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Influence of gestational overfeeding on cardiac morphometry and hypertrophic protein markers in fetal sheep

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Abstract

Intrauterine overnutrition is associated with development of cardiovascular disease in adulthood although the underlying mechanism has not been precisely elucidated. This study evaluated the effects of maternal overnutrition on fetal cardiac morphometry and hypertrophy-related mRNA/protein expression. Multiparous ewes were fed either 150% of National Research Council (NRC) nutrient requirements (overfed group) or 100% of NRC requirements (control group) from 60 days before mating to Day 75 (D75) of gestation, when ewes were euthanized. Cardiac morphometry, histology and expression of Akt, forkhead-3a (Foxo3a), glycogen synthase kinase-3β (GSK3β), mammalian target of rapamycin (mTOR), NFATc3 and GATA4, atrial natriuretic factor (ANF), calcineurin A and caspase-8 were examined. Crown rump length, left and right ventricular free wall weights and left ventricular wall thickness were increased in D75 overnourished fetuses. Hematoxylin and eosin staining revealed irregular myofiber orientation and increased interstitial space in heart tissues from overfed group. Masson's trichrome staining displayed myofiber hypertrophy and fascicular disarray in heart tissues from overfed group. Overfeeding significantly enhanced Foxo3a phosphorylation in both ventricles, while protein expression of Akt, Foxo3a, GSK3β and caspase-8 as well as phosphorylated Akt and GSK3β in either ventricle was unaffected. Overfeeding increased left ventricular mTOR, NFATc3 (both total and phosphorylated) and calcineurin A. GATA4, pGATA4 and ANF expression were unchanged in both ventricles. Collectively, our data suggested that overfeeding during early to mid gestation (D75) leads to morphometric changes without overt pathology which may be related to elevated expression of mTOR, NFATc3, calcineurin A and phosphorylation of Foxo3a, mTOR and NFATc3.

Keywords: Fetus; Heart; Hypertrophy; Morphometry; Morphology

1. Introduction

Cardiac hypertrophy, characterized by increased ventricular mass and wall thickness, is accompanied by maladaptive responses such as fibrosis, chamber dilatation, and hemodynamic decompensation. Both clinical and experimental evidence has demonstrated that hypertrophic responses may be triggered by pressure overload or neuroendocrine stimulation [1–3]. Cardiac hypertrophy is usually a consequence of activation of cell signaling cascades, up-regulation of fetal gene expression programs, increased protein synthesis and increased sarcomere assembly and modulation of cellular energy sources [4]. A number of signaling molecules have been implicated in the reactive and negative regulation of myocardial hypertrophy such as Akt and its downstream targets forkhead-3a (Foxo3a), mammalian target of rapamycin (mTOR) and glycogen synthase kinase-3β (GSK3β) [5]. Several of these hypertrophy regulatory proteins including Foxo and GSK3β may also exert antihypertrophic functions [2,5–7].

Maternal obesity, which represents as a major challenge to obstetric practice, often results in negative outcomes for both women and fetuses. In particular, the fetus is at risk for stillbirth and congenital anomalies [8]. Despite the apparent tie between gestational obesity and health issues later on in life for both mother and child, how maternal obesity leads to the increased prevalence of heart disease is still not clear. We recently reported that intrauterine overfeeding leads to increased fetal weight, fetal heart and ventricular weights and increased plasma insulin and insulin-like growth factor-1 levels in fetal sheep [9,10]. However, little is known in regards to the mechanism of action involved in

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Table 1
Morphometric data in fetus on D75 and D135 of gestation

	Control	Overfed
D75 Fetal weight (g)	294.1±8.0	318.1±8.3
D75 Crown rump length (cm)	21.4±0.5	23.6±0.5*
D75 Heart weight (g)	2.46±0.16	3.32±0.16*
D75 Heart weight/fetal weight (mg/g)	8.91±0.60	10.60±0.60*
D75 Left ventricular weight (g)	0.74±0.09	1.14±0.09*
D75 Left ventricle/fetal weight (mg/g)	2.70±0.30	3.60±0.30*
D75 Right ventricular weight (g)	0.54±0.06	0.90±0.06*
D75 Right ventricle/fetal weight (mg/g)	2.00±0.20	2.90±.20*
D75 Left ventricular thickness (mm)	2.49±0.16	3.31±0.13*
D75 Right ventricular thickness (mm)	2.12±0.06	2.30±0.19
D135 Fetal weight (g)	5257±201	4861±153
D135 Crown rump length (cm)	57.9±0.8	57.3±.6
D135 Heart weight (g)	39.9±1.9	35.5±1.1*
D135 Heart weight/fetal weight (mg/g)	7.62±0.32	7.31±0.13
D135 Left ventricular weight (g)	14.12±1.60	16.99±1.26
D135 Left ventricle/fetal weight (mg/g)	2.77±0.43	3.56±0.32
D135 Right ventricular weight (g)	12.04±2.18	10.55±1.53
D135 Right ventricle/fetal weight (mg/g)	2.31±0.41	2.24±0.31
D135 Left ventricle thickness (mm)	5.37±0.45	5.05±0.28
D135 Right ventricle thickness (mm)	4.39±0.25	4.26±0.27

Mean±S.E.M., *P<.05 vs. control, n=8–10 and 11–15 for D75 and D135 groups, respectively.

overnutrition-induced changes in fetal cardiac structure and function, especially cardiac hypertrophy. Therefore, the purpose of this study was to examine fetal cardiac morphometry and hypertrophic signaling following fetal overnutrition in the first half of pregnancy. Numerous animal models have been developed for assessment of human fetal development. The pregnant sheep model has been extensively used to study fetal development throughout the United States and other parts of the world. Without doubt, humans and other primates are distinctly different from laboratory animals such as rodents and sheep in fetal development. Rodents are polytocous and generate a larger biomass of products of conception relative to maternal weight compared to pregnant women. A rodent weighing 250 g with 16 pups and a 5-g placenta is equivalent to a woman baring a 13-kg baby. However, sheep are monotocous (rarely carry more than twins) and are precocial as are pregnant women [11]. To this end, sheep is a much better model for the study of fetal development and maternal obesity due to its similarity to human pregnancy.

2. Materials and methods

2.1. Experimental animals and tissue collection

All animal procedures were approved by the University of Wyoming Animal Care and Use Committee (Laramie, WY, USA). From 60 days before conception (day of mating denotes Day 0) to Day 75 (D75) of gestation (term, 149 days). Multiparous ewes (Rambouillet/Columbia cross) were fed either a highly palatable diet at 100% (control group) of National Research Council (NRC) recommendations or 150% (overnourished group) of NRC recommendations on a metabolic body weight (BW) basis ($BW^{0.75}$). On day 45 of gestation, ultrasonography was used to determine the number of fetuses present and only twin bearing ewes were utilized in this study. All ewes were weighed at weekly intervals and rations adjusted for weight gain.

