dewerchin M, Collen D, Carmeliet P. (2003) Cardia bifida, defective heart development and abnormal neural crest migration in embryos lacking hypoxia-inducible factor-1alpha. Cardiovasc Res. 60, 569-79.

Covassin LD, V.J., Kacergis MC, Weinstein BM, Lawson ND. (2006) Distinct genetic interactions between multiple Vegf receptors are required for development of different blood vessel types in zebrafish. Proc Natl Acad Sci U S A 103, 6554-9.

Covello, K.L., Kehler, J., Yu, H., Gordan, J.D., Arsham, A.M., Hu, C.J., Labosky, P.A., Simon, M.C. and Keith, B. (2006) HIF-2alpha regulates Oct-4: effects of hypoxia on stem cell function, embryonic development, and tumor growth. Genes Dev 20, 557-70.

Cowden Dahl, K.D., Fryer, B.H., Mack, F.A., Compernolle, V., Maltepe, E., Adelman, D.M., Carmeliet, P. and Simon, M.C. (2005) Hypoxia-inducible factors 1alpha and 2alpha regulate trophoblast differentiation. Mol Cell Biol 25, 10479-91.

Cross, H.R., Murphy, E., Koch, W.J. and Steenbergen, C. (2002) Male and female mice overexpressing the beta(2)-adrenergic receptor exhibit differences in ischemia/reperfusion injury: role of nitric oxide. Cardiovasc Res 53, 662-71.

Csonka, C., Szilvassy, Z., Fulop, F., Pali, T., Blasig, I.E., Tosaki, A., Schulz, R. and Ferdinandy, P. (1999) Classic preconditioning decreases the harmful

المنارة للاستشارات
www.manaraa.com

accumulation of nitric oxide during ischemia and reperfusion in rat hearts. Circulation 100, 2260-6.

Dai, D.F., Chen, T., Szeto, H., Nieves-Cintron, M., Kutyavin, V., Santana, L.F. and Rabinovitch, P.S. (2011) Mitochondrial Targeted Antioxidant Peptide Ameliorates Hypertensive Cardiomyopathy. J Am Coll Cardiol.

Das, B. and Sarkar, C. (2003) Cardiomyocyte mitochondrial KATP channels participate in the antiarrhythmic and antiinfarct effects of KATP activators during ischemia and reperfusion in an intact anesthetized rabbit model. Pol J Pharmacol 55, 771-86.

de Herreros, A.G., Peiro, S., Nassour, M. and Savagner, P. (2010) Snail family regulation and epithelial mesenchymal transitions in breast cancer progression. J Mammary Gland Biol Neoplasia 15, 135-47.

Delcuve, G.P., Rastegar, M. and Davie, J.R. (2009) Epigenetic control. J Cell Physiol 219, 243-50.

Dewing, P., Shi, T., Horvath, S. and Vilain, E. (2003) Sexually dimorphic gene expression in mouse brain precedes gonadal differentiation. Brain Res Mol Brain Res 118, 82-90.

Dolinoy, D.C., Weidman, J.R., Waterland, R.A. and Jirtle, R.L. (2006) Maternal genistein alters coat color and protects Avy mouse offspring from obesity by modifying the fetal epigenome. Environ Health Perspect 114, 567-72.

Donkena, K.V., Karnes, R.J. and Young, C.Y. (2010) Vitamins and prostate cancer risk. Molecules 15, 1762-83.

Druyan S, C.A., Ashwell CM. (2007) The expression patterns of hypoxia-inducing factor subunit alpha-1, heme oxygenase, hypoxia upregulated protein 1, and cardiac troponin T during development of the chicken heart. Poult Sci. 86, 2384-9.

Duranteau, J., Chandel, N.S., Kulisz, A., Shao, Z. and Schumacker, P.T. (1998) Intracellular signaling by reactive oxygen species during hypoxia in cardiomyocytes. J Biol Chem 273, 11619-24.

Edelberg, J.M., Aird, W.C., Wu, W., Rayburn, H., Mamuya, W.S., Mercola, M. and Rosenberg, R.D. (1998) PDGF mediates cardiac microvascular communication. J Clin Invest 102, 837-43.

Elmes, M.J., McMullen, S., Gardner, D.S. and Langley-Evans, S.C. (2008) Prenatal diet determines susceptibility to cardiac ischaemia-reperfusion injury following treatment with diethylmaleic acid and N-acetylcysteine. Life Sci 82, 149-55.

المنارة للاستشارات
www.manaraa.com

Endo, K., Ueda, T., Ueyama, J., Ohta, T. and Terada, T. (2000) Immunoreactive E-cadherin, alpha-catenin, beta-catenin, and gamma-catenin proteins in hepatocellular carcinoma: relationships with tumor grade, clinicopathologic parameters, and patients' survival. Hum Pathol 31, 558-65.

Engel, F.B., Schebesta, M., Duong, M.T., Lu, G., Ren, S., Madwed, J.B., Jiang, H., Wang, Y. and Keating, M.T. (2005) p38 MAP kinase inhibition enables proliferation of adult mammalian cardiomyocytes. Genes Dev 19, 1175-87.

Evans SM, D.K., Chalian AA, Mick R, Zhang PJ, Hahn SM, Quon H, Lustig R, Weinstein GS, Koch CJ. (2007) Patterns and levels of hypoxia in head and neck squamous cell carcinomas and their relationship to patient outcome. Int J Radiat Oncol Biol Phys 69, 1024-31.

Evans SM, J.K., Jenkins WT, Dilling T, Judy KD, Schrlau A, Judkins A, Hahn SM, Koch CJ. (2008) Imaging and analytical methods as applied to the evaluation of vasculature and hypoxia in human brain tumors. Radiat Res 170, 677-90.

Fisher SA, B.W. (2007) Role of hypoxia in the evolution and development of the cardiovascular system. Antioxid Redox Signal. 9, 1339-52.

Fishman, M.C. and Chien, K.R. (1997) Fashioning the vertebrate heart: earliest embryonic decisions. Development 124, 2099-117.

Forsythe, J.A., Jiang, B.H., Iyer, N.V., Agani, F., Leung, S.W., Koos, R.D. and Semenza, G.L. (1996) Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. Mol Cell Biol 16, 4604-13.

Flueck, M. (2009) Plasticity of the muscle proteome to exercise at altitude. High Alt Med Biol 10, 183-93.

Galanis, A., Pappa, A., Giannakakis, A., Lanitis, E., Dangaj, D. and Sandaltzopoulos, R. (2008) Reactive oxygen species and HIF-1 signalling in cancer. Cancer Lett 266, 12-20.

