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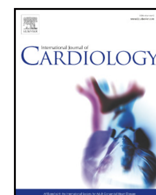

Long-term exposure to high altitude hypoxia during pregnancy increases fetal heart susceptibility to ischemia/reperfusion injury and cardiac dysfunction

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ABSTRACT

Background: High altitude hypoxia (HAH) exposure affects fetal development. However, the fetal cardiovascular responses to the HAH are not well understood. We have tested the hypothesis that long-term HAH exposure alters the hypoxia/ischemia-sensitive gene expressions, leading to an increase in fetal heart susceptibility to ischemia/reperfusion (I/R) injury and cardiac dysfunction.
Methods: Time-dated pregnant sheep were exposed to high-altitude (3820 m) or were maintained at sea level (~300 m) for 110 days. Fetal hearts were isolated from the near-term ewes and subjected to I/R in a Langendorff preparation.
Results: HAH decreased the fetal body and heart weights in the female but not male fetuses. HAH had no effect on the left ventricle (LV) function at baseline, but increased the LV infarct size and attenuated the post-ischemic recovery of LV function in both male and female fetuses, as compared with the normoxic groups. HAH increased the protein levels of hypoxia-inducible factor (HIF)-1α and DNA methyltransferases type 3b (DNMT3b), but attenuated protein kinase C epsilon (PKCε) levels in the fetal hearts. AHA induced a 4.3 fold increase of miR-210 in the males and a 2.9 fold increase in female hearts. In addition, HAH had no effect on mTOR protein and phosphorylation levels but increased the autophagy biomarker, LC3B-II protein levels and LC3B-II/LC3B-I ratio in the fetal hearts.
Conclusion: The results suggest that gestational HAH exposure induces in utero programming of the hypoxia/ischemia-sensitive gene expression pattern in the developing heart and increases cardiac susceptibility to I/R injury.

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1. Introduction

High altitude is defined as an elevation of >2500 m, which is mainly characterized by a lower partial pressure of O_2 relative to sea level at the same latitudes. A high altitude environment exerts a potential challenge to humans who are living or traveling at high altitude hypoxic regions [1]. Notably, pregnancy at high altitude is a significant burden for both the mother and fetus. Epidemiological and animal studies have shown that high altitude hypoxia (HAH) increases the incidence of pregnancy complications and neonatal morbidity, such as intrauterine growth restriction (IUGR), aberrant organ development and neuro behave disorder in neonates [1–3]. Of importance, HAH exposure during pregnancy has been well recognized as a fetal stress that affects fetal cardiovascular programming [4,5]. However, the changes of the cardiovascular system to HAH are variable, depending on individual predisposition, oxygen level and the exposed time at high altitude. In acute short-term exposure to HAH, the initial response of the fetal cardiovascular system is characterized by an increased systemic vascular resistance, blood pressure, heart rate and cardiac output, which were mainly regulated by higher sympathetic activity and hyperventilation [6]. In response to long-term HAH exposure, the fetal cardiovascular system may promote compensatory adaptation changes in cardiovascular structure and functional proteins through various molecular mechanisms including the epigenetic regulatory mechanism. Furthermore, the fetal cardiovasculatures may also progress a pathologic adaptation to long-term HAH, resulting in the development of cardiac hypertrophy, heart failure or hypertensive phenotype.

Our research center has developed a model using pregnant sheep that are transported to high altitude (3820 m) during the gestational

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period and have demonstrated that the fetal heart shows a decrease in cardiac output that is associated with a decrease in myocardial contractile function in response to long-term HAH exposure [4]. However, how the molecular mechanisms underlying with the long-term HAH-mediated fetal heart dysfunction are not fully understood. Previous studies have shown that long-term HAH exposure during pregnancy has profound effects on utero-placental circulation including an altered utero-placental and fetal volumetric blood flow [7]. This suggests that the fetus is experiencing a long-term ischemic environment. Therefore, we take the position to test a novel hypothesis that long-term HAH exposure during pregnancy alters the hypoxia/ischemia-sensitive gene expression patterns, leading to an increase in the fetal heart susceptibility to ischemia/reperfusion (I/R) injury and cardiac dysfunction. In the present study, we employed a well-established Langendorff heart perfusion system in our lab to examine the effects of long-term HAH exposure on ischemia/reperfusion (I/R)-induced heart injury and contractile contraction in ovine fetuses to see whether long-term HAH increases I/R-induced heart injury and dysfunction. In addition, we examined the hypoxia/ischemia-sensitive genes expression patterns in both HAH and normoxic fetal hearts.

2. Materials and methods

2.1. Experimental animals

Time-dated pregnant sheep of a homogeneous-mixed western breed were obtained from a single supplier (Nebeker Ranch, Lancaster, CA) and allocated to long-term hypoxic or control (normoxic) groups. At 30 days gestation, the ewes in the long-term hypoxic group were transported to the Barcroft Laboratory White Mountain Research Station at Bishop, CA (elevation 3820 m, barometric pressure ~480 Torr, maternal PaO_2: ~60 mm Hg) for 110 days (from 30 days of gestation to 140 days of gestation). The ewes in the control (normoxic) group were maintained at ~300 m above sea level (low altitude, PaO_2: ~102 mm Hg). At near-term pregnancy (~140 days), ewes from the high altitude were transported to our laboratory. Immediately after arrival, hypoxia (maternal PaO_2: ~60 mm Hg) was reestablished by administering nitrogen gas through a maternal tracheal catheter as described previously [4,8]. This was maintained during the entire experimental period. All procedures and protocols were approved by the Institutional Animal Care and Use Committee of Loma Linda University and followed the guidelines in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. After tissue collections, animals were killed via intravenous injection of 15 mL T-61 solution (Hoechst-Roussel, Somerville, NJ), according to American Veterinary Medical Association guidelines.

2.2. Measurement of cardiac function and ischemia-reperfusion injury

The ewes were anesthetized with intravenous injection of propofol (2 mg/kg) followed by intubation, and anesthesia was maintained on 1.5% to 3.0% isoflurane balanced in O_2 throughout the surgery. An incision was made in the abdomen, the fetuses were lifted and removed from the uterus. The chest of the fetuses were opened by a midline incision and the hearts were removed from the fetuses and retrogradely perfused via the aorta in a modified Langendorff apparatus under constant pressure (70 mm Hg) with gassed (95% O_2, 5% CO_2) Krebs-Henseleit buffer at 37 °C, as we described previously [9]. A pressure transducer connected to a saline-filled balloon inserted into the left ventricular (LV) was used to assess ventricular function by measuring LV pressure (mm Hg) and its first derivative (dP/dt). LV end diastolic pressure (LVEDP) was set at approximately 5 mm Hg. After baseline recording for 60 min, hearts were subjected to 20 min of global ischemia by stopping the perfusion followed by 60 min of reperfusion. Left ventricular developed pressure (LVDP), heart rate (HR), dp/dt_{max}, dp/dt_{min}, and LV end-diastolic pressure (LVEDP) were continuously recorded. Myocardial infarct size was measured as described previously [9]. Briefly, at the end of reperfusion, left ventricles were collected, cut into slices, incubated with 1% triphenyltetrazolium chloride solution for 15 min at 37 °C, and immersed in formalin for 30 min. Each slice was then photographed separately, and the areas of myocardial infarction in each slice were analyzed by computerized planimetry and expressed as a percentage of the left ventricle weight.

2.3. Real-time reverse transcription PCR

Total RNA was isolated from the left ventricle (LV) tissues from both HAH and normoxic groups using TRIzol reagent (Invitrogen, CA) and subjected to reverse transcription with the iScript cDNA Synthesis system (Bio-Rad, Hercules, CA). Quantification of mature miR-210 was performed using the miScript II kit and miScript SYBR Green PCR kit with miScript Primer Assay kit (Qiagen) according to the manufacturer's instructions as described previously [10]. Primers included miScript Universal Primer, miR-210 miScript Primer Assay, and SNORD61 miScript Primer Assay (Qiagen). Serial dilutions of the positive control were done on each plate to create a standard curve for the quantification. PCR was done in triplicate, and threshold cycle numbers were averaged for each sample. SNORD61 miScript Primer was used as the internal control. The relative expressions levels of mature miR-210 were computed and expressed as fold of SNORD61.

2.4. Western immunoblotting

Protein abundances in the LV tissues were measured as described previously [9]. Briefly, tissues were homogenized in a lysis buffer followed by centrifugation at 4 °C for 10 min at 10,000*g*, and the supernatants were collected. Samples with equal proteins were loaded onto 7.5% polyacrylamide gel with 0.1% sodium dodecyl sulfate and separated by electrophoresis at 100 V for 2 h. Proteins were then transferred onto nitrocellulose membranes. After blocking nonspecific binding sites by dry milk, membranes were incubated with primary antibodies against HIF-1α (Santa Cruz; 1:200 dilution), PKCε (Santa Cruz; 1:1000 dilution), DNMT3b (Millipore Inc.; 1:500 dilution), phospho-mTOR (Ser 2448, Cell Signaling Technology; 1:500 dilution), t-mTOR (Cell Signaling Technology; 1:1000 dilution) and LC3B (Cell Signaling Technology; 1:200 dilution). After washing, membranes were incubated with secondary horseradish peroxidase-conjugated antibodies. Proteins were visualized with enhanced chemiluminescence reagents, and blots were exposed to Hyperfilm. Results were quantified with the Kodak electrophoresis analysis system and Kodak ID image analysis software (Kodak, Rochester, NY). The target protein abundance was normalized to the abundance of GAPDH or β-actin as a protein loading control.

2.5. Statistical analysis

Data are expressed as the mean ± SEM obtained from the number of experimental animals given. Experimental numbers (n) represents fetuses from different ewes. Difference between the groups was compared by Student's *t*-test or analysis of variance (ANOVA) using the Graph-Pad Prism software (GraphPad Software Version 4, San Diego, CA, USA) where appropriate. For all comparisons, P-values < 0.05 indicated statistical significance.

3. Results

3.1. Effect of long-term HAH exposure on fetal body and heart weight

In male fetuses, both the body weight and heart weight were not affected by long-term HAH. Their body weights in the near term pregnancy (~140 days) were 4.0 ± 0.24 kg (n = 10) for control and 4.11 ± 0.30 kg (n = 7) for HAH (P > 0.05), and their heart weights were 37.32 ± 2.28 g (n = 10) for control and 35.45 ± 2.55 g for HAH (n = 7) (P > 0.05). However, in female fetuses, both their body weight and heart weight were attenuated by long-term HAH. Their body weights were 4.41 ± 0.23 kg (n = 4) for control and 3.40 ± 0.25 kg (n = 10) for HAH (P < 0.05), and their heart weights were 42.75 ± 3.54 g (n = 4) for control and 34.72 ± 1.81 g (n = 10) for HAH (P < 0.05).

3.2. Effect of long-term HAH exposure on baseline LV function and post-ischemic recovery of LV function

Table 1 (Supplementary file) shows the pre-ischemic values of LV function and coronary flow rate in the isolated hearts from male and female fetuses in a Langendorff preparation. Long-term HAH showed no significant effects on baseline LV function and coronary flow in fetal hearts. As shown in Fig. 1, global ischemia for 20 min resulted in a remarkable impairment in LV function in both male and female hearts. Long-term HAH exposure resulted in decreases in post-ischemic recovery of LVDP (Fig. 1A & E), dP/dt_{max} (Fig. 1B & F), dP/dt_{min} (Fig. 1C & G) and heart rate (HR) (Fig. 1D) in both male and female fetuses after 20 min of global ischemia as compared to the normoxic control. In addition, long-term HAH exposure attenuated the post-ischemic recovery of coronary flow in both male (Fig. 2A) and female (Fig. 2D) fetuses. However, long-term HAH exposure enhanced the post-ischemic recovery of LVEDP in both male (Fig. 2B) and female (Fig. 2E) fetuses. The infarct size of LV at the end of 60-min reperfusion after 20-min ischemia is shown in Fig. 2C (male) and Fig. 2F (female). Ischemia and reperfusion caused LV myocardial infarction in both male and female fetal hearts. Long-term HAH exposure increased infarct size in both male (Fig. 2C) and female (Fig. 2F) fetal hearts.