Immediately prior to necropsy, on D75, ewes were sedated with ketamine and euthanized with an overdose of sodium pentobarbital (Abbott Laboratories, Abbott Park, IL, USA) and exanguinated and the gravid uterus quickly removed. Fetal weight, crown rump length, fetal organ weights including fetal heart weight and left and right ventricular weights were determined for each twin fetus. Left and right ventricular free wall thickness were measured with an Electronic Digital Caliper (Cen-Tech) for both control and overfed groups, and samples of right and left ventricular tissue were snap-frozen in liquid nitrogen for later analysis [9,10]. A cohort of control and overfed ewes were maintained to near term [Day 135 (D135)] before fetal weight, crown rump length, fetal heart weight, left and right ventricular weights as well as left and right ventricular free wall thickness were collected and measured.

2.2. Histopathological analysis

On D75 of gestation, fetal ventricular tissues from control and overfed ewes were collected and fixed in formalin for 24 h. The tissues were then dehydrated through serial alcohols, cleared in xylenes and embedded in paraffin. Paraffin sections with a thickness 5 μm were cut and stained with hematoxylin and eosin (H&E) or Masson's trichrome as described previously [12].

2.3. Western analysis of Akt, Foxo3a, GSK3β, mTOR, nuclear factor of activated T cells (NFATc3), GATA4, ANF and calcineurin A

For Western blot analysis, left and right ventricular tissues were homogenized and lysed in a lysis buffer containing 20 mmol/L HEPES (pH 7.4), 50 mmol/L β-glycerol phosphate, 2 mmol/L EGTA, 1 mmol/L dithiothreitol (DTT), 10 mM NaF, 1 mmol/L sodium orthovanadate, 1% Triton-100, 10% glycerol with 1% protease inhibitor cocktail. Lysates were clarified by centrifugation at 12,000×g for 30 min at 4°C and protein concentrations were determined using the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Richmond, CA, USA). Equal amounts of

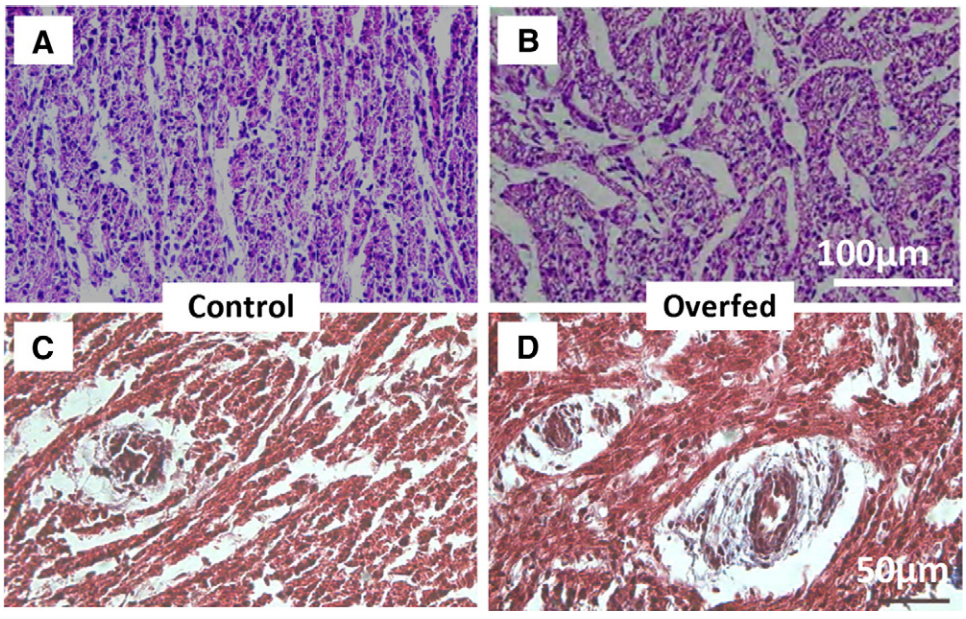

Fig. 1. Representative micrographs of H&E (A and B) and Masson's trichrome (C & D) stained sections from fetal left ventricular tissues from control and overfed groups (original magnification 400×).

protein samples (40 μg/lane) were separated on a 10% or 7% or 15% sodium dodecyl sulfate-polyacrylamide gels based on the molecular weight of corresponding hypertrophic proteins. SeeBlue plus2 Prestained markers (Invitrogen, Carlsbad, CA) were used as standards. Electrophoretic transfer of proteins to nitrocellulose membranes (0.2 μm pore size, Bio-Rad Laboratories, Hercules, CA, USA) was accomplished in a transfer buffer consisting of 25 mmol/l Tris–HCl, 192 mM glycine and 20% or 10% ethanol for 60 min. Membranes were blocked for 60 min at room temperature in TBS-T (0.1% Tris-buffered saline Tween-20) with 5% nonfat dry milk. Membranes were incubated overnight with anti-Akt (1:1,000, Cell Signaling, Beverly, MA, USA), anti-Foxo3a (1:1,000, Upstate Biotechnology, Lake Placid, NY, USA), anti-pAkt (Ser473, 1:1,000, Cell Signaling), anti-pFoxo3a (Thr32, 1:1,000, Upstate Biotechnology), anti-GSK3β (1:1,000, Cell Signaling), anti-mTOR (1:1,000, Cell Signaling), anti-pGSK3β (Ser9, 1:1,000, Cell Signaling), anti-pmTOR (Ser2448, 1:1,000, Cell Signaling), anti-NFATc3 (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-pNFATc3 (Ser265, 1:500, Santa Cruz), anti-GATA4 (1:500, Santa Cruz), anti-pGATA4 (Ser105, 1:500, Santa Cruz), anti-ANF (1:1,000, Bachem Americas, Torrance, CA, USA), anti-calcineurin A (1:1,000, Abcam, Cambridge, MA, USA) and anti-caspase-8 (1:1,000, Cell Signaling) antibodies at 4°C. After incubation with the primary antibody, blots were incubated with the anti-rabbit or anti-mouse IgG horseradish peroxidase–linked antibodies (1:5,000 or 1:3,000) for 60 min at room temperature. After three washes in TBS-T, immunoreactive bands were detected using the Super Signal west Dura Extended Duration Substrate (Pierce, Milwaukee, WI, USA). The intensity of bands was measured with a scanning densitometer (Model GS-800; Bio-Rad, Hercules, CA, USA) coupled with Bio-Rad PC analysis software [9].