Ghatpande SK, B.C.J., Rivkees SA, Wendler CC. (2008) Hypoxia induces cardiac malformations via A1 adenosine receptor activation in chicken embryos. Birth Defects Res A Clin Mol Teratol. 82, 121-30.

Gilbert, R.D. (1998) Fetal myocardial responses to long-term hypoxemia. Comp Biochem Physiol A Mol Integr Physiol 119, 669-74.

Giordano FJ, G.H., Williams SP, VanBruggen N, Bunting S, Ruiz-Lozano P, Gu Y, Nath AK, Huang Y, Hickey R, Dalton N, Peterson KL, Ross J Jr, Chien KR, Ferrara N. (2001) A cardiac myocyte vascular endothelial growth factor paracrine pathway is required to maintain cardiac function. Proc Natl Acad Sci U S A. 98, 5780-5.

المنارة للاستشارات

www.manaraa.com

Gobbi, G., Masselli, E., Micheloni, C., Nouvenne, A., Russo, D., Santi, P., Matteucci, A., Cocco, L., Vitale, M. and Mirandola, P. (2010) Hypoxia-induced down-modulation of PKCepsilon promotes trail-mediated apoptosis of tumor cells. Int J Oncol 37, 719-29.

Goldberg, M., Zhang, H.L. and Steinberg, S.F. (1997) Hypoxia alters the subcellular distribution of protein kinase C isoforms in neonatal rat ventricular myocytes. J Clin Invest 99, 55-61.

Gonzalez-Reyes, S., Martinez, L. and Tovar, J.A. (2005) Effects of prenatal vitamins A, E, and C on the hypoplastic hearts of fetal rats with diaphragmatic hernia. J Pediatr Surg 40, 1269-74.

Gordan, J.D. and Simon, M.C. (2007) Hypoxia-inducible factors: central regulators of the tumor phenotype. Curr Opin Genet Dev 17, 71-7.

Gordan, J.D., Lal, P., Dondeti, V.R., Letrero, R., Parekh, K.N., Oquendo, C.E., Greenberg, R.A., Flaherty, K.T., Rathmell, W.K., Keith, B., Simon, M.C. and Nathanson, K.L. (2008) HIF-alpha effects on c-Myc distinguish two subtypes of sporadic VHL-deficient clear cell renal carcinoma. Cancer Cell 14, 435-46.

Graf AV, M.M., Maklakova AS, Sokolova NA, Kudryashova NY, Krushinskaya YV, Goncharenko EN, Neverova ME, Fidelina OV. (2006) Effect of hypoxia during early organogenesis on cardiac activity and noradrenergic regulation in the postnatal period. Bull Exp Biol Med. 142, 543-5.

Graham RM, F.D., Thompson JW, Haliko S, Li H, Wasserlauf BJ, Spiga MG, Bishopric NH, Webster KA. (2004) A unique pathway of cardiac myocyte death caused by hypoxia-acidosis. J Exp Biol. 207, 3189-200.

Granger, A., Abdullah, I., Huebner, F., Stout, A., Wang, T., Huebner, T., Epstein, J.A. and Gruber, P.J. (2008) Histone deacetylase inhibition reduces myocardial ischemia-reperfusion injury in mice. FASEB J 22, 3549-60.

Gray, M.O., Karliner, J.S. and Mochly-Rosen, D. (1997) A selective epsilon-protein kinase C antagonist inhibits protection of cardiac myocytes from hypoxia-induced cell death. J Biol Chem 272, 30945-51.

Gross MW, K.U., Groebe K, Franko AJ, Mueller-Klieser W. (1995) Calibration of misonidazole labeling by simultaneous measurement of oxygen tension and labeling density in multicellular spheroids. Int J Cancer. 61, 567-73.

Guzy RD, S.P. (2006) Oxygen sensing by mitochondria at complex III: the paradox of increased reactive oxygen species during hypoxia. Exp Physiol 91, 807-19.

المنارة للاستشارات
www.manaraa.com

Harper, M.T. and Poole, A.W. (2010) Diverse functions of protein kinase C isoforms in platelet activation and thrombus formation. J Thromb Haemost 8, 454-62.

Harris, C.M., Sanders, S.A. and Massey, V. (1999) Role of the flavin midpoint potential and NAD binding in determining NAD versus oxygen reactivity of xanthine oxidoreductase. J Biol Chem 274, 4561-9.

Hausenloy DJ, D.M., Yellon DM. (2003) Inhibiting mitochondrial permeability transition pore opening at reperfusion protects against ischaemia-reperfusion injury. Cardiovasc Res 60, 617-25.

He, M., Vanaja, D.K., Karnes, R.J. and Young, C.Y. (2009) Epigenetic regulation of Myc on retinoic acid receptor beta and PDLIM4 in RWPE1 cells. Prostate 69, 1643-50.

Hescheler, J., Meyer, R., Plant, S., Krautwurst, D., Rosenthal, W. and Schultz, G. (1991) Morphological, biochemical, and electrophysiological characterization of a clonal cell (H9c2) line from rat heart. Circ Res 69, 1476-86.

Hirst, M. and Marra, M.A. (2009) Epigenetics and human disease. Int J Biochem Cell Biol 41, 136-46.

Hlavackova, M., Kozichova, K., Neckar, J., Kolar, F., Musters, R.J., Novak, F. and Novakova, O. (2010) Up-regulation and redistribution of protein kinase C-delta in chronically hypoxic heart. Mol Cell Biochem 345, 271-82.

Hoerter, J.A. and Vassort, G. (1982) Participation of the sarcolemma in the control of relaxation of the mammalian heart during perinatal development. Adv Myocardiol 3, 373-80.

Hongpaisan, J., Sun, M.K. and Alkon, D.L. (2011) PKC epsilon activation prevents synaptic loss, Abeta elevation, and cognitive deficits in Alzheimer's disease transgenic mice. J Neurosci 31, 630-43.

Hu, C.J., Wang, L.Y., Chodosh, L.A., Keith, B. and Simon, M.C. (2003) Differential roles of hypoxia-inducible factor 1alpha (HIF-1alpha) and HIF-2alpha in hypoxic gene regulation. Mol Cell Biol 23, 9361-74.

Hurn PD, V.S., Hagberg H. (2005) Adult or perinatal brain injury: does sex matter? Stroke 36, 193-5.

Hutter MM, S.R., Barbosa V, Wolfe CL. (1994) Heat-shock protein induction in rat hearts. A direct correlation between the amount of heat-shock protein induced and the degree of myocardial protection. Circulation 89, 355-60.