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3.3. Effect of long-term HAH exposure on hypoxia-sensitive biomarkers

Hypoxia-inducible factor-1α (HIF-1α) is considered as the master transcriptional regulator of cellular and developmental response to hypoxia. The dysregulation and overexpression of HIF-1α by either hypoxia or genetic alternations have been implicated in a number of pathophysiologies. To determine whether high altitude exposure altered HIF-1α expression in the developing heart, we measured the protein level of HIF-1α in fetal hearts. As shown in Fig. 3, the protein levels of HIF-1α in heart tissues were higher in the long-term HAH exposed group than in the normoxic exposed group of both male (Fig. 3A) and female (Fig. 3E) animals.

Similar to HIF-1α, miRNA-210 (miR-210) is also considered as one of the important biomarkers for hypoxia-mediated cellular responses. Therefore, in the present study we measured the miR-210 expression levels in the fetal hearts using RT-PCR analysis. As shown in Fig. 2, long-term HAH exposure up-regulated miR-210 expression levels and induced about a 3.4-fold increase in male hearts (Fig. 3B) and 3.0-fold increase in female hearts (Fig. 3F), as compared with normoxia exposed groups.

3.4. Effect of long-term HAH on cardiac ischemia-sensitive PKCε protein expression

Protein kinas C epsilon (PKCε) gene expression pattern has a significant impact in the regulation of heart hypertrophy and plays a vital role in cardio-protection in the setting of heart ischemia and reperfusion injury [11]. In the present study, we measured the protein abundance of PKCε in fetal hearts. As shown in Fig. 3, long-term HAH exposure decreased PKCε protein expression in both male (Fig. 3D) and female (Fig. 3H) hearts, as compared with the normoxic control groups.

3.5. Effect of long-term HAH on the protein expression of DNA-methyltransferase 3 beta (DNMT3b)

DNA methylation is an epigenetic modification that plays an important role in genomic imprinting, specific gene regulation, and embryonic development. DNMT3b is a de novo methyltransferase that is essential for the establishment of DNA methylation. Overexpression or repression of DNMT3b results in aberrant embryonic development. Therefore, we evaluated the protein abundance of DNMT3b in the fetal heart. As shown in Fig. 3, in both male (Fig. 3C) and female (Fig. 3G) fetal hearts, long-term HAH exposure enhanced the protein expressions of DNMT3b in the hearts, as compared with the normoxic groups.

3.6. Effect of long-term HAH exposure on autophagy markers

Autophagy is essential for basal homeostasis. However, prolonged autophagy activation may lead to a high turnover rate of proteins and organelles, resulting in organ dysfunction. In the present study, we measured some key autophagy-related genes expressions by Western blot. As shown in Fig. 4, the protein expressions of p-mTOR and t-mTOR in both male and female hearts were not altered by long-term HAH exposure, as compared with the normoxia exposed group. However, long-term HAH exposure substantially enhanced LC3B-II expression and increased the ratio of LC3B-II/LC3B-I in both male (Fig. 4C) and female (Fig. 4F) hearts, as compared with the normoxia exposed group.

4. Discussion

The present study shows that long-term high altitude hypoxia exposure induces an aberrant development of ischemic sensitive phenotype of the heart in the ovine fetus. The major findings in the present study are that: 1) long-term HAH caused a gender dependent reduction of fetal body and heart weight in the females but not in male fetuses; 2) long-term HAH exposure had no effect on the basal LV function but decreased post-ischemic recovery of the LV function after global ischemia in both male and female fetuses; 3) HAH-mediated LV dysfunction was associated with a remarkable increase in LV end diastolic pressure and myocardial infarct size; 4) in response to the long-term HAH exposure, the expression levels of both HIF-1α and miRNA-210 were increased in the developing hearts; 5) long-term HAH exposure enhanced DNMT3b expression but attenuated PKCε protein abundance in the developing hearts; 6) HAH exposure had no effect on total mTOR and Phospho-mTOR protein levels but increased the autophagy marker light chain 3B-II (LC3B-II) expression and the ratio of LC3B-II/LC3B-I in the developing hearts.

There is conflicting evidence that long-term high altitude hypoxia induces a decrease in birth weight or has no effect on it when compared with sea-level counterparts. Although epidemiological and animal studies have shown that high altitude hypoxia induces fetal growth restriction [3,5,12,13], the magnitude of the birth-weight fall varies among populations. Furthermore, multigenerational high altitude populations are protected from the altitude-associated fetal growth restrictions [13]. Previous study in our research group has shown that the ovine fetal weight is not affected by long-term high altitude hypoxia [14]. However, in the present study, we separated the male from female fetuses and found that long-term high altitude hypoxia had no effect on the fetal body and heart weight in the males but significantly decreased the body and heart weight in female fetuses. These observations suggest that the effect of HAH on the fetal growth can be partly compensated in the male but not female fetuses. The precise mechanisms underlying the gender different adaptation of fetal growth to high altitude remain uncertain, but it deserves future studies.

The present study showed that long term HAH had no effect on pre-ischemic baseline values of heart function but increased the LV myocardial infarct size and decreased the post-ischemic recovery of LV function after 20 min of global ischemia/reperfusion in both male and female fetuses. These data suggest that high altitude hypoxia may not impair fetal heart function at resting condition but alters the heart function when it encounters an ischemic stress challenge. Similar findings have been reported in different animal models where prenatal exposure to hypoxia have had no effect on cardiac function at resting condition while enhances the heart ischemic injury and dysfunction after ischemia stimulation [15,16]. These findings suggest that moderate fetal stress such as chronic hypoxia may not affect baseline cardiac function but have made the heart less adaptable and more vulnerable for life to challenge by a stressor. The programming of an organ adaptive capability and vulnerability to a stressor appears to be a common mechanism for developmental programming of health and disease. In the present study we found that post-ischemic coronary flow rates were significantly decreased in HAH exposed fetal hearts as compared with the sea level exposed hearts. This decrease in coronary perfusion, and subsequent decrease in the delivery of nutrient and oxygen, may be one of the important patho-physiological mechanisms underlying HAH-mediated enhanced heart ischemic injury and attenuated post-ischemic recovery of heart function. Mechanisms potentially responsible for the development of heart ischemia-sensitive phenotype in the long-term HAH exposed sheep fetuses could involve extrinsic factors. Indeed, previous studies have reported that, in response to long-term HAH, the ovine fetus shows an increase in arterial blood pressure and a decrease in cardiac output that is secondary to a decrease in cardiac contractile function [4]. These findings suggest that the causes and mechanisms underlying the HAH-mediated heart ischemic injury and I/R-induced dysfunction are complex.

Growing evidences have shown that the intrinsic changes in cardiomyocytes play an important role in cardiac programming in response to an adverse intrauterine environment [17–20]. PKCε gene is one of the major intrinsic cardio-protective proteins, which has been shown to play a key role in cardioprotection during ischemia/reperfusion injury [11,21]. Previous studies have demonstrated that prenatal stress

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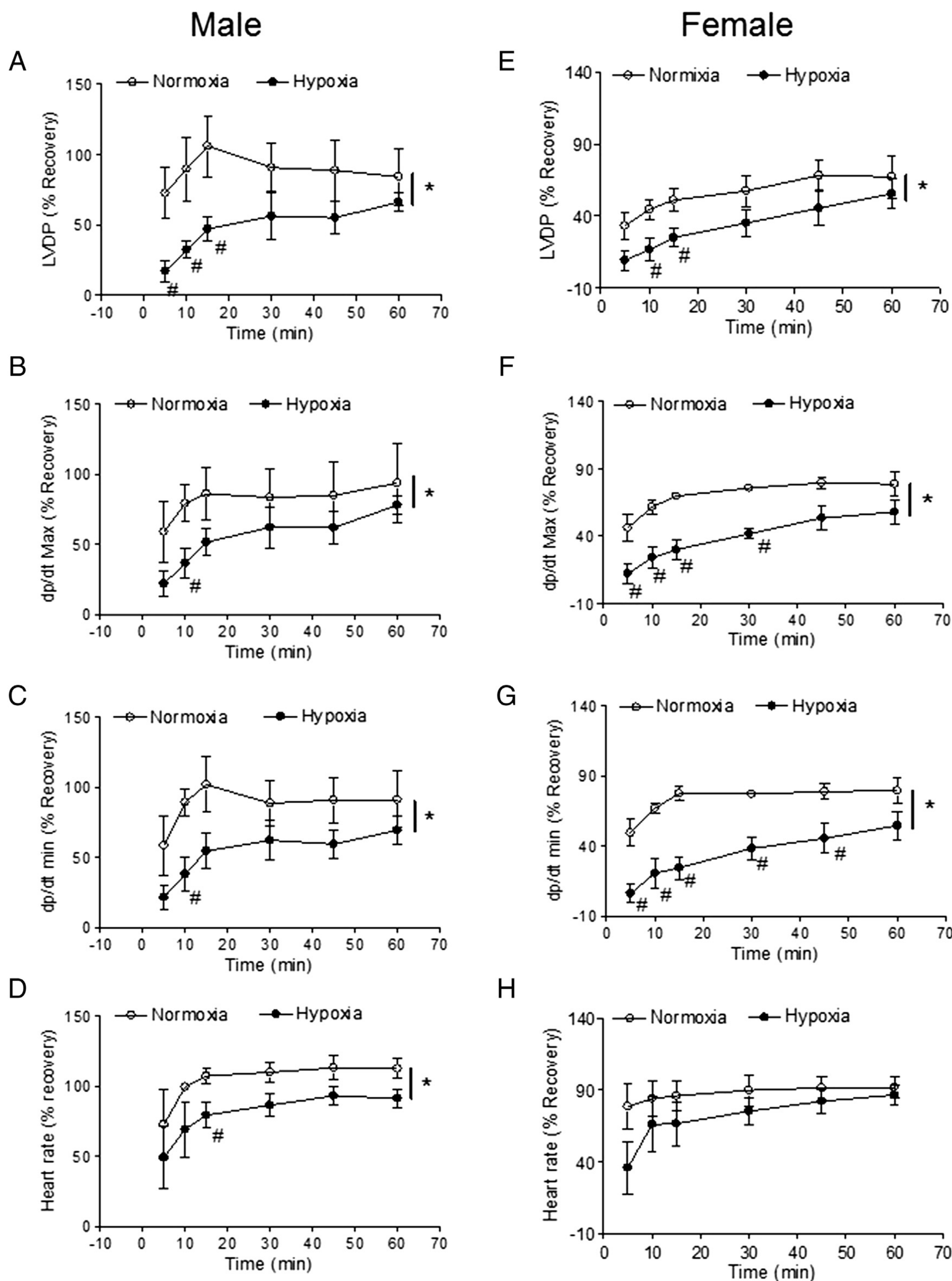

Fig. 1. Effect of HAH on post ischemic recovery of LV function in both male and female fetuses. Hearts were isolated from the male and female fetuses. The hearts were subjected to 20 min of ischemia and 60 min of reperfusion in a Langendorff preparation. Post-ischemic recoveries of the left ventricular diastolic pressures (LVDP) in male (A) and female (E). dP/dpmax in male (B) and female (F). dP/dpmin in male (C) and female (G). Heart rate in male (D) and female (H). Data are means ± SEM of animals from each group. Data were analyzed by 2-way repeated measures ANOVA (*P < 0.05 vs. control group). Then, compare the two group at every time point using multiple *t*-test comparison (#P < 0.05 vs. control at each time point).

exposure increases ischemia/reperfusion-induced cardiac injury which is associated with PKCε gene repression in the cardiomyocytes of fetuses and adult offspring [15,17,22,23]. In agreement with previous studies, our current data that long-term high altitude hypoxia exposure decreased cardiac PKCε protein expression in the fetus, suggest that the PKCε gene repression may be one of the common mechanisms underlying prenatal insults-mediated cardiac ischemic injury. Although the molecular mechanisms underlying HAH-mediated cardiac PKCε gene expression remain unclear, our present data that long-term HAH increased the DNA methyltransferases 3b (DNMT3b) expression, suggest that the increased methylation levels of CpG dinucleotides in the promoter region of PKCε gene may be one of the key epigenetic mechanisms. Indeed, previous studies in different animal models have demonstrated that cardiac PKCε expression patterns are directly regulated through the DNA methylation

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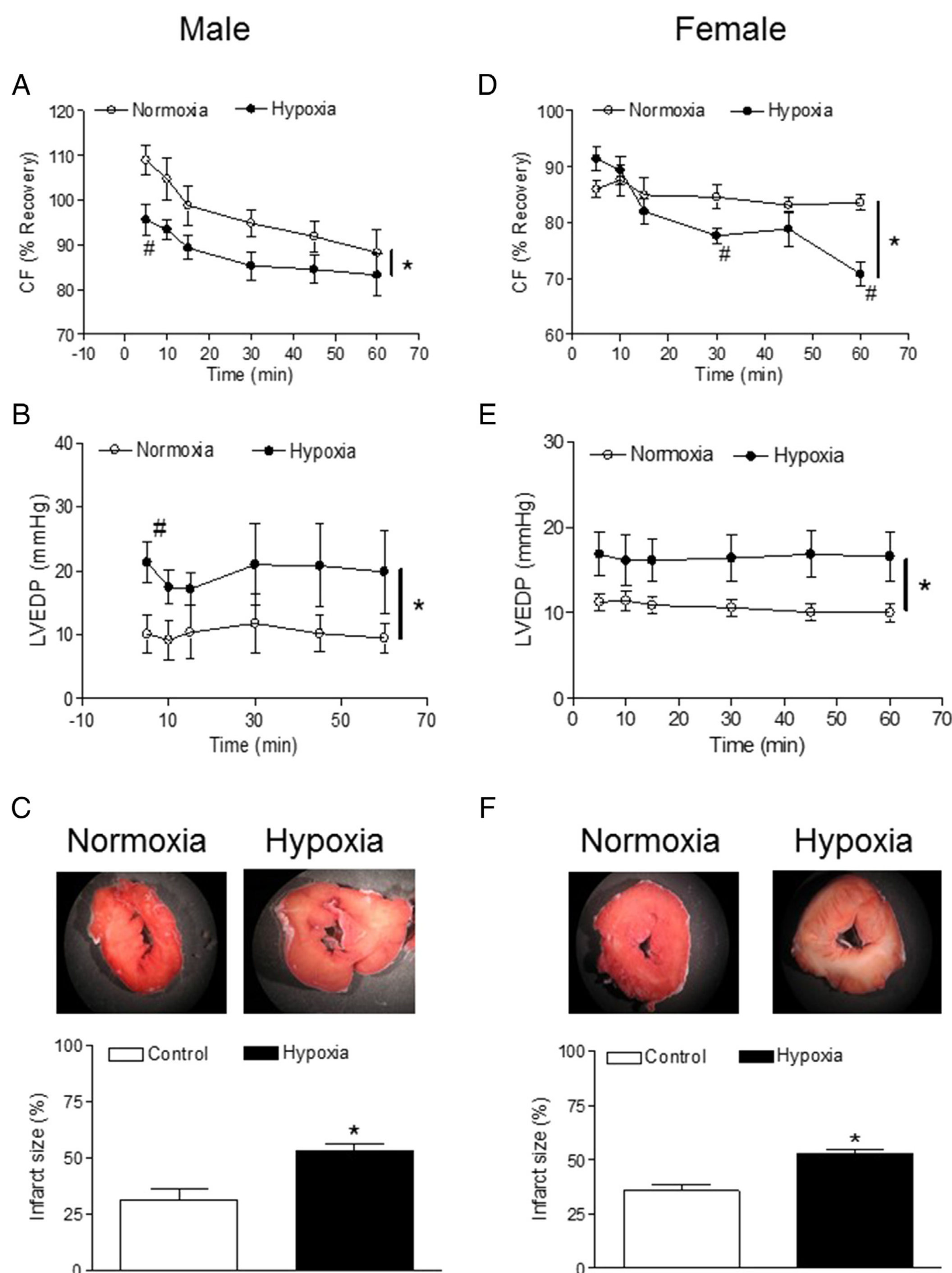

Fig. 2. Effect of HAH on I/R-induced coronary flow rate (CF), LVEDP and myocardial infarction in both male and female fetuses. Hearts were isolated from the male and female fetuses. The hearts were subjected to 20 min of ischemia and 60 min of reperfusion in a Langendorff preparation. During ischemia/reperfusion (I/R), the pulmonary artery effluent was collected from both male (A) and female (D) as an index of coronary flow (milliliters per minute per gram of heart wet weigh). Post-ischemic recovery of the left ventricular end-diastolic pressures (LVEDP) was determined during the course of reperfusion in both male (B) and female (E) fetuses. The left ventricular tissue were collected from both male (C) and female (F) fetuses at the end of reperfusion, and the myocardial infarct size was determined with 1% triphenyltetrazolium chloride (TTC) staining and expressed as a percentage of the total ventricular weight. Data are means ± SEM of animals from each group. Data for CF and LEVDP were analyzed with 2-way repeated measures ANOVA (*P < 0.05 vs. control group). Then, compare the two group at every time point using multiple *t*-test comparison (#P < 0.05 vs. control at each time point). Data for infarct size were analyzed by Student *t*-test. *P < 0.05 vs. control (normoxia).

mechanism in response to fetal stresses [22,23]. In addition, the HAH-mediated DNMT3b over-expression may not only alter specific CpG methylation in PKCε gene, but also impair global genomic DNA methylation levels in the developing hearts. Given the facts that change of global genomic DNA methylation plays a key role in the development of heart ischemia-sensitive phenotype [9], in our future studies, we will determine whether long-term high altitude hypoxia exposure alters cardiac global genomic DNA methylation patterns and whether this change directly contributes to the development of a heart ischemia-sensitive phenotype in the fetus.

Hypoxia-inducible factor-1α (HIF-1α), a unique regulator of the cellular response to hypoxia, functions as a master regulator that coordinately regulates the expression of a large number of genes whose products play important roles in mediating cardiovascular responses

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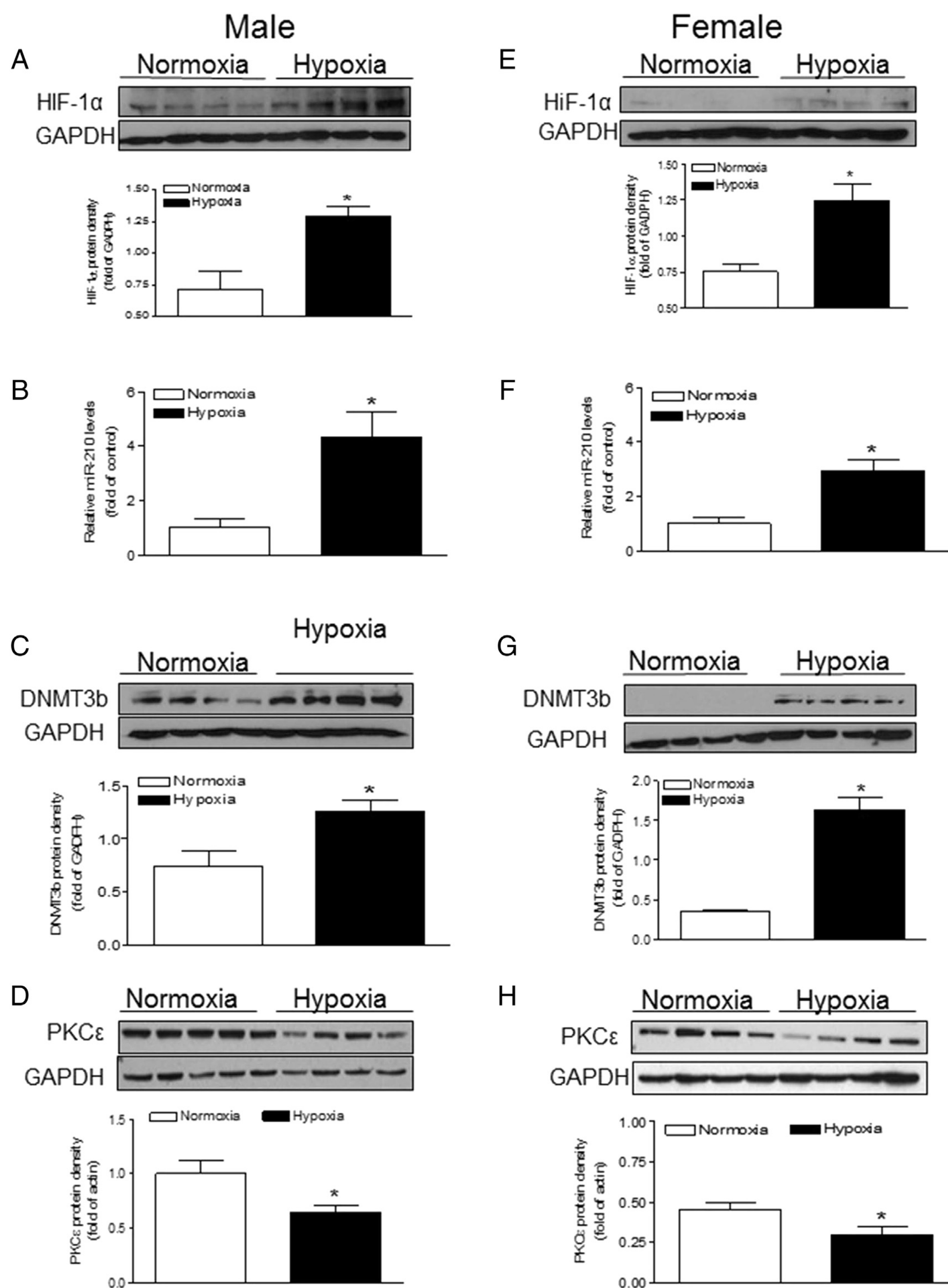

Fig. 3. HAH-mediated changes of hypoxic biomarkers and protein expressions. Heart were isolated from fetuses from near-term pregnant sheep maintained at sea level (control) or exposed to high altitude hypoxia (HAH). Protein abundances in the left ventricle (LV) tissues were determined by Western blot analyses. The protein levels of HIF-1α in male (A) and female (E) LV tissues. The protein levels of DNMT3b in male (C) and female (G) LV tissues. The protein levels of PKCε in male (D) and female (H) LV tissues. The protein levels are expressed as fold of GAPDH (loading control). MiRNA-210 levels in the LV tissues isolated from male (B) and female (F) fetuses were measured by qRT-PCR analysis, as described under Material and methods. The expression of miR-210 is expressed as percentage of SNORD61 (internal control). Data are means ± SEM of animals from each group and were analyzed by Student *t*-test. *P < 0.05 vs. control (normoxia).

to hypoxia and ischemia [24]. In the current study, we found that the HIF-1α expression in cardiac tissue is significantly increased in both male and female fetuses in response to long-term high altitude hypoxia, which suggests that the enhanced HIF-1α expression may be the initial upstream regulatory signaling protein underlying the HAH-mediated cardiac dysfunction. MiR-210 is one of these genes that is directly regulated by HIF-1α. Consisting with the present finding of increased HIF-1α, in the present study, we also found a 3- to 4-fold increase in miR-210 with both male and female fetal hearts from ovine fetuses acclimatized to high-altitude hypoxia. Similarly, high altitude hypoxia exposure also increases expression of the miR-210 in circulating plasma, uterine artery and placenta [25–27]. These findings suggest that long-

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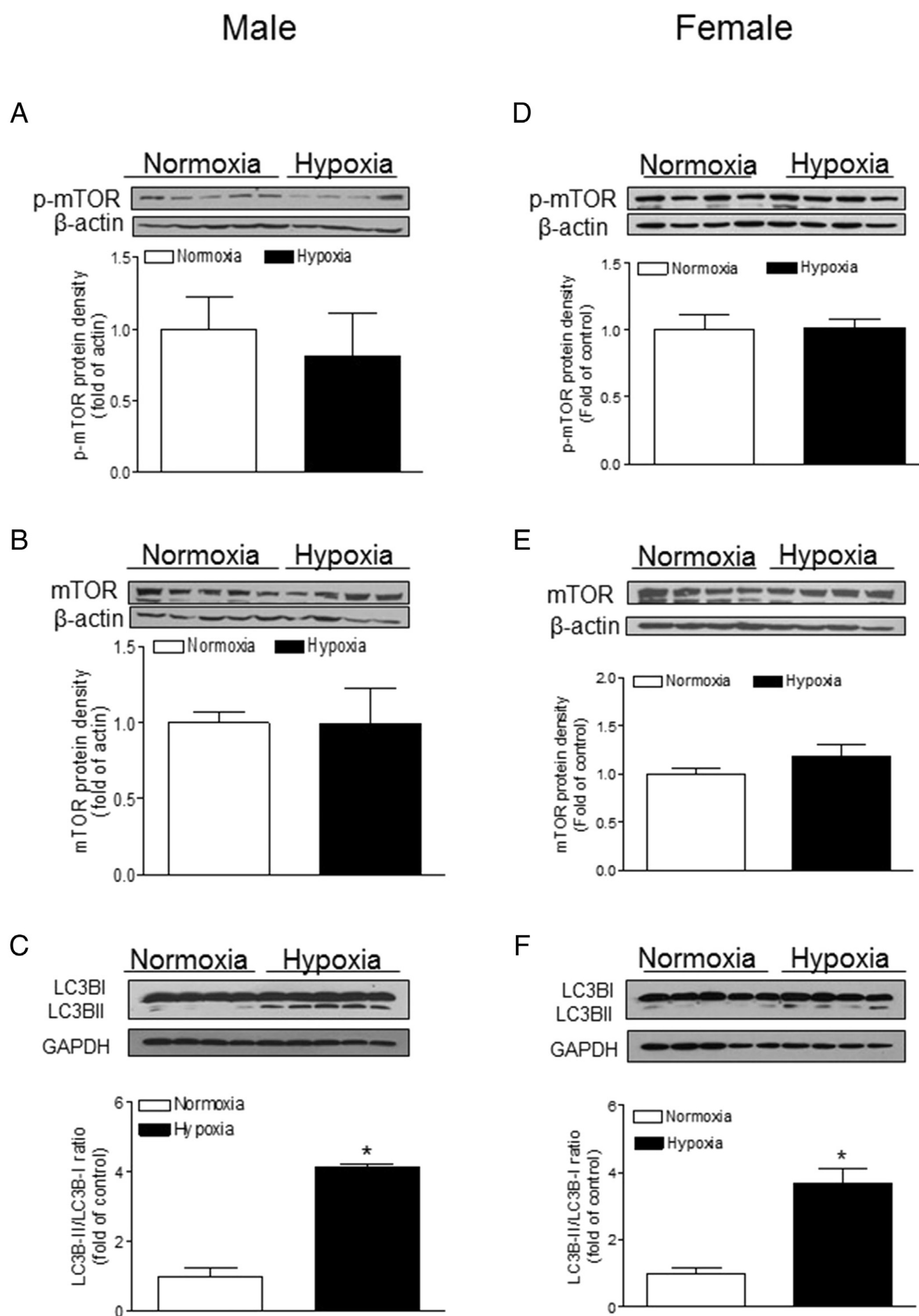

Fig. 4. Effect of HAH on autophagy-related protein expressions. Heart were isolated from fetuses from near-term pregnant sheep maintained at sea level (control) or exposed to high altitude hypoxia (HAH). Protein abundances in the left ventricle (LV) tissues were determined by Western blot analyses. The protein levels of phosphor-mTOR in male (A) and female (D) LV tissues. The total protein levels of mTOR in male (B) and female (E) LV tissues. The ratio of LC3B-II to LC3B-I protein levels in male (C) and female (F) LV tissues. Data are means ± SEM of animals from each group and were analyzed by Student *t*-test. *P < 0.05 vs. control (normoxia).