2.4. Assessment of mRNA expression by quantitative real-time PCR

Total RNA was extracted from left and right ventricular tissues before cDNA was generated. Quantitative real-time reverse transcriptase-PCR analysis was performed for atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP) and 18S (used as the housekeeping gene). The experiments were performed using a QuantiTect SYBR

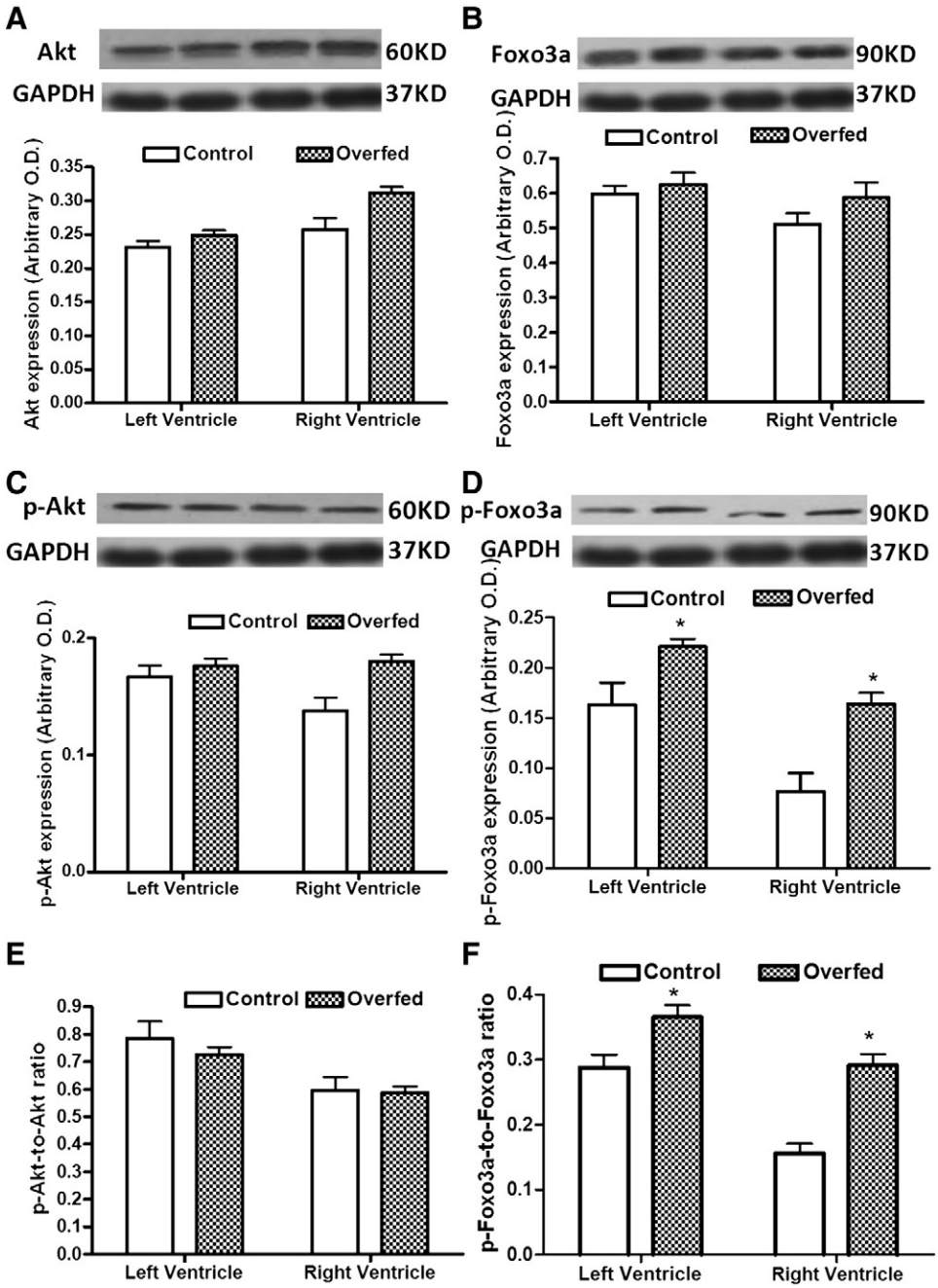

Fig. 2. Protein expression of total and phosphorylated Akt and Foxo3a in left and right ventricles from fetus of control and overfed ewes. (A) Akt. (B) Foxo3a. (C) Phosphorylated Akt (p-Akt). (D) Phosphorylated Foxo3a (p-Foxo3a); (E) p-Akt-to-Akt ratio. (F) p-Foxo3a-to-Foxo3a ratio. (Inset) representative gels depicting Akt, Foxo3a, p-Akt and p-Foxo3a using specific antibodies; Mean±S.E.M., *P<.05 vs. respective control group, n=6–7 per group.

Green Real-Time PCR kit (Bio-Rad, Hercules, CA, USA). The primer (Integrated DNA Technologies, Coralville, IA, USA) sequences of ANP were: forward: 5′-CCC AAT CCA CTC TGG GCT-3′; reverse: 5′-TTT GGA GGA CAA GAT GCC T-3′. The primer sequences for BNP were: forward: 5′-TTG CAG CCC AGG CCA CTG A-3′; reverse: 5′-AGC TGT TGG ACC GTC TAC GA-3′ [13]. The primer sequences for 18S were: forward: 5′-AGC CTG CGG CTT AAT TTG AC-3′; reverse: 5′-CAA CTA AGA ACG GCC ATG CA-3′ [14].

2.5. Statistical analysis

Data are presented as mean±S.E.M. Mean differences between groups were assessed using a one-way analysis of variance. When an overall significance was determined, a Dunnett's post hoc analysis was incorporated. $P<.05$ was considered significant.

3. Results

3.1. General features of fetuses under gestational overfeeding

Ewes in the overfed group increased their BW by ~30% from diet initiation to mating (71.56±3.23 and 92.84±2.97 kg, respectively; $P<.05$) and increased an additional ~10% in BW from mating to necropsy on D75 of gestation (102.22±2.41 kg). In contrast, ewes in the control group exhibited a modest nonsignificant increase in BW from diet initiation to necropsy (68.32±2.91 and 72.18±3.27 kg, respectively; $P>.10$). On D75, although fetal weight failed to reach a statistically significant level between the overfed and control groups, crown rump length, fetal heart and left and right ventricular weight were increased ($P<.05$) by 10.3%, 35.0%, 54.1% and 66.7%, respectively, in the overfed group compared with the control group. The sizes of left and right ventricles (normalized to fetal weight) were significantly elevated ($P<.05$) in the overfed group. Left ventricular wall thickness on D75 was also increased ($P<.05$) by approximately 33.1% in the overfed group compared with the control group while there was no difference in right ventricular wall thickness between overfed and control groups. Interestingly, the fetal weight was comparable between the control and overfed groups near term (D135). Fetal heart exhibited slight however significant atrophy

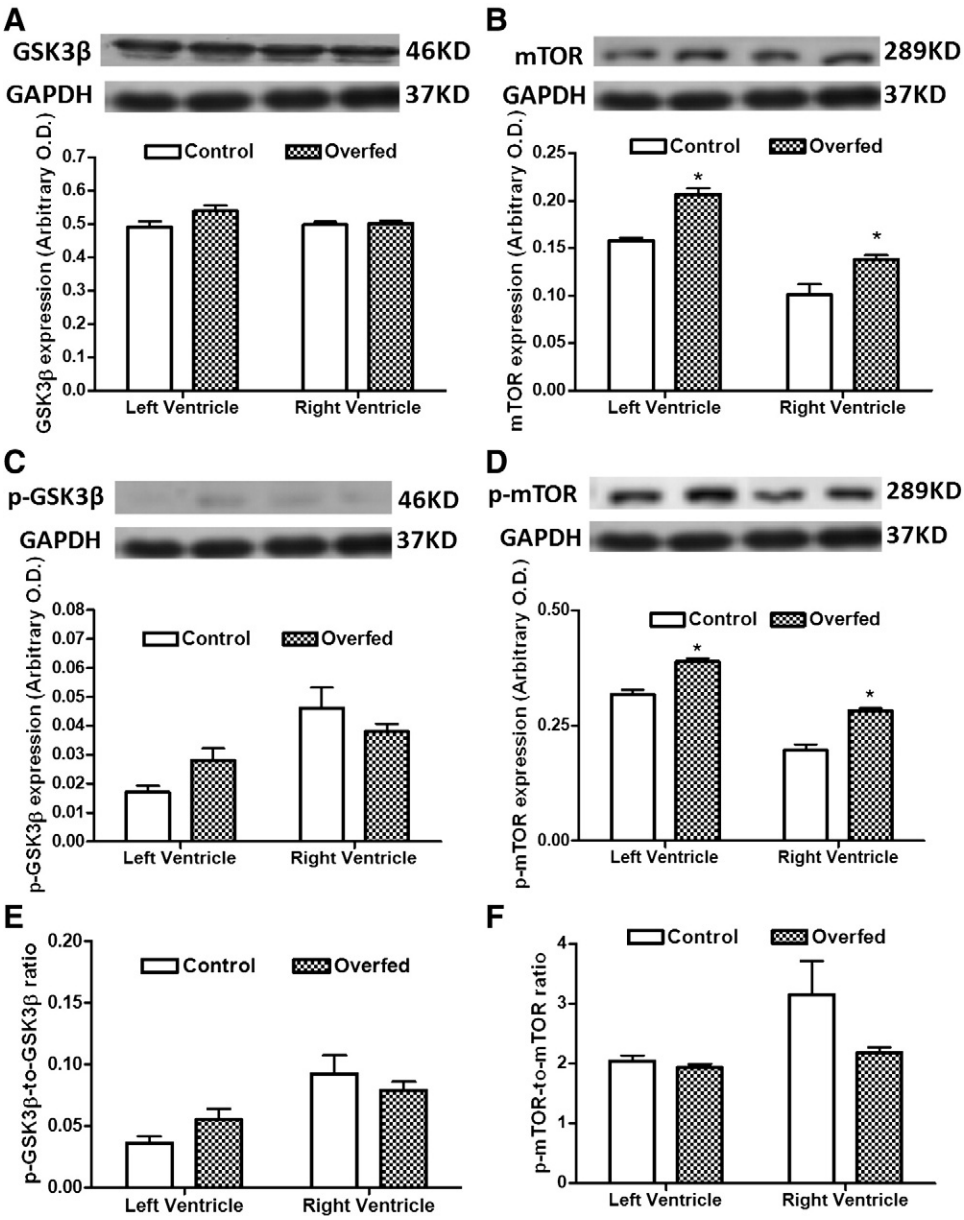

Fig. 3. Total and phosphorylated GSK3β and mTOR protein in left and right ventricles from fetus of control and overfed ewes. (A) GSK3β. (B) mTOR. (C) Phosphorylated GSK3β (p-GSK3β). (D) Phosphorylated mTOR (p-mTOR). (E) p-GSK3β-to-GSK3β ratio. (F) p-mTOR-to-mTOR ratio. (Inset) representative gels depicting GSK3β, mTOR, p-GSK3β and p-mTOR using specific antibodies. Mean±S.E.M. *$P<.05$ vs. respective control, $n=6–7$ per group.

near term (by 11.0%). Left and right ventricles were essentially unchanged near term (D135). The left and right ventricular size (ventricular weight normalized to fetal weight) and wall thickness were comparable between the control and overfed groups near term (D135) (Table 1).

3.2. Histopathological analysis

H&E staining revealed irregular myofiber orientation and increased interstitial space in ventricular tissues from the overfed group compared with the control group. Masson's trichrome staining demonstrates myofiber hypertrophy and fascicular disarray in tissues from the overfed group compared with those from the control group. Although no obvious interstitial fibrosis was identified in both groups, profound perivascular fibrosis was observed in tissues from the overfed group (Fig. 1).

3.3. Expression of total and phosphorylated Akt, Foxo3a, GSK3β and mTOR

To explore the potential mechanism(s) involved in the gestational overnutrition-elicited cardiac hypertrophy, basal expression and phosphorylation of a number of hypertrophic signaling molecules were examined including Akt, Foxo3a, GSK3β and mTOR. Fetal overnutrition significantly enhanced phosphorylation of Foxo3a in both ventricles without affecting total or phosphorylated Akt or Foxo3a protein expression in either ventricle (Fig. 2). Fetal overnutrition also increased total and phosphorylated mTOR in both ventricles although the p-mTOR-to-mTOR ratio remained unchanged in both ventricles. Neither total nor phosphorylated GSK3β was affected by fetal overnutrition in either ventricle (Fig. 3).