المنارة للاستشارات
www.manaraa.com

Hwang JM, W.Y., Lin JA, Bau DT, Ko FY, Tsai FJ, Tsai CH, Wu CH, Lin PC, Huang CY, Kuo WW. (2008) Hypoxia-induced compensatory effect as related to Shh and HIF-1alpha in ischemia embryo rat heart. Mol Cell Biochem. 311, 179-87.

Imai, T., Horiuchi, A., Wang, C., Oka, K., Ohira, S., Nikaido, T. and Konishi, I. (2003) Hypoxia attenuates the expression of E-cadherin via up-regulation of SNAIL in ovarian carcinoma cells. Am J Pathol 163, 1437-47.

Inagaki, K., Begley, R., Ikeno, F. and Mochly-Rosen, D. (2005) Cardioprotection by epsilon-protein kinase C activation from ischemia: continuous delivery and antiarrhythmic effect of an epsilon-protein kinase C-activating peptide. Circulation 111, 44-50.

Inagaki K, C.E., Mochly-Rosen D. (2006) Epsilon protein kinase C as a potential therapeutic target for the ischemic heart. Cardiovasc Res. 70, 222-30.

Ivanovic, Z. (2009) Hypoxia or in situ normoxia: The stem cell paradigm. J Cell Physiol 219, 271-5.

Iyer NV, K.L., Agani F, Leung SW, Laughner E, Wenger RH, Gassmann M, Gearhart JD, Lawler AM, Yu AY, Semenza GL. (1998) Cellular and developmental control of O2 homeostasis by hypoxia-inducible factor 1 alpha. Genes Dev. 12, 149-62.

Jaburek, M., Costa, A.D., Burton, J.R., Costa, C.L. and Garlid, K.D. (2006) Mitochondrial PKC epsilon and mitochondrial ATP-sensitive K+ channel copurify and coreconstitute to form a functioning signaling module in proteoliposomes. Circ Res 99, 878-83.

Jauniaux E, W.A., Burton G. (2001) Evaluation of respiratory gases and acid-base gradients in human fetal fluids and uteroplacental tissue between 7 and 16 weeks' gestation. Am J Obstet Gynecol 184, 998-1003.

Jayakumar, J., Suzuki, K., Sammut, I.A., Smolenski, R.T., Khan, M., Latif, N., Abunasra, H., Murtuza, B., Amrani, M. and Yacoub, M.H. (2001) Heat shock protein 70 gene transfection protects mitochondrial and ventricular function against ischemia-reperfusion injury. Circulation 104, I303-7.

Jelinek, J. and Jensen, A. (1991) Catecholamine concentrations in plasma and organs of the fetal guinea pig during normoxemia, hypoxemia, and asphyxia. J Dev Physiol 15, 145-52.

Jensen, A., Garnier, Y. and Berger, R. (1999) Dynamics of fetal circulatory responses to hypoxia and asphyxia. Eur J Obstet Gynecol Reprod Biol 84, 155-72.

Jensen GM, M.L. (1997) The effect of high altitude and other risk factors on birthweight: independent or interactive effects? Am J Public health 87, 1003-1007.

المنارة للاستشارات
www.manaraa.com

Jiang, B.H., Rue, E., Wang, G.L., Roe, R. and Semenza, G.L. (1996a) Dimerization, DNA binding, and transactivation properties of hypoxia-inducible factor 1. J Biol Chem 271, 17771-8.

Jiang, B.H., Semenza, G.L., Bauer, C. and Marti, H.H. (1996b) Hypoxia-inducible factor 1 levels vary exponentially over a physiologically relevant range of O2 tension. Am J Physiol 271, C1172-80.

Jones, S.P. and Bolli, R. (2006) The ubiquitous role of nitric oxide in cardioprotection. J Mol Cell Cardiol 40, 16-23.

Jonker, S.S., Zhang, L., Louey, S., Giraud, G.D., Thornburg, K.L. and Faber, J.J. (2007a) Myocyte enlargement, differentiation, and proliferation kinetics in the fetal sheep heart. J Appl Physiol 102, 1130-42.

Jonker, S.S., Faber, J.J., Anderson, D.F., Thornburg, K.L., Louey, S. and Giraud, G.D. (2007b) Sequential growth of fetal sheep cardiac myocytes in response to simultaneous arterial and venous hypertension. Am J Physiol Regul Integr Comp Physiol 292, R913-9.

Josko, J., Gwozdz, B., Jedrzejowska-Szypulka, H. and Hendryk, S. (2000) Vascular endothelial growth factor (VEGF) and its effect on angiogenesis. Med Sci Monit 6, 1047-52.

Kabir AM, C.J., Tanno M, Cao X, Hothersall JS, Dashnyam S, Gorog DA, Bellahcene M, Shattock MJ, Marber MS. (2006) Cardioprotection initiated by reactive oxygen species is dependent on activation of PKCepsilon. Am J Physiol Heart Circ Physiol 291, H1893-9.

Kalinich, J.F., Catravas, G.N. and Snyder, S.L. (1989) The effect of gamma radiation on DNA methylation. Radiat Res 117, 185-97.

Kamitomo, M., Longo, L.D. and Gilbert, R.D. (1994) Cardiac function in fetal sheep during two weeks of hypoxemia. Am J Physiol 266, R1778-85.

Kamitomo, M., Onishi, J., Gutierrez, I., Stiffel, V.M. and Gilbert, R.D. (2002) Effects of long-term hypoxia and development on cardiac contractile proteins in fetal and adult sheep. J Soc Gynecol Investig 9, 335-41.

Kayyali, U.S., Donaldson, C., Huang, H., Abdelnour, R. and Hassoun, P.M. (2001) Phosphorylation of xanthine dehydrogenase/oxidase in hypoxia. J Biol Chem 276, 14359-65.

Kim, E., Raval, A.P., Defazio, R.A. and Perez-Pinzon, M.A. (2007) Ischemic preconditioning via epsilon protein kinase C activation requires cyclooxygenase-2 activation in vitro. Neuroscience 145, 931-41.

المنارة للاستشارات
www.manaraa.com

Kim, M.H., Jung, Y.S., Moon, C.H., Jeong, E.M., Lee, S.H., Baik, E.J. and Moon, C.K. (2003) Isoform-specific induction of PKC-epsilon by high glucose protects heart-derived H9c2 cells against hypoxic injury. Biochem Biophys Res Commun 309, 1-6.