term high altitude hypoxia-mediated HIF-1α may be one of the major driving forces behind the increased expression of miR-210. The functional significance of miR-210 is complex and might be highly tissue- and time-dependent. It was reported that miR-210 overexpression, induced by the injection of minicircle non-viral vectors in the peri-infarct region, can inhibit apoptosis and improve cardiac functions [28]. On the other hand, miR-210 was reported to be upregulated in animal models having cardiac hypertrophy, heart failure, transient focal ischemia in the brain, and in ischemic wounds [29–32]. In addition, previous studies have reported that miR-210 impairs trophoblast invasion, resulting in the development of IUGR in high altitude exposed animals [33]. Furthermore, in a model of perinatal hypoxic-ischemic encephalopathy, inhibition of miR-210 by local administration of LNA-anti-miR-210 reduces brain infarct size and improves neurological

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function recovery [10]. In the present animal model, whether the increased miR-210 expression plays a compensatory role or detrimental role in the development of fetal heart ischemia-sensitive phenotype remains to be determined. MicroRNAs play an important role in the epigenetic control of gene expression patterns by targeting the mRNA 3′UTR, resulting in the degradation of mRNAs of translational inhibition of the target transcripts. PKCε may be one of the potential targeting genes of miR-210 in the fetal heart and the down-regulation of PKCε by miR-210 may also contribute to the development of hypoxia/ischemia-sensitive phenotype.

Autophagy is an important mechanism in numerous pathophysiological processes, including development, tumorigenesis, cell death, and survival. When cells encounter environmental stresses, such as starvation, oxidative stress, hypoxia, radiation, or pathogen infection, the level of autophagy can be dramatically elevated as a protective response, resulting in adaptation and survival [34]. However, inadequate repairs or constant stress stimulation can lead to cell death, named autophagic death. In the present study, we found that protein expression levels of LC3II and the ratio of LC3II/LC3I in fetal hearts were significantly higher in the HAH exposed fetuses than in control groups. LC3II, generated by the proteolytic cleavage of LC3, is associated with the formation of autophagosomes. Levels of LC3II correlate with the amount of autophagosomes and is considered a marker of autophagosome formation [35]. There are two important signaling pathways in the regulation of autophagy. One is the hypoxia inducible HIF-1α/BNIP3/Beclin1/LC3II pathway and the other one is the PI3K/AKT/mTOR/LC3II signaling pathway [36]. Our current findings show high altitude hypoxia increased HIF-1α expression but had no effect on both the total protein and phosphorylation levels of mTOR in the fetal hearts as compared with the control. This suggests that the HIF-1α/BNIP3/Beclin1/LC3II pathway may be the major signaling pathway underlying the high altitude hypoxia-induced over-autophagy in our current model. Indeed, recent studies have provided a novel link between hypoxia and the induction of autophagy, and have indicated that the hypoxia factor HIF-1 directly activates the transcription of the BNIP3 gene protein, resulting in the induction of autophagy [37]. Previous studies suggest that cardiac autophagy-induced by ischemia/reperfusion, can be adaptive or detrimental [38]. Reperfusion induces excessive autophagy by activation of the Beclin1 gene, which is an essential molecule for inducing autophagy and adapting to cardiac stress. However, during reperfusion, persistent activation of Beclin1 can be detrimental, leading to cardiomyocyte damage and increasing cardiac injury [39].

4.1. Study limitations

Most epidemiological and animal studies have shown that high altitude hypoxia decreases birth weight and induces fetal growth restriction [3,5,12,13]. In our current studies we found that high altitude hypoxia only decreased the fetal weights in female but not male fetuses. One of the limitations in this study is that we measured the fetal body weights from different pregnant sheep whose gestational ages were about 140–145 days. Therefore, the fetal age might affect the accuracy of fetal weight measurement. To overcome this limitation, in our future studies we will increase the sample size of the fetal body weights and make a scatter plot with the gestational ages as x-axis. A linear regression for control-males, control-females, HAH-males, and HAH-females will be determined. Then we will decide whether there is a significance among those groups from the regression parameters. In present studies we showed that high altitude hypoxia induced an aberrant development of ischemic sensitive phenotype of the heart in ovine fetus, which was associated with changes in some of the key ischemia-sensitive gene expressions (such as, HIF-1α, miR-210, PKCε, LC3II etc.). However, there is a lack of direct cause-effect evidence to show that the alterations of these genes are contributed to the development of heart ischemia-sensitive phenotype in response to high altitude hypoxia exposure.

4.2. Conclusions and future directions

In conclusion, our data showed that long-term high altitude hypoxia exposure induced cardiac programming during fetal development and indicated that the fetal cardiac function was normal at resting condition but was markedly blunted in response to an ischemia/reperfusion challenge in sheep at high altitude. We found that, in response to long-term high altitude hypoxia exposure, the fetal hearts were under-going developmental programming. First, there was an intrinsic increase in hypoxic biomarkers, such as HIF-1α and miR-210 in the developing hearts. Then, the increased hypoxic biomarkers induced an excess of autophagy. In addition, the long-term high altitude hypoxia induced an epigenetic down-regulation of the cardio protective gene (PKCε) expression via DNA methylation or the miR-201 signaling pathway. Finally, the intrinsic molecular changes in cardiomyocytes lead to the development of the heart ischemia-sensitive phenotype. However, how the changes of these hypoxic biomarkers and genes link to development of the heart ischemia-sensitive phenotype is still unclear. Although the present findings reinforce the notion that high altitude hypoxia exposure during pregnancy could alter heart development in utero, whether the fetal heart functional change is compensatory beneficial or pathologic adaptation to high altitude is unclear. Whether this aberrant fetal heart development will persist into postnatal life and increase the risk of cardiovascular disease in adulthood remains unknown. Therefore, more studies are required to understand those adaptation progresses during fetal development and insight into the mechanisms will help us to develop interventions to counteract adverse pathologic changes and simultaneously preserve the beneficial adaptation at a high altitude.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijcard.2018.07.046.

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Conflict of interest

The authors have declared that no competing interest exist in this work.

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