3.4. Expression of NFATc3, GATA4, ANF and calcineurin A

Fetal overnutrition significantly up-regulated protein expression of the hypertrophic marker NFATc3 in the left but not the right ventricle. Phosphorylation of NFATc3 was elevated in both ventricles following fetal overnutrition. However, the p-NFATc3-to-NFATc3 ratio remained unchanged in both ventricles (Fig. 4). Expression of total and phosphorylated GATA4 and the hypertrophic marker ANF were unchanged in both ventricles, but fetal overnutrition enhanced expression of calcineurin A in the left but not right ventricle (Fig. 5).

3.5. mRNA expression of ANP and BNP

To further examine the possible mechanism(s) of action involved in cardiac hypertrophy, mRNA levels of the hypertrophic factors ANP and BNP were evaluated. Our results revealed comparable ANP mRNA level in either ventricle between the two groups. Similarly, BNP mRNA level was also similar in either ventricle between the control and overfed groups (Fig. 6).

3.6. Expression of apoptotic marker caspase-8

To examine if apoptosis participated in the development of cardiac hypertrophy, protein expression of the apoptosis marker caspase-8 was evaluated. Our result shown in Fig. 7 displayed comparable

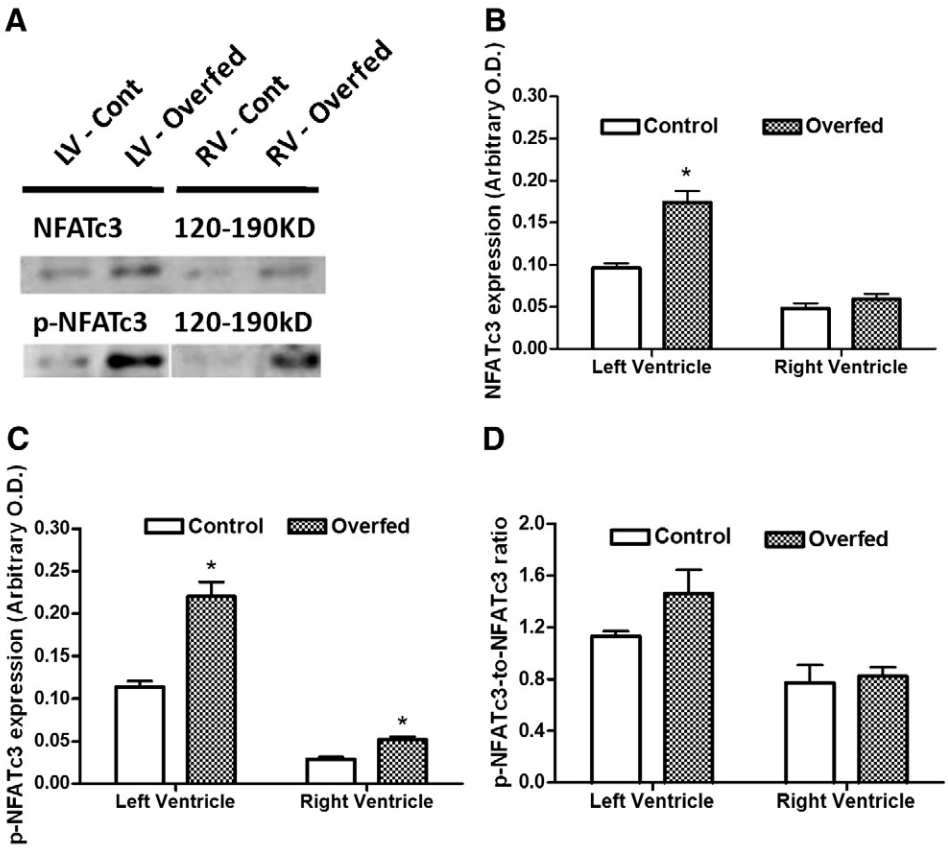

Fig. 4. Protein expression of NFATc3 and phosphorylated NFATc3 (pNFATc3) in ventricles from fetus of control and overfed ewes. (A) Actual gel blotting depicting NFATc3 and pNFATc3 using specific antibodies. (B) NFATc3. (C) pNFATc3; and Panel D: pNFATc3-to-NFATc3 ratio. Mean±S.E.M. *P<.05 vs. respective control, n=6–7 per group.

caspase-8 expression in either ventricle between the two groups, thus not favoring any involvement of apoptosis in the development of cardiac hypertrophy in our current experimental setting.

4. Discussion

The major findings of our present study indicated that intrauterine overnutrition during early to mid gestation leads to accelerated cardiac (ventricular) growth, irregular myofiber orientation, increased interstitial space, myofiber hypertrophy, fascicular disarray and perivascular fibrosis in the absence of changes in the pathological cardiac hypertrophic markers ANF, ANP and BNP. Fetal crown rump length (but not fetal weight), left and right ventricular weights as well as left ventricular wall thickness were all significantly increased following fetal overnutrition in the first half of pregnancy (D75), indicating overt gross morphometric changes during early gestational overfeeding. Our data suggested possible contribution of elevated expression of mTOR, NFATc3, calcineurin A and phosphorylation of Foxo3a, mTOR and NFATc3 in overfeeding-associated accelerated cardiac growth during early to mid gestation. Interestingly, left ventricular weight displayed a trend of increase (although non-significantly) near term (D135) in the overfed group. Left ventricular wall thickness and size in the overfed group were essentially reverted back to normal levels near term while the right ventricular weight exhibited a nonsignificant decline associated with a significant reduction in the fetal weight in the overfed group compared with the controls.

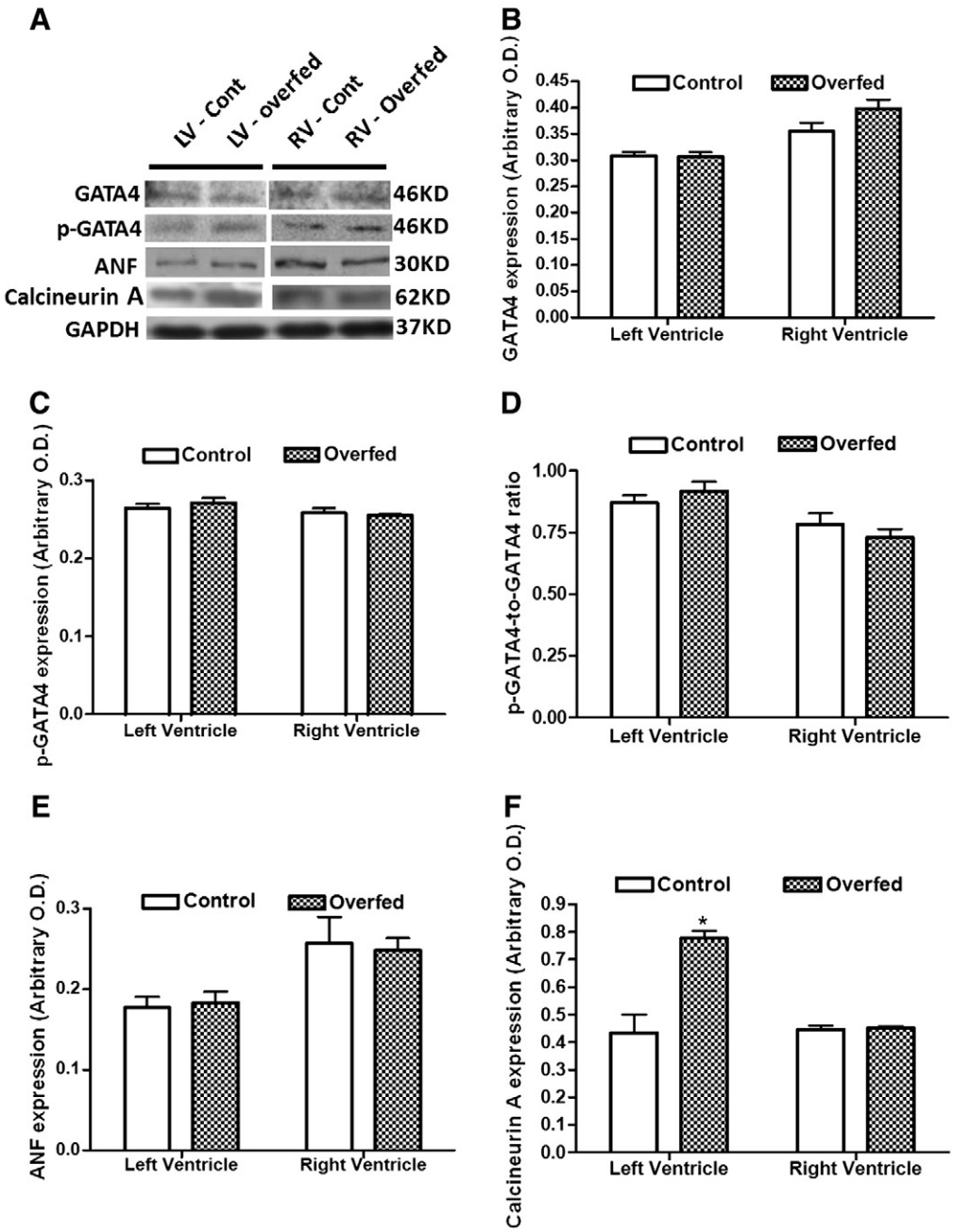

Fig. 5. Protein expression of GATA4, ANF and calcineurin A in ventricles from fetus of control and overfed ewes. (A) Representative GATA4, pGATA4, ANF and calcineurin A gel bands using specific antibodies. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the loading control (1:1,000). (B) GATA4. (C) p-GATA4. (D) p-GATA4-to-GATA4 ratio. (E) ANF. (F) Calcineurin A from both ventricles in control and overfed groups. Mean±S.E.M. *P<.05 vs. respective control, n=6–7 per group.

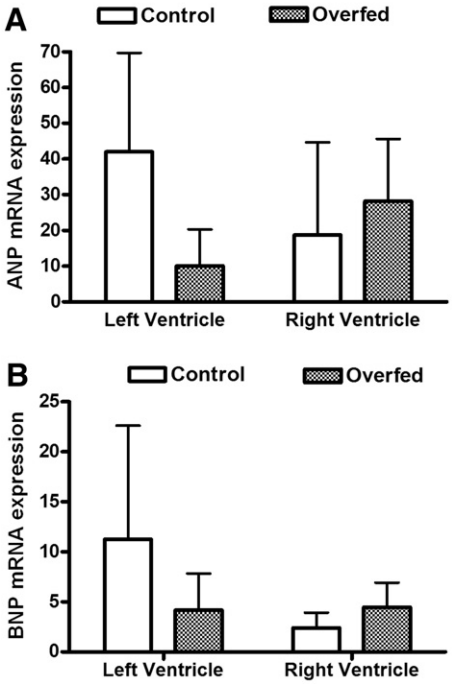

Fig. 6. mRNA levels of ANP and BNP in left and right ventricles from fetus of control and overfed ewes. (A) mRNA expression of ANP. (B) mRNA expression of BNP. Mean±S.E.M., n=5–6 per group.

Several rationales may be considered for the overfeeding-induced cardiac growth in the absence of overt cardiac hypertrophic pathology (e.g., levels of ANF, ANP and BNP). Data from our current study revealed that fetal overnutrition resulted in a marked increase in the phosphorylation of Foxo3a and p-Foxo3a/Foxo3a ratio in both ventricles. Phosphorylation of Foxo3a and consequently Foxo3a inactivation have been shown to contribute to cardiac hypertrophic growth in the absence of change of total Foxo3 protein levels [2]. In fetuses from overfed ewes, the phosphorylation of Foxo3a in ventricular tissue is markedly increased, which would reduce Foxo3a activity and nuclear translocation of Foxo3a thereby favoring antiapoptosis and an enlarged ventricular mass. It is worth mentioning that expression of caspase-8, a protein marker for apoptosis, was unchanged in either ventricle following overfeeding. mTOR and p-mTOR were elevated in both ventricles. mTOR is a known hypertrophic signaling molecule responsible for enhanced protein synthesis induced cardiac hypertrophy [15]. A central role has been suggested for mTOR signaling in sensing and responding to intracellular nutrient availability [16]. Cell regulation by mTOR includes up-regulation of vascular endothelial growth factor (VEGF) signaling and protein translation, and down-regulation of programmed cell death by autophagy. Thus, the increase we have observed in mTOR is compatible with fetal overnutrition and cardiac hypertrophy. We have previously shown that nutrient restriction in the first half of gestation in the pregnant baboon results in down regulation of mTOR in the fetal kidney [17].

The intracellular protein phosphatase calcineurin and NFAT promote translocation of transcription factors from cytoplasm to nucleus and cardiac hypertrophy [18]. Calcineurin/NFATc–dependent signaling pathway and its downstream molecules MCIP1.4, ANF, GATA, ANP and BNP are involved in pathological hypertrophic signaling pathway in the heart [1,19]. The increased calcineurin A/NFATc3 in the left ventricle of overnourished fetuses supports an important role for the calcineurin A-NFATc3 cascade in the accelerated cardiac growth [20-23]. There were no differences in GATA4 expression and its phosphorylation between the two groups indicating that GATA4 may not be a downstream, rate-limiting mediator of calcineurin/NFATc and may be regulated by another pathway. Increased expression of fetal ANF has been reported to accompany cardiac hypertrophy [24]. Natriuretic peptides are a family of structurally related hormone/paracrine factors which play a central role in cardiovascular, endocrine and renal homeostasis. ANP and BNP are secreted from atria and ventricles, respectively, to serve as biomarkers of myocardial volume [25]. It is believed that ANP participates in the regulation of blood pressure and cardiac hypertrophy whereas BNP acts locally to reduce ventricular fibrosis [19]. More recent finding suggests that the increased levels of natriuretic peptides are related to the blood pressure elevation rather than left ventricular hypertrophy [26]. Measurement of the mRNA levels of ANP and BNP as well as the ANF protein levels did not favor the presence of an overt pathological cardiac hypertrophy (despite of accelerated cardiac growth) during early to mid gestation, at least in our current experimental setting.

We observed little changes in the levels of Akt, pAkt, GSK3β or phosphorylated GSK3β between the two groups. These data, in conjunction with the increased Foxo3a and mTOR (total protein and the phosporylated forms), indicated that certain Akt-independent mechanism(s) may be responsible for changes in Foxo3a and mTOR, known downstream targets of Akt. Recent evidence demonstrated that mTOR/p70S6K and nitric oxide/PKG may play a role in pressure overload-induced cardiac hypertrophy [21,27]. Increased expression of phosphorylated NFATc3 in left and right ventricles is consistent with unchanged GSK3β, which is regulated by Akt [28,29]. GSK-3β is capable of phosphorylating NFAT proteins and antagonizing the actions of calcineurin through facilitated NFAT nuclear export [7]. Last but not the least, although gestational obesity is known to be linked to the increased risks of diabetes and metabolic syndrome for both mother (such as gestational diabetes and preeclampsia) and offspring (i.e., fetal origins of obesity, cardiovascular disease and insulin resistance) [8,30,31], recent evidence from our group using a similar sheep maternal obesity model did not favor the presence of full-blown diabetes in either the ewe and fetus [32]. Therefore, it is less likely that full-blown diabetes contributes to the accelerated cardiac growth in our current experimental setting.

In summary, our findings demonstrate that fetal overnutrition in the first half of pregnancy promotes fetal cardiac growth and

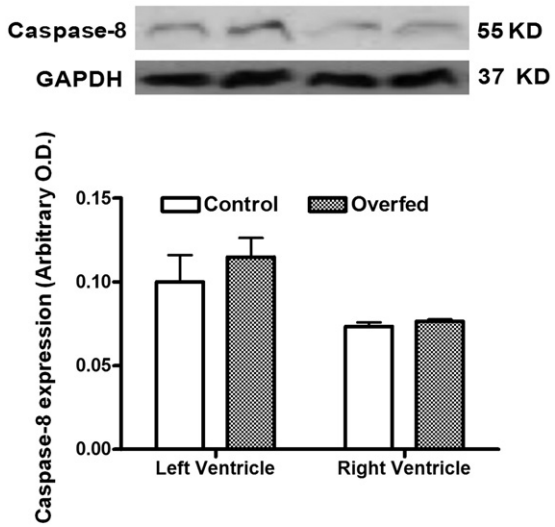

Fig. 7. Caspase-8 expression in left and right ventricles from fetus of control and overfed ewes. (Inset) Representative gel blot of caspase-8 using specific anti-caspase-8 antibody. Mean±S.E.M., n=6–7 per group.

morphometric changes in the absence of overt cardiac hypertrophic pathology. Enhanced protein expression (and/or its phosphorylation) of mTOR and NFATc3 in both ventricles accompanies the overnutrition-induced accelerated ventricular growth. Increased phosphorylation of Foxo3a in both may also contribute to the significantly increased fetal left and right ventricular weights, albeit in the absence of overt apoptosis. These findings suggested that the accelerated cardiac growth triggered by intrauterine overnutrition may be related, at least in part, to elevated protein expression of mTOR, NFATc3, pFoxo3a, pmTOR and pNFATc3. Interestingly, these morphometric changes may occur in the absence of overt pathological cardiac hypertrophic alteration as evidence by unchanged ANF, ANP and BNP in response the gestational overfeeding. One rather interesting observation from our study is that gestational overfeeding-elicited fetal gross morphometric changes during early to mid gestation (D75) that were somewhat reverted to normal levels by term. Although this is beyond the scope of the current study, possible maternal and fetal compensatory mechanisms may be present to counteract the detrimental effects of overnutrition. A recent study from our laboratory demonstrated that placental vascularity was markedly decreased at mid gestation in obese overnourished ewes compared with control ewes, which may slow maternal to fetal nutrient transfer and fetal growth rate [33]. Further study is warranted to understand precisely the nature (increase in cell number versus cell volume) and progression of accelerated cardiac growth and hypertrophic pathology, if any, in response to gestational overnutrition.

Acknowledgments

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References

[1] Bueno OF, Wilkins BJ, Tymitz KM, Glascock BJ, Kimball TF, Lorenz JN, et al. Impaired cardiac hypertrophic response in Calcineurin Abeta-deficient mice. Proc Natl Acad Sci USA 2002;99:4586–91.
[2] Ni YG, Berenji K, Wang N, Oh M, Sachan N, Dey A, et al. Foxo transcription factors blunt cardiac hypertrophy by inhibiting calcineurin signaling. Circulation 2006;114:1159–68.
[3] Ravingerova T, Barancik M, Strniskova M. Mitogen-activated protein kinases: a new therapeutic target in cardiac pathology. Mol Cell Biochem 2003;247:127–38.
[4] Li HH, Willis MS, Lockyer P, Miller N, McDonough H, Glass DJ, et al. Atrogin-1 inhibits Akt-dependent cardiac hypertrophy in mice via ubiquitin-dependent coactivation of Forkhead proteins. J Clin Invest 2007;117:3211–23.
[5] Proud CG. Ras, PI3-kinase and mTOR signaling in cardiac hypertrophy. Cardiovasc Res 2004;63:403–13.
[6] Haq S, Choukroun G, Kang ZB, Ranu H, Matsui T, Rosenzweig A, et al. Glycogen synthase kinase-3beta is a negative regulator of cardiomyocyte hypertrophy. J Cell Biol 2000;151:117–30.
[7] Antos CL, McKinsey TA, Frey N, Kutschke W, McAnally J, Shelton JM, et al. Activated glycogen synthase-3 beta suppresses cardiac hypertrophy in vivo. Proc Natl Acad Sci USA 2002;99:907–12.
[8] Leddy MA, Power ML, Schulkin J. The impact of maternal obesity on maternal and fetal health. Rev Obstet Gynecol 2008;1:170–8.
[9] Dong F, Ford SP, Nijland MJ, Nathanielsz PW, Ren J. Influence of maternal undernutrition and overfeeding on cardiac ciliary neurotrophic factor receptor and ventricular size in fetal sheep. J Nutr Biochem 2008;19:409–14.
[10] Zhu MJ, Han B, Tong J, Ma C, Kimzey JM, Underwood KR, et al. AMP-activated protein kinase signalling pathways are down regulated and skeletal muscle development impaired in fetuses of obese, over-nourished sheep. J Physiol 2008;586:2651–64.
[11] Nathanielsz PW. A time to be born: implications of animal studies in maternal-fetal medicine. Birth 1994;21:163–9.
[12] Doser TA, Turdi S, Thomas DP, Epstein PN, Li SY, Ren J. Transgenic overexpression of aldehyde dehydrogenase-2 rescues chronic alcohol intake-induced myocardial hypertrophy and contractile dysfunction. Circulation 2009;119:1941–9.
[13] Cameron VA, Rademaker MT, Ellmers LJ, Espiner EA, Nicholls MG, Richards AM. Atrial (ANP) and brain natriuretic peptide (BNP) expression after myocardial infarction in sheep: ANP is synthesized by fibroblasts infiltrating the infarct. Endocrinology 2000;141:4690–7.
[14] Lopez-Andreo M, Lugo L, Garrido-Pertierra A, Prieto MI, Puyet A. Identification and quantitation of species in complex DNA mixtures by real-time polymerase chain reaction. Anal Biochem 2005;339:73–82.
[15] Balasubramanian S, Johnston RK, Moschella PC, Mani SK, Tuxworth Jr WJ, Kuppuswamy D. mTOR in growth and protection of hypertrophying myocardium. Cardiovasc Hematol Agents Med Chem 2009;7:52–63.
[16] Marshall S. Role of insulin, adipocyte hormones, and nutrient-sensing pathways in regulating fuel metabolism and energy homeostasis: a nutritional perspective of diabetes, obesity, and cancer. Sci STKE 2006;2006:re7.
[17] Nijland MJ, Schlabritz-Loutsevitch NE, Hubbard GB, Nathanielsz PW, Cox LA. Non-human primate fetal kidney transcriptome analysis indicates mammalian target of rapamycin (mTOR) is a central nutrient-responsive pathway. J Physiol 2007;579:643–56.
[18] Clerk A, Cullingford TE, Fuller SJ, Giraldo A, Markou T, Pikkarainen S, et al. Signaling pathways mediating cardiac myocyte gene expression in physiological and stress responses. J Cell Physiol 2007;212:311–22.
[19] Potter LR, Yoder AR, Flora DR, Antos LK, Dickey DM. Natriuretic peptides: their structures, receptors, physiologic functions and therapeutic applications. Handb Exp Pharmacol 2009:341–66.
[20] Fiedler B, Lohmann SM, Smolenski A, Linnemuller S, Pieske B, Schroder F, et al. Inhibition of calcineurin-NFAT hypertrophy signaling by cGMP-dependent protein kinase type I in cardiac myocytes. Proc Natl Acad Sci USA 2002;99:11363–8.
[21] Gonzalez Bosc LV, Wilkerson MK, Bradley KN, Eckman DM, Hill-Eubanks DC, Nelson MT. Intraluminal pressure is a stimulus for NFATc3 nuclear accumulation: role of calcium, endothelium-derived nitric oxide, and cGMP-dependent protein kinase. J Biol Chem 2004;279:10702–9.
[22] Tokudome T, Horio T, Kishimoto I, Soeki T, Mori K, Kawano Y, et al. Calcineurin-nuclear factor of activated T cells pathway-dependent cardiac remodeling in mice deficient in guanylyl cyclase A, a receptor for atrial and brain natriuretic peptides. Circulation 2005;111:3095–104.
[23] Wilkins BJ, De Windt LJ, Bueno OF, Braz JC, Glascock BJ, Kimball TF, et al. Targeted disruption of NFATc3, but not NFATc4, reveals an intrinsic defect in calcineurin-mediated cardiac hypertrophic growth. Mol Cell Biol 2002;22:7603–13.
[24] Chien KR, Zhu H, Knowlton KU, Miller-Hance W, van-Bilsen M, O'Brien TX, et al. Transcriptional regulation during cardiac growth and development. Annu Rev Physiol 1993;55:77–95.
[25] Tripepi G, Mattace-Raso F, Mallamaci F, Benedetto FA, Witteman J, Malatino L, et al. Biomarkers of left atrial volume: a longitudinal study in patients with end stage renal disease1. Hypertension 2009;54:818–24.
[26] Soualmia H, Ayadi I, Omar S, Feki M, Drissa H, Mebazaa A, et al. Atrial natriuretic peptide and brain natriuretic peptide release in human essential hypertension. Clin Lab 2009;55:120–7.
[27] Boluyt MO, Li ZB, Loyd AM, Scalia AF, Cirrincione GM, Jackson RR. The mTOR/p70S6K signal transduction pathway plays a role in cardiac hypertrophy and influences expression of myosin heavy chain genes in vivo. Cardiovasc Drugs Ther 2004;18:257–67.
[28] Kim HS, Skurk C, Maatz H, Shiojima I, Ivashchenko Y, Yoon SW, et al. Akt/FOXO3a signaling modulates the endothelial stress response through regulation of heat shock protein 70 expression. FASEB J 2005;19:1042–4.
[29] Hardt SE, Sadoshima J. Glycogen synthase kinase-3beta: a novel regulator of cardiac hypertrophy and development. Circ Res 2002;90:1055–63.
[30] King KB, Gerich JE, Guzick DS, King KU, McDermott MP. Is a history of gestational diabetes related to risk factors for coronary heart disease? Res Nurs Health 2009;32:298–306.
[31] Symonds ME, Sebert SP, Hyatt MA, Budge H. Nutritional programming of the metabolic syndrome. Nat Rev Endocrinol 2009;5:604–10.
[32] Ford SP, Zhang L, Zhu M, Miller MM, Smith DT, Hess BW, et al. Maternal obesity accelerates fetal pancreatic beta-cell but not alpha-cell development in sheep: prenatal consequences. Am J Physiol Regul Integr Comp Physiol 2009;297:R835–43.
[33] Zhu MJ, Du M, Nijland MJ, Nathanielsz PW, Hess BW, Moss GE, et al. Down-regulation of growth signaling pathways linked to a reduced cotyledonary vascularity in placentomes of over-nourished, obese pregnant ewes. Placenta 2009;30:405–10.