Kim, M.J., Moon, C.H., Kim, M.Y., Kim, M.H., Lee, S.H., Baik, E.J. and Jung, Y.S. (2004) Role of PKC-delta during hypoxia in heart-derived H9c2 cells. Jpn J Physiol 54, 405-14.

Krieg M, H.R., Brauch H, Acker T, Flamme I, Plate KH. (2000) Up-regulation of hypoxia-inducible factors HIF-1alpha and HIF-2alpha under normoxic conditions in renal carcinoma cells by von Hippel-Lindau tumor suppressor gene loss of function. Oncogene 19, 5435-43.

Koch CJ, E.S. (2003) Non-invasive PET and SPECT imaging of tissue hypoxia using isotopically labeled 2-nitroimidazoles. Adv Exp Med Biol 510, 285-92.

Kondo K, K.J., Nakamura E, Lechpammer M, Kaelin WG Jr. (2002) Inhibition of HIF is necessary for tumor suppression by the von Hippel-Lindau protein. Cancer Cell 1, 237-46.

Koshiji, M., Kageyama, Y., Pete, E.A., Horikawa, I., Barrett, J.C. and Huang, L.E. (2004) HIF-1alpha induces cell cycle arrest by functionally counteracting Myc. EMBO J 23, 1949-56.

Kotch LE, I.N., Laughner E, Semenza GL. (1999) Defective vascularization of HIF-1alpha-null embryos is not associated with VEGF deficiency but with mesenchymal cell death. Dev Biol. 209, 254-67.

Kumar S, S.X., Wiseman DA, Tian J, Umapathy NS, Verin AD, Black SM. (2009) Hydrogen peroxide decreases endothelial nitric oxide synthase promoter activity through the inhibition of Sp1 activity. DNA Cell Biol 28, 119-29.

Ladoux, A. and Frelin, C. (1997) Cardiac expressions of HIF-1 alpha and HLF/EPAS, two basic loop helix/PAS domain transcription factors involved in adaptative responses to hypoxic stresses. Biochem Biophys Res Commun 240, 552-6.

Lainakis, G. and Bamias, A. (2008) Targeting angiogenesis in renal cell carcinoma. Curr Cancer Drug Targets 8, 349-58.

Lawrence, J., Xiao, D., Xue, Q., Rejali, M., Yang, S. and Zhang, L. (2008) Prenatal nicotine exposure increases heart susceptibility to ischemia/reperfusion injury in adult offspring. J Pharmacol Exp Ther 324, 331-41.

المنارة للاستشارات
www.manaraa.com

Lawrence, J., Chen, M., Xiong, F., Xiao, D., Zhang, H., Buchholz, J.N. and Zhang, L. (2011) Foetal nicotine exposure causes PKCepsilon gene repression by promoter methylation in rat hearts. Cardiovasc Res 89, 89-97.

Lee, Y.M., Jeong, C.H., Koo, S.Y., Son, M.J., Song, H.S., Bae, S.K., Raleigh, J.A., Chung, H.Y., Yoo, M.A. and Kim, K.W. (2001) Determination of hypoxic region by hypoxia marker in developing mouse embryos in vivo: a possible signal for vessel development. Dev Dyn 220, 175-86.

Lesscher, H.M., Wallace, M.J., Zeng, L., Wang, V., Deitchman, J.K., McMahon, T., Messing, R.O. and Newton, P.M. (2009) Amygdala protein kinase C epsilon controls alcohol consumption. Genes Brain Behav 8, 493-9.

Levitt, N.S., Lindsay, R.S., Holmes, M.C. and Seckl, J.R. (1996) Dexamethasone in the last week of pregnancy attenuates hippocampal glucocorticoid receptor gene expression and elevates blood pressure in the adult offspring in the rat. Neuroendocrinology 64, 412-8.

Li G, X.Y., Estrella JL, Ducsay CA, Gilbert RD, Zhang L. (2003) Effect of fetal hypoxia on heart susceptibility to ischemia and reperfusion injury in the adult rat. J Soc Gynecol Investig. 10, 265-74.

Li G, B.S., Zhang L. (2004) Effect of prenatal hypoxia on heat stress-mediated cardioprotection in adult rat heart. Am J Physiol Heart Circ Physiol. 286, 1717-9.

Lim, S.O., Gu, J.M., Kim, M.S., Kim, H.S., Park, Y.N., Park, C.K., Cho, J.W., Park, Y.M. and Jung, G. (2008) Epigenetic changes induced by reactive oxygen species in hepatocellular carcinoma: methylation of the E-cadherin promoter. Gastroenterology 135, 2128-40, 2140 e1-8.

Liu H, F.S. (2008) Hypoxia-inducible transcription factor-1alpha triggers an autocrine survival pathway during embryonic cardiac outflow tract remodeling. Circ Res 102, 1331-9.

Lucas, A. (1991) Programming by early nutrition in man. Ciba Found Symp 156, 38-50; discussion 50-5.

Lueder, F.L., Kim, S.B., Buroker, C.A., Bangalore, S.A. and Ogata, E.S. (1995) Chronic maternal hypoxia retards fetal growth and increases glucose utilization of select fetal tissues in the rat. Metabolism 44, 532-7.

Mahon PC, H.K., Semenza GL. (2001) FIH-1: a novel protein that interacts with HIF-1alpha and VHL to mediate repression of HIF-1 transcriptional activity. Genes Dev 15, 2675-86.

المنارة للاستشارات
www.manaraa.com

Maloyan, A., Eli-Berchoer, L., Semenza, G.L., Gerstenblith, G., Stern, M.D. and Horowitz, M. (2005) HIF-1alpha-targeted pathways are activated by heat acclimation and contribute to acclimation-ischemic cross-tolerance in the heart. Physiol Genomics 23, 79-88.

Maltepe E, K.G., Okazaki KM, Red-Horse K, Mak W, Simon MC, Fisher SJ. (2005) Hypoxia-inducible factor-dependent histone deacetylase activity determines stem cell fate in the placenta. Development 132, 3393-403.

Mansfield, K.D., Simon, M.C. and Keith, B. (2004) Hypoxic reduction in cellular glutathione levels requires mitochondrial reactive oxygen species. J Appl Physiol 97, 1358-66.

Martin C, Y.A., Jiang BH, Davis L, Kimberly D, Hohimer AR, Semenza GL (1998) Cardiac hypertrophy in chronically anemic fetal sheep: Increased vascularization is associated with increased myocardial expression of vascular endothelial growth factor and hypoxia-inducible factor 1. Am J Obstet Gynecol 178, 527-34.

Martyn, U. and Schulte-Merker, S. (2004) Zebrafish neuropilins are differentially expressed and interact with vascular endothelial growth factor during embryonic vascular development. Dev Dyn 231, 33-42.

Marzo F, L.A., Coluzzi G, Santucci E, Tarantino F, Rio T, Conti E, Autore C, Agati L, Andreotti F. (2008) Erythropoietin in heart and vessels: focus on transcription and signalling pathways. J Thromb Thrombolysis 26, 183-7.

Mao C, H.J., Ge J, Hu Y, Ding Y, Zhou Y, Zhang H, Xu Z, Zhang L. (2009) Changes of Renal AT(1)/AT(2) Receptors and Structures in Ovine Fetuses following Exposure to Long-Term Hypoxia. Am J Nephrol. 31, 141-150.

Mao, C., Hou, J., Ge, J., Hu, Y., Ding, Y., Zhou, Y., Zhang, H., Xu, Z. and Zhang, L. (2010) Changes of renal AT1/AT2 receptors and structures in ovine fetuses following exposure to long-term hypoxia. Am J Nephrol 31, 141-50.

Maynard, M.A., Evans, A.J., Shi, W., Kim, W.Y., Liu, F.F. and Ohh, M. (2007) Dominant-negative HIF-3 alpha 4 suppresses VHL-null renal cell carcinoma progression. Cell Cycle 6, 2810-6.

McAlees, J.W., Smith, L.T., Erbe, R.S., Jarjoura, D., Ponzio, N.M. and Sanders, V.M. (2011) Epigenetic regulation of beta2-adrenergic receptor expression in T(H)1 and T(H)2 cells. Brain Behav Immun 25, 408-15.

McKenna ES, and Roberts.C. (2009) Epigenetics and cancer without genomic instability. Cell Cycle 8, 23-6.

المنارة للاستشارات

www.manaraa.com

Meyer K, and Zhang L. (2007) Fetal programming of cardiac function and disease. Reprod Sci. 14, 209-16.

Meyer KD, Z.H., Zhang L. (2009A) Prenatal cocaine exposure abolished ischemic preconditioning-induced protection in adult male rat hearts: role of PKC{varepsilon}. Am J Physiol Heart Circ Physiol. 296, H1566-76.

Meyer, K., Zhang, H. and Zhang, L. (2009B) Direct effect of cocaine on epigenetic regulation of PKCvarepsilon gene repression in the fetal rat heart. J Mol Cell Cardiol.

Miranda, T.B. and Jones, P.A. (2007) DNA methylation: the nuts and bolts of repression. J Cell Physiol 213, 384-90.

Mitchell, J.A. and Van Kainen, B.R. (1992) Effects of alcohol on intrauterine oxygen tension in the rat. Alcohol Clin Exp Res 16, 308-10.

Moore, L.G., Niermeyer, S. and Zamudio, S. (1998) Human adaptation to high altitude: regional and life-cycle perspectives. Am J Phys Anthropol Suppl 27, 25-64.

Moore LG, Z.S., Zhuang J, Sun S, Droma T. (2001) Oxygen transport in tibetan women during pregnancy at 3,658 m. Am J Phys Anthropol 114, 42-53.

Morgan, W.D. (1989) Transcription factor Sp1 binds to and activates a human hsp70 gene promoter. Mol Cell Biol 9, 4099-104.

Myatt, L. (2006) Placental adaptive responses and fetal programming. J Physiol 572, 25-30.

Myers, D.A., Hyatt, K., Mlynarczyk, M., Bird, I.M. and Ducsay, C.A. (2005) Long-term hypoxia represses the expression of key genes regulating cortisol biosynthesis in the near-term ovine fetus. Am J Physiol Regul Integr Comp Physiol 289, R1707-14.

Nafee, T.M., Farrell, W.E., Carroll, W.D., Fryer, A.A. and Ismail, K.M. (2008) Epigenetic control of fetal gene expression. BJOG 115, 158-68.

Nanka, O., Valasek, P., Dvorakova, M. and Grim, M. (2006) Experimental hypoxia and embryonic angiogenesis. Dev Dyn 235, 723-33.

Nasevicius, A., Larson, J. and Ekker, S.C. (2000) Distinct requirements for zebrafish angiogenesis revealed by a VEGF-A morphant. Yeast 17, 294-301.

Netuka I, S.O., Maly J, Besik J, Neckar J, Kolar F, Ostadalova I, Pirk J, Ostadal B. (2006) Effect of perinatal hypoxia on cardiac tolerance to acute ischaemia in adult male and female rats. Clin Exp Pharmacol Physiol. 33, 714-9.

المنارة للاستشارات
www.manaraa.com

Newton, A.C. (1995) Protein kinase C: structure, function, and regulation. J Biol Chem 270, 28495-8.

Nichols, J., Zevnik, B., Anastassiadis, K., Niwa, H., Klewe-Nebenius, D., Chambers, I., Scholer, H. and Smith, A. (1998) Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. Cell 95, 379-91.

Nicholson, D.W., Ali, A., Thornberry, N.A., Vaillancourt, J.P., Ding, C.K., Gallant, M., Gareau, Y., Griffin, P.R., Labelle, M., Lazebnik, Y.A. and et al. (1995) Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. Nature 376, 37-43.

NCT01255384: (2010) Possible Epigenetic Changes in Offspring of Women With Pregestational and Gestational Diabetes: Molecular Studies of the Placenta and Cord Blood and Possible Correlation to Postnatal Development.

NCT00568113: (2007) Oral N-Acetyl Cysteine Can Prevent Preterm Labour in Multiparae With Previous Preterm Labour.

NCT00785954: (2008) Inhibition of Delta-protein Kinase C for the Reduction of Infarct Size in Acute Myocardial Infarction

NCT01015235: (2008) A Double-Blind, Randomized, Placebo- and Active-Comparator-Controlled, Single-Dose Study to Assess the Efficacy of KAI-1678 Administered by Subcutaneous Infusion for the Treatment of Postoperative Pain.

NCT00606164: (2008) A Randomized, Double-Blind, Placebo-Controlled, Parallel Groups, Study to Evaluate the Safety, Efficacy, Pharmacokinetics and Pharmacodynamics of Bryostatin 1 in Patients With Mild to Moderate Alzheimer's Disease.

NCT00546390: (2007) Protection of the Heart With Remote Ischemic Preconditioning During Heart Surgery: A Pilot Study.

Oberpriller, J.O. and Oberpriller, J.C. (1974) Response of the adult newt ventricle to injury. J Exp Zool 187, 249-53.

Okamura, T., Miura, T., Takemura, G., Fujiwara, H., Iwamoto, H., Kawamura, S., Kimura, M., Ikeda, Y., Iwatate, M. and Matsuzaki, M. (2000) Effect of caspase inhibitors on myocardial infarct size and myocyte DNA fragmentation in the ischemia-reperfused rat heart. Cardiovasc Res 45, 642-50.

المنارة للاستشارات
www.manaraa.com

Okano, M., Bell, D.W., Haber, D.A. and Li, E. (1999) DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell 99, 247-57.

Okubo, S., Wildner, O., Shah, M.R., Chelliah, J.C., Hess, M.L. and Kukreja, R.C. (2001) Gene transfer of heat-shock protein 70 reduces infarct size in vivo after ischemia/reperfusion in the rabbit heart. Circulation 103, 877-81.

Onishi, J., Browne, V.A., Kono, S., Stiffel, V.M. and Gilbert, R.D. (2004) Effects of long-term high-altitude hypoxia and troponin I phosphorylation on cardiac myofilament calcium responses in fetal and nonpregnant sheep. J Soc Gynecol Investig 11, 1-8.

Ornish D, W.G., Fair WR, Marlin R, Pettengill EB, Raisin CJ, Dunn-Emke S, Crutchfield L, Jacobs FN, Barnard RJ, Aronson WJ, McCormac P, McKnight DJ, Fein JD, Dnistrian AM, Weinstein J, Ngo TH, Mendell NR, Carroll PR. (2005) Intensive lifestyle changes may affect the progression of prostate cancer. J Urol 174, 1069-70.

Papetti, M. and Herman, I.M. (2002) Mechanisms of normal and tumor-derived angiogenesis. Am J Physiol Cell Physiol 282, C947-70.

Patterson, A.J. and Zhang, L. (2010) Hypoxia and fetal heart development. Curr Mol Med 10, 653-66.

Peng J, Z.L., Drysdale L, Fong GH. (2000) The transcription factor EPAS-1/hypoxia-inducible factor 2α plays an important role in vascular remodeling. Proc. Natl. Acad. Sci. 97, 8386-8391.

Peng, Y.W., Buller, C.L. and Charpie, J.R. (2011) Impact of N-acetylcysteine on Neonatal Cardiomyocyte Ischemia-Reperfusion Injury. Pediatr Res.

Ping, P., Song, C., Zhang, J., Guo, Y., Cao, X., Li, R.C., Wu, W., Vondriska, T.M., Pass, J.M., Tang, X.L., Pierce, W.M. and Bolli, R. (2002) Formation of protein kinase C(epsilon)-Lck signaling modules confers cardioprotection. J Clin Invest 109, 499-507.

Pollard, P.J., Loenarz, C., Mole, D.R., McDonough, M.A., Gleadle, J.M., Schofield, C.J. and Ratcliffe, P.J. (2008) Regulation of Jumonji-domain-containing histone demethylases by hypoxia-inducible factor (HIF)-1alpha. Biochem J 416, 387-94.

Portbury AL, C.R., Groelle M, McMillian MK, Elias A, Herlong JR, Rios M, Roffler-Tarlov S, Chikaraishi DM. (2003) Catecholamines act via a beta-adrenergic receptor to maintain fetal heart rate and survival. Am J Physiol Heart Circ Physiol. 284, 2069-77.

المنارة للاستشارات
www.manaraa.com

Poss, K.D., Wilson, L.G. and Keating, M.T. (2002) Heart regeneration in zebrafish. Science 298, 2188-90.

Pringle, K.G., Kind, K.L., Sferruzzi-Perri, A.N., Thompson, J.G. and Roberts, C.T. (2010) Beyond oxygen: complex regulation and activity of hypoxia inducible factors in pregnancy. Hum Reprod Update 16, 415-31.

Raleigh JA, C.-A.D., Rinker LH, Ballenger CA, Weissler MC, Fowler WC Jr, Novotny DB, Varia MA. (1998) Hypoxia and vascular endothelial growth factor expression in human squamous cell carcinomas using pimonidazole as a hypoxia marker. Cancer Res 58, 3765-8.

Raval AP, D.K., Mochly-Rosen D, Sick TJ, Pérez-Pinzón MA. (2003) Epsilon PKC is required for the induction of tolerance by ischemic and NMDA-mediated preconditioning in the organotypic hippocampal slice. J Neurosci 23, 384-91.

Ream M, R.A., Chandra R, Chikaraishi DM (2008) Early fetal hypoxia leads to growth restriction and myocardial thinning. Am J Physiol Regul Integr Comp Physiol. 292, 583-95.

Reichling, D.B. and Levine, J.D. (2009) Critical role of nociceptor plasticity in chronic pain. Trends Neurosci 32, 611-8.

Reneman, R.S. (1998) [Intrinsic and endogenous mechanisms for protecting the heart against ischemia]. Verh K Acad Geneeskd Belg 60, 147-60; discussion 160-1.

Reynolds, J.D., Penning, D.H., Dexter, F., Atkins, B., Hrdy, J., Poduska, D. and Brien, J.F. (1996) Ethanol increases uterine blood flow and fetal arterial blood oxygen tension in the near-term pregnant ewe. Alcohol 13, 251-6.

Rodesch, F., Simon, P., Donner, C. and Jauniaux, E. (1992) Oxygen measurements in endometrial and trophoblastic tissues during early pregnancy. Obstet Gynecol 80, 283-5.

Roseboom, T.J., van der Meulen, J.H., Ravelli, A.C., Osmond, C., Barker, D.J. and Bleker, O.P. (2001) Effects of prenatal exposure to the Dutch famine on adult disease in later life: an overview. Twin Res 4, 293-8.

Roskoski, R., Jr. (2008) VEGF receptor protein-tyrosine kinases: structure and regulation. Biochem Biophys Res Commun 375, 287-91.

Rudolph, A.M. (2000) Myocardial growth before and after birth: clinical implications. Acta Paediatr 89, 129-33.

المنارة للاستشارات
www.manaraa.com

Ruijtenbeek, K., le Noble, F.A., Janssen, G.M., Kessels, C.G., Fazzi, G.E., Blanco, C.E. and De Mey, J.G. (2000) Chronic hypoxia stimulates periarterial sympathetic nerve development in chicken embryo. Circulation 102, 2892-7.

Safe S, K.K. (2008) Non-classical genomic estrogen receptor (ER)/specificity protein and ER/activating protein-1 signaling pathways. J Mol Endocrinol 41, 263-75.

Sauer H, W.M. (2005) Reactive oxygen species as signaling molecules in cardiovascular differentiation of embryonic stem cells and tumor-induced angiogenesis. Antioxid Redox Signal 7, 1423-34.

Saurin AT, P.D., Raat NJ, Latchman DS, Owen MJ, Marber MS. (2002) Targeted disruption of the protein kinase C epsilon gene abolishes the infarct size reduction that follows ischaemic preconditioning of isolated buffer-perfused mouse hearts. Cardiovasc Res. 55, 672-80.

Scholer, H.R., Ruppert, S., Suzuki, N., Chowdhury, K. and Gruss, P. (1990) New type of POU domain in germ line-specific protein Oct-4. Nature 344, 435-9.

Seckl, J.R. (2008) Glucocorticoids, developmental 'programming' and the risk of affective dysfunction. Prog Brain Res 167, 17-34.

Sedmera, D., Pexieder, T., Vuillemin, M., Thompson, R.P. and Anderson, R.H. (2000) Developmental patterning of the myocardium. Anat Rec 258, 319-37.

Sedmera, D., Pexieder, T., Vuillemin, M., Thompson, R.P. and Anderson, R.H. (2000) Developmental patterning of the myocardium. Anat Rec 258, 319-37.

Semenza, G.L. (1998) Hypoxia-inducible factor 1: master regulator of O2 homeostasis. Curr Opin Genet Dev 8, 588-94.

Semenza, G.L., Agani, F., Iyer, N., Kotch, L., Laughner, E., Leung, S. and Yu, A. (1999) Regulation of cardiovascular development and physiology by hypoxia-inducible factor 1. Ann N Y Acad Sci 874, 262-8.

Semenza, G.L. (2007) Hypoxia-inducible factor 1 (HIF-1) pathway. Sci STKE 2007, cm8.

Sharma SK, L.J., Nordman C, Tinney JP, Tobita K, Keller BB. (2006) Impact of hypoxia on early chick embryo growth and cardiovascular function. Pediatr Res. 59, 116-20.

Sharp, B.R., Jones, S.P., Rimmer, D.M. and Lefer, D.J. (2002) Differential response to myocardial reperfusion injury in eNOS-deficient mice. Am J Physiol Heart Circ Physiol 282, H2422-6.

المنارة للاستشارات
www.manaraa.com

Sisci D, M.E., Morelli C, Lanzino M, Aquila S, Rizza P, Catalano S, Casaburi I, Maggiolini M, Andò S. (2010) 17β-estradiol enhances α(5) integrin subunit gene expression through ERα-Sp1 interaction and reduces cell motility and invasion of ERα-positive breast cancer cells. Breast Cancer Res Treat 124, 63-77.

Snoeckx, L.H., Cornelussen, R.N., Van Nieuwenhoven, F.A., Reneman, R.S. and Van Der Vusse, G.J. (2001) Heat shock proteins and cardiovascular pathophysiology. Physiol Rev 81, 1461-97.

Soothill, P.W., Nicolaides, K.H., Rodeck, C.H. and Campbell, S. (1986) Effect of gestational age on fetal and intervillous blood gas and acid-base values in human pregnancy. Fetal Ther 1, 168-75.

Stroka DM, B.T., Desbaillets I, Wenger RH, Neil DA, Bauer C, Gassmann M, Candinas D. (2001) HIF-1 is expressed in normoxic tissue and displays an organ-specific regulation under systemic hypoxia. FASEB J. 15, 2445-53.

Sugishita Y, L.D., Agani F, Watanabe M, Fisher SA. (2004A) Hypoxia-responsive signaling regulates the apoptosis-dependent remodeling of the embryonic avian cardiac outflow tract. Dev Biol. 273, 285-96.

Sugishita Y, W.M., Fisher SA. (2004B) Role of myocardial hypoxia in the remodeling of the embryonic avian cardiac outflow tract. Dev Biol. 267, 294-308.

Suzuki, N., Rohdewohld, H., Neuman, T., Gruss, P. and Scholer, H.R. (1990) Oct-6: a POU transcription factor expressed in embryonal stem cells and in the developing brain. EMBO J 9, 3723-32.

Takeuchi S, T.K., Oishi I, Nomi M, Ikeya M, Itoh K, Tamura S, Ueda T, Hatta T, Otani H, Terashima T, Takada S, Yamamura H, Akira S, Minami Y. (2000) Mouse Ror2 receptor tyrosine kinase is required for the heart development and limb formation. Genes Cells 5, 71-8.

Takano, H., Zou, Y., Hasegawa, H., Akazawa, H., Nagai, T. and Komuro, I. (2003) Oxidative stress-induced signal transduction pathways in cardiac myocytes: involvement of ROS in heart diseases. Antioxid Redox Signal 5, 789-94.

Thornburg, K.L. (1991) Fetal response to intrauterine stress. Ciba Found Symp 156, 17-29; discussion 29-37.

Tillmanns, J., Rota, M., Hosoda, T., Misao, Y., Esposito, G., Gonzalez, A., Vitale, S., Parolin, C., Yasuzawa-Amano, S., Muraski, J., De Angelis, A., Lecapitaine, N., Siggins, R.W., Loredo, M., Bearzi, C., Bolli, R., Urbanek, K., Leri, A., Kajstura, J. and Anversa, P. (2008) Formation of large coronary arteries by cardiac progenitor cells. Proc Natl Acad Sci U S A 105, 1668-73.

المنارة للاستشارات

www.manaraa.com

Tintu A, R.E., Verlohren S, Brinkmann J, Ahmad S, Crispi F, van Bilsen M, Carmeliet P, Staff AC, Tjwa M, Cetin I, Gratacos E, Hernandez-Andrade E, Hofstra L, Jacobs M, Lamers WH, Morano I, Safak E, Ahmed A, le Noble F. Hypoxia induces dilated cardiomyopathy in the chick embryo: mechanism, intervention, and long-term consequences. PLoS ONE. 4, e5155.

Tomanek, R.J., Lotun, K., Clark, E.B., Suvarna, P.R. and Hu, N. (1998) VEGF and bFGF stimulate myocardial vascularization in embryonic chick. Am J Physiol 274, H1620-6.

Tomanek, R.J., Ratajska, A., Kitten, G.T., Yue, X. and Sandra, A. (1999) Vascular endothelial growth factor expression coincides with coronary vasculogenesis and angiogenesis. Dev Dyn 215, 54-61.

Tomanek, R.J., Lund, D.D. and Yue, X. (2003) Hypoxic induction of myocardial vascularization during development. Adv Exp Med Biol 543, 139-49.

Turk, P.W., Laayoun, A., Smith, S.S. and Weitzman, S.A. (1995) DNA adduct 8-hydroxyl-2'-deoxyguanosine (8-hydroxyguanine) affects function of human DNA methyltransferase. Carcinogenesis 16, 1253-5.

Val'kovich EI, M.V., Davydova MK, Davydova OK. (1986) [Changes in the myocardium of fetuses and newborn infants as a result of hypoxia]. Arkh Anat Gistol Embriol. 90, 35-9.

Vornehm, N.D., Wang, M., Abarbanell, A., Herrmann, J., Weil, B., Tan, J., Wang, Y., Kelly, M. and Meldrum, D.R. (2009) Acute postischemic treatment with estrogen receptor-alpha agonist or estrogen receptor-beta agonist improves myocardial recovery. Surgery 146, 145-54.

Ward, J. (2008) Oxygen sensors in context. Biochim Biophys Acta. 1777, 1-14.

Watanabe M, J.A., Fisher SA. (2001) Apoptosis is required for the proper formation of the ventriculo-arterial connections. Dev Biol. 240, 274-88.

Waterland RA, J.R. (2004) Early nutrition, epigenetic changes at transposons and imprinted genes, and enhanced susceptibility to adult chronic diseases. Nutrition 20, 63-8.

Watson, J.A., Watson, C.J., McCrohan, A.M., Woodfine, K., Tosetto, M., McDaid, J., Gallagher, E., Betts, D., Baugh, J., O'Sullivan, J., Murrell, A., Watson, R.W. and McCann, A. (2009) Generation of an epigenetic signature by chronic hypoxia in prostate cells. Hum Mol Genet.

Webster WS, A.D. (2007) The effect of hypoxia in development. Birth Defects Res C Embryo Today. 81, 215-28.

المنارة للاستشارات
www.manaraa.com

Weitzman, S.A., Turk, P.W., Milkowski, D.H. and Kozlowski, K. (1994) Free radical adducts induce alterations in DNA cytosine methylation. Proc Natl Acad Sci U S A 91, 1261-4.

Wellmann, S., Bettkober, M., Zelmer, A., Seeger, K., Faigle, M., Eltzschig, H.K. and Buhrer, C. (2008) Hypoxia upregulates the histone demethylase JMJD1A via HIF-1. Biochem Biophys Res Commun 372, 892-7.

Wenger, R.H. and Gassmann, M. (1997) Oxygen(es) and the hypoxia-inducible factor-1. Biol Chem 378, 609-16.

Weinstein, B.M. and Lawson, N.D. (2002) Arteries, veins, Notch, and VEGF. Cold Spring Harb Symp Quant Biol 67, 155-62.

Wiesener, M.S., Jurgensen, J.S., Rosenberger, C., Scholze, C.K., Horstrup, J.H., Warnecke, C., Mandriota, S., Bechmann, I., Frei, U.A., Pugh, C.W., Ratcliffe, P.J., Bachmann, S., Maxwell, P.H. and Eckardt, K.U. (2003) Widespread hypoxia-inducible expression of HIF-2alpha in distinct cell populations of different organs. FASEB J 17, 271-3.

Wikenheiser J, D.Y., Fisher SA, Watanabe M. (2006) Differential levels of tissue hypoxia in the developing chicken heart. Dev Dyn. 235, 115-23.

Wilson, V.L. and Jones, P.A. (1983) Inhibition of DNA methylation by chemical carcinogens in vitro. Cell 32, 239-46.

Woods, J.R., Jr., Plessinger, M.A. and Clark, K.E. (1987) Effect of cocaine on uterine blood flow and fetal oxygenation. JAMA 257, 957-61.

Wu, H. and Sun, Y.E. (2006) Epigenetic regulation of stem cell differentiation. Pediatr Res 59, 21R-5R.

Xia, X., Lemieux, M.E., Li, W., Carroll, J.S., Brown, M., Liu, X.S. and Kung, A.L. (2009) Integrative analysis of HIF binding and transactivation reveals its role in maintaining histone methylation homeostasis. Proc Natl Acad Sci U S A 106, 4260-5.

Xiao, D., Ducsay, C.A. and Zhang, L. (2000) Chronic hypoxia and developmental regulation of cytochrome c expression in rats. J Soc Gynecol Investig 7, 279-83.

Xu, Y., Williams, S.J., O'Brien, D. and Davidge, S.T. (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, 1251-3.

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Xu, Y., Arenas, I.A., Armstrong, S.J., Plahta, W.C., Xu, H. and Davidge, S.T. (2006) Estrogen improves cardiac recovery after ischemia/reperfusion by decreasing tumor necrosis factor-alpha. Cardiovasc Res 69, 836-44.

Xue, Q. and Zhang, L. (2009) Prenatal hypoxia causes a sex-dependent increase in heart susceptibility to ischemia and reperfusion injury in adult male offspring: Role of PKC(Ardehali). J Pharmacol Exp Ther.

Yla-Herttuala, S., Rissanen, T.T., Vajanto, I. and Hartikainen, J. (2007) Vascular endothelial growth factors: biology and current status of clinical applications in cardiovascular medicine. J Am Coll Cardiol 49, 1015-26.

Yue, X. and Tomanek, R.J. (1999) Stimulation of coronary vasculogenesis/angiogenesis by hypoxia in cultured embryonic hearts. Dev Dyn 216, 28-36.

Zamudio, S. (2003) The placenta at high altitude. High Alt Med Biol 4, 171-91.

Zhai, P., Eurell, T.E., Cotthaus, R., Jeffery, E.H., Bahr, J.M. and Gross, D.R. (2000) Effect of estrogen on global myocardial ischemia-reperfusion injury in female rats. Am J Physiol Heart Circ Physiol 279, H2766-75.

Zhang, R., Min, W. and Sessa, W.C. (1995) Functional analysis of the human endothelial nitric oxide synthase promoter. Sp1 and GATA factors are necessary for basal transcription in endothelial cells. J Biol Chem 270, 15320-6.

Zhang, H., Meyer, K.D. and Zhang, L. (2009) Fetal exposure to cocaine causes programming of Prkce gene repression in the left ventricle of adult rat offspring. Biol Reprod 80, 440-8.

Zhang, L. (2005) Prenatal hypoxia and cardiac programming. J Soc Gynecol Investig 12, 2-13.

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