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Original Article

Maternal hypoxia has an influence on Sfrp5 expression of offspring rats and negatively correlated with Wnt/JNK signaling pathway

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Abstract: Aim: Evaluate the long-term effects of reduced fetal oxygen supply on adipokine secreted frizzled-related protein 5 (Sfrp5) and the contribution to insulin signaling pathway. The programming of responses to high-fat feeding was also investigated. Methods: According to a 2×2 factorial design, consisting of two factors [maternal hypoxia (MH) and postnatal high-fat diet (HFD)]; offspring were randomly assigned into four groups. Characteristics of growth, metabolic parameters, insulin levels, and expression of Sfrp5, as well as Wnt5a and the molecular mechanisms involved in insulin signaling pathway were evaluated in Sprague-Dawley rat offspring. Results: Maternal hypoxia caused a relative increased insulin levels and P-IRS-1 protein expression in offspring compared to the normoxic counterparts (all $P<0.05$). In addition, a significantly decreased Sfrp5 mRNA and protein concentrations in adipose tissue and increased Wnt5a protein was found in the same maternal hypoxia offspring compared to controls (all $P<0.05$); and those changes were accompanied by increased P-JNK-1, P-C-Jun in hypoxia offspring (all $P<0.05$). Moreover, a positive interaction effect between prenatal hypoxia and hyperlipidemia was found ($P<0.05$). Conclusions: Maternal hypoxia during pregnancy has an effect on postnatal Sfrp5 expression in offspring adipose tissue and may provide an underlying mechanism for the development of IR by through Wnt/JNK signaling. Furthermore, the effects of prenatal hypoxia were augmented by hyperlipidemia.

Keywords: Maternal hypoxia, secreted frizzled-related protein 5, P-IRS-1 protein expression, Wnt5a protein

Introduction

The "developmental origins of health and disease" hypothesis, coupled with the emerging role of adipose tissue as a dynamic endocrine organ, has generated numerous studies linking early-life events with long-term alterations in adipose tissue structure and function, which proposed an association between a suboptimal fetal environment and later deleterious consequences in offspring [1-7]. The growing body studies [8-16] addressed that a maternal hypoxic environment may modify the expression of adipokines in fetal adipocytes, leading to the subsequent development of long-term complications including IR, in adulthood, one of key symptoms of MetS. So far, studies about adipokines in a maternal hypoxic environment and the role of early programming of adipose tissue has pointed out that it may provide an insight into potential mechanisms. However, the mechanisms underlying the prenatal regulation of adipokines and the programming of adipose tissue, which influences the postnatal insulin signaling pathways that are involved in the development of adult diseases, are still poorly understood.

Recently, Ouchi et al. [17, 18] reported secreted frizzled-related protein 5 (Sfrp5) is a "new" adipokine, but data regarding the role of Sfrp5 as adipokine are limited [17-20], and the others role has not been investigated, including under prenatal hypoxia. Therefore, to examine the

long-term effects of stress experiencing early in gestation on the programming of offspring adipose tissue function, pregnant rats were exposed to hypoxia and offspring were fed either a standard or a high-fat diet, previously used in our studies [21, 22], were utilized in a 2×2 full factorial design to investigate the impact of maternal hypoxia during pregnancy on changing the expression of adipokine Sfrp5 and the role of Wnt/JNK signaling pathway. In addition, the programmings of responses to high-fat feeding in offspring were investigated.

Materials and methods

Animals

All experimental procedures were performed in accordance with the National Institutes of Health guidelines and the approval by the Standing Committee on Ethics and Animal Experimentation at the Fujian Medical University (China). Sprague-Dawley rats (Shanghai Experimental Animal Center, Shanghai, China) were used in this study. Three month old female rats were housed overnight with male rats, and mating was confirmed by the presence of spermatozoa in the vaginal smear the following morning (day 0, term 21 days). On day 7, rats were randomly assigned to either the maternal hypoxia (MH) or control group. During pregnancy and lactation, all rats were fed with standard lab rat chow ad libitum.

Models of maternal hypoxia

From Day 7 to Day 21 of pregnancy, rats (n=10) in the MH group were put into a Plexiglas chamber (volume, 140 L) and oxygen concentration was maintained at 10 ± 1% by continuous infusion of a nitrogen gas and compressed air mixture. A portable oxygen analyzer (S-450; IST-AIM) was used to monitor the oxygen concentration of the chamber (calibrated daily). The expired CO_2 was eliminated by circulating chamber air through soda lime and the water contained in the expired gas was trapped in a chilled glass tank. Blood gas and pH were measured by a blood gas analyzer (Rapidlab 850; Bayer). Arterial blood samples were withdrawn from a cannulated left femoral artery after 1 h of exposure to the hypoxic environment in six randomly-selected rats. Pregnant rats were removed from the chamber and housed in room air after 3 h of hypoxia. Control animals (n=10) were put into an identical Plexiglas chamber which compressed air was continuously infused.

Offspring

In order to standardize postnatal nutrition, litters were reduced to eight pups at birth. A randomly-culled pup per litter was weighed again after weaning (at 21 days). According to a 2×2 factorial design, consisting of two factors [maternal hypoxia (MH) and postnatal high-fat diet (HFD)], each with two levels, male offspring were randomly assigned into four groups (each containing 12 animals) as follows: maternal hypoxia offspring with postnatal high-fat diet [MH+HFD]; maternal hypoxia offspring with postnatal normal diet [MH]; maternal normoxia offspring with postnatal high-fat diet [HFD]; maternal normoxia offspring with normal diet [Control].

After weaning, male offspring assigned to the high-fat diet groups were fed with high-fat diet (92.3% standard lab rat chow supplemented with 2% cholesterol, 5% lard, 0.5% sodium cholate, and 0.2% propylthiouracil) for 12 weeks. Blood samples were collected from the jugular vein to detect serum lipids in order to confirm the induction of hyperlipidemia.

Tissue collection

Adipose tissue was collected at the end of Week 3 and Week 15. Rats were sacrificed under deep anesthesia and epididymal adipose tissue was collected immediately. Tissues for Western Blot and PCR detection were frozen in liquid nitrogen and then refrigerated at -80°C freezer until processing.

Metabolic measurements

Blood samples collected from the jugular vein were centrifuged at 3,000 rpm (1,500× g) for 15 min and then stored at -20°C. Plasma fasting insulin (FINS) concentrations were determined using a radioimmunoassay kit for insulin. Fasting plasma glucose (FPG), total cholesterol (TC), triglycerides (TG) and free fatty acid concentrations (FFA) were measured in the clinical biochemistry department using a standard automatic biochemistry analyzer (Olympus AU2700, Hamburg, Germany) and commercial ELISA kits were used to measure proteins (Raybiotech, USA). The formula for HOMA-IR

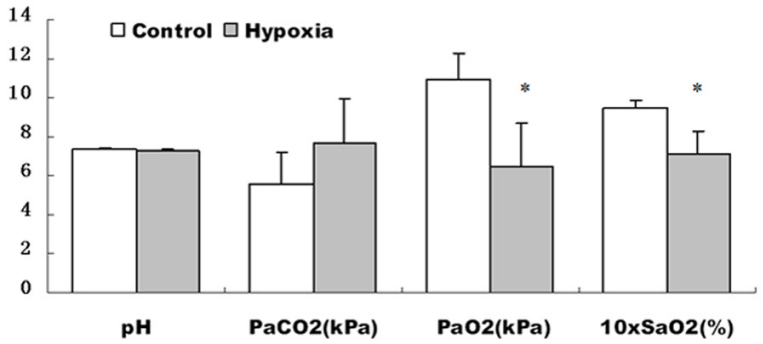

Figure 1. Arterial blood gases in the control and MH groups. The MH group was exposed to hypoxia for 1 h. All data are expressed as means ± SEM (n=6 in each group). *P<0.05, compared with control. PaO2: arterial O2 partial pressure; PaCO2: arterial CO2 partial pressure.

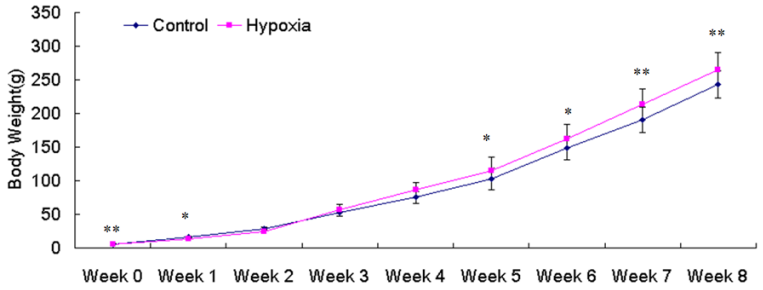

Figure 2. Weight of offspring in the control and MH groups during lactation. All data are expressed as means ± SEM (n=8 in each group) for control and hypoxia rats. *P<0.05, **P<0.01 compared with control.

measurement was: FPG (mmol/L) × FINS (mIU/L)/22.5.

Quantitative real-time PCR

Gene expression levels were quantified by real-time PCR. Total RNA from adipose tissues was isolated using the TRIzol reagent (Invitrogen) and treated with RNase-free DNase (Promega, Madison, WI) at 37°C for 30 mins to remove genomic DNA. RNA was then subjected to reverse transcription and real-time PCR analysis on an ABI Prism 7000 instrument (Applied Biosystems), as described [23]. All samples were run in triplicate and the mean values were used for calculations. Each sample was normalized to the levels of β-actin, a housekeeping gene, detected and the results were expressed as 'fold change' relative to controls using the $2^{-\Delta\Delta Ct}$ method. Primers used for real-time PCR were designed using Beacon Designer software (Premier Biosoft Int.): 5'-TTCCAGCCTTCCTTCCTG-3' and 5'-GGCATAGAGGTCTTTACGG-3' for β-actin, 5'-TCGCTCTTCGCTCCTGTC-3' and 5'-GCTGTCCCTCCACTTTGC-3' for Sfrp5.

Western bolt analysis

Adipose tissue samples (0.5 g each) for Western Blot analysis were homogenized in 5 ml buffer containing 25 mM HEPES, 250 mM sucrose, 4 mM EDTA, 1 μM leupeptin, 1 μM aprotinine and 1 μM pepstatin, pH 7.4. Homogenates were centrifuged at 12,000 g for 10 min at 4°C and the supernatant was collected, aliquoted and stored at -20°C. The proteins were measured by the Coomassie Protein Assay Reagent (Pierce) using BSA as a standard. Samples were incubated for 5 min at 95°C and 20 μg protein was separated by SDS-PAGE on 4-15% running gel. Proteins were transferred to pure polyvinylidene fluoride (PVDF) membranes, which were incubated for 1 hour in blocking buffer (5% non-fat dry milk in Tris buffered saline solution (TBST)) with the primary antibody diluted (1:1000) in blocking buffer. Following incubation with the primary antibody membranes were washed 3 times in TBS and incubated with the horse radish peroxidase-conjugated secondary antibody. The ECL Western Blotting Detection kit (Amersham Pharmacia Biotech) was used for detection. Western blots were quantified by scanning densitometry (Phoretix 1D Quantifier, Nonlinear Dynamics) with Image J program. The anti-phospho-JNK (Thr183/Tyr185), anti-phospho-C-Jun (Ser63) and anti-phospho-IRS-1 (Ser307) antibodies were purchased from Cell Signaling Technology. The Sfrp5 antibody was purchased from Abcam. The Wnt5a antibody was purchased from R&D Systems. The β-actin antibody was purchased from Santa Cruz.

Statistical analysis

All data analysis was performed with commercially available statistical analysis software packages (SPSS 19.0; SPSS, Chicago, IL). All data were expressed as means ± SEM. Differences were analyzed by Student's unpaired t test or analysis of variance (ANOVA) for

Table 1. Metabolic parameters of offsprings at Week 15

Group	Control (n=6)	MH (n=6)	HFD (n=6)	MH+HFD (n=6)
TC (mmol/L)	1.92 ± 0.26	1.95 ± 0.20	2.21 ± 0.16*	2.88 ± 0.23*
TG (mmol/L)	0.30 ± 0.41	0.32 ± 0.24*	0.49 ± 0.19*	0.50 ± 0.29*
FFA (uEq/L)	332.10 ± 75.50	386.40 ± 96.80*	582.30 ± 217.70**	1036.70 ± 127.70***
HDL-C (mmol/L)	0.58 ± 0.05	0.59 ± 0.01	0.45 ± 0.54*	0.41±0.08*
LDL-C (mmol/L)	0.29 ± 0.11	0.34±0.23*	2.47 ± 1.40*	5.97 ± 1.57*

All data are expressed as means ± SEM. *P<0.05; **P<0.01; ***P<0.001 compared with control group.

Table 2. Metabolic parameters of offspring at Week 3 and Week 15

	Week 3		Week 15			
	Control (n=8)	MH (n=8)	Control (n=8)	MH (n=8)	HFD (n=8)	MH+HFD (n=8)
FPG (mmol/L)	5.61± 0.03	5.63 ± 0.05	5.56 ± 0.03	5.62 ± 0.04	5.67 ± 0.05	6.06 ± 0.14*
FINS (mIU/L)	6.63± 0.21	14.66 ± 0.68***	9.59 ± 0.24	11.91 ± 0.40**	14.97 ± 0.85**	41.14 ± 1.10***
HOMA-IR	1.65± 0.05	3.66 ± 0.16***	2.37 ± 0.06	2.97 ± 0.10**	3.77± 0.21**	11.07 ± 0.31***

All data are expressed as means ± SEM. *P<0.05; **P<0.01; ***P<0.001 compared with control group.

multiple comparisons. A general linear model of univariate process was performed to analyze the main effects and the interaction effect of prenatal hypoxia and hyperlipidemia on changes in Sfrp5 expression in a completely randomized factorial design experiment. A value of *P* <0.05 was accepted as statistically significant.

Results

Arterial blood gas analysis

After 1 h of hypoxia, there was a significant decrease in blood PaO_2 and SaO_2 in rats exposed to the hypoxic environment compared with the control group (all P<0.05), and no significant differences in mean $PaCO_2$ and pH values were found between them (**Figure 1**). There was no significant difference in daily food intake or every other day weight gain between pregnant rats in the MH and control groups during the treatment period (P>0.05, date not shown), confirming that this model can be used to study the effects of hypoxia alone.

Characteristics of offspring

There was no significant difference in the number of pups per litter between the MH and control groups (12.5 ± 2.5 vs. 13.3 ± 2.3; P>0.05). However, the birth weights of offspring in the MH group were significantly reduced compared with control offspring (4.82 ± 0.58 g vs. 5.86 ± 0.39g; P<0.01). Prior to lactation, the number of pups was reduced to eight. At the end of lactation there were no difference in body weight between the groups (42.8 ± 2.92 g vs. 45.1 ± 2.67 g; P>0.05); however, a significant increase in body weight at the end of lactation (day 21) was observed in the MH group when compared to the control group (**Figure 2**). In addition, at birth, the organ weight-to-body weight ratios were also significantly different between the MH and control groups (See in our published data, Ref. [24, 25]). Maternal exposure to hypoxia resulted in offspring that were small for gestational age (SGA), but exhibited a postnatal catch-up in growth.

Metabolic parameters of offspring

Offspring fed the high-fat diet had serum total cholesterol (TC) and triglyceride (TG) levels that were persistently increased as compared to those fed a normal diet, which confirmed the state of hyperlipidemia (**Table 1**). FINS concentrations were elevated in the offspring of the MH group at the end of Week 3 and Week 15 and the HFD and MH+HFD groups at Week 15, compared with offspring of the control group (all P<0.01). In contrast, there were no significant differences in FPG levels amongst the groups, other than the MH+HFD group compared with the control group (P=0.677 and 0.842 at Week 3 and Week 15, respectively). Maternal hypoxia and postnatal hyperlipidemia led to significantly increased HOMA-IR (all P<0.01). The FINS concentrations and HOMA-IR were significantly higher in offspring of the MH+HFD group than in the HFD group (*P*<0.05) and there was a positive interaction between

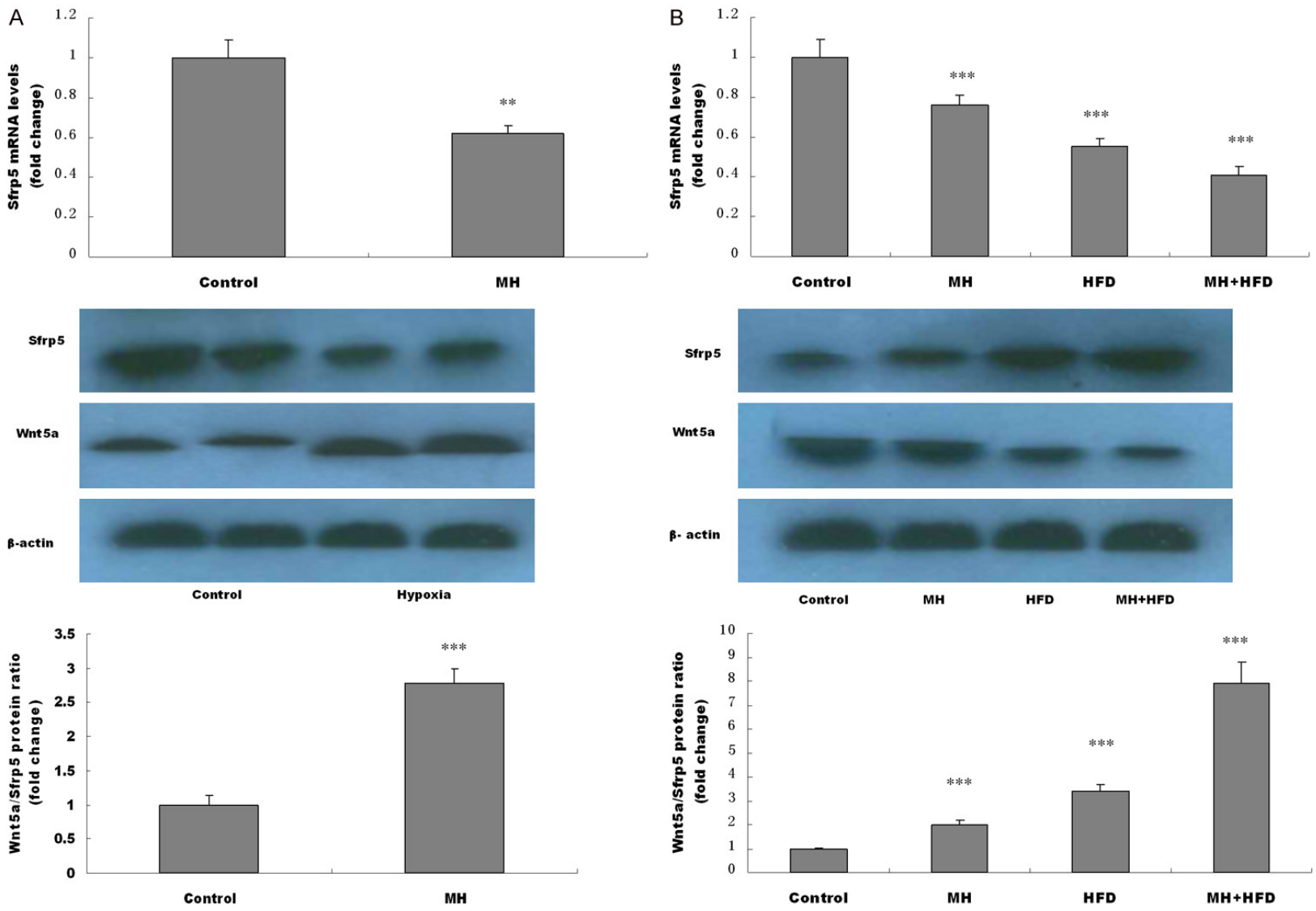

Figure 3. Expression of Sfrp5 and Wnt5a in epididymal adipose tissue at the end of Week 3 (A) and Week 15 (B). All data are expressed as means ± SEM (n=6 or 8 in each group). *P<0.05; **P<0.01; ***P<0.001 compared with control group.

the MH group and the HFD group for plasma insulin concentrations and HOMA-IR (*P*<0.05) (**Table 2**).

Effect of maternal hypoxia on Sfrp5 and Wnt5a expression

Sfrp5 mRNA expression was reduced at Week 3 and Week 15 in the offspring of the MH group compared with control group by a factor of 0.62 ± 0.04 and 0.76 ± 0.05, respectively (all P<0.001). In addition, in the MH+HFD group, Sfrp5 mRNA expression was reduced by 0.41 ± 0.04 compared with control group, which was significant (P<0.001). The effect of maternal hypoxia on Sfrp5 and Wnt5a protein expression were also evaluated and maternal hypoxia led to decreased Sfrp5 protein expression, higher levels of Wnt5a protein expression, and an increase in the ratio of Wnt5a to Sfrp5 in the offspring (**Figure 3**).

The general linear model of univariate process was used to analyze the main effects and the interaction effects of prenatal hypoxia and hyperlipidemia on the integrated optical density (IOD) of Sfrp5 protein expression at the end of Week 15. Using this model there was a statistical difference (F=379.724; P<0.001; R^2= 0.973). Prenatal hypoxia caused a significant decrease in Sfrp5 protein expression, with a main effect of 0.1 IOD (P<0.001). In addition, there was a positive interaction effect between prenatal hypoxia and hyperlipidemia on Sfrp5 protein expression (P<0.001), with an IOD of 0.06 for hypoxia and 0.13 for hypoxia with hyperlipidemia. These results revealed that prenatal hypoxia and postnatal hyperlipidemia exerted a synergistic effect on the expression of Sfrp5 protein in offspring. Similarly, the analysis model on Wnt5a protein expression had statistical significance (F=474.471; P< 0.001; R2=0.979); prenatal hypoxia and hyperlipidemia caused a significant increase in expression of Wnt5a protein (P<0.001), which also exhibited a positive interaction to hyperlipidemia (P<0.001).

Effect of maternal hypoxia on Wnt/JNK signaling pathway activation

Phosphorylation of C-jun N-terminal kinase (JNK), a downstream target of the noncanonical Wnt signaling, was elevated 2.14 ± 0.31 and 1.75 ± 0.18, at Week 3 and Week 15, respectively, in the offspring of the MH group compared with control group (all P<0.05). Also, the phosphorylation of C-jun, a downstream substrate of JNK, was also elevated in the offspring of the MH group by a factor of 2.72 ± 0.32 and 1.83 ± 0.14 (all P<0.05). Activation of JNK-1 promotes IR through serine phosphorylation of the insulin receptor substrate-1 (IRS-1). IRS-1 phosphorylation at residue Ser 307 was increased in adipose tissue of the offspring of the MH group by a factor of 2.51 ± 0.36 and 1.75 ± 0.23 (all P<0.05) (**Figure 4**).

In addition, after 12 weeks of a postnatal high-fat diet (Week 15) phosphorylation of JNK, C-Jun, and IRS-1 were significantly increased in the offspring of the MH group by 3.05 ± 0.41, 5.50 ± 0.38 and 4.89 ± 0.35, respectively, compared with control group (all P<0.01) (**Figure 4**).

Discussion

Prenatal hypoxia presents a major threat to the well-being of fetal development [1, 3, 8, 9]. A number of animal models have been developed, all of which result in the long-term programming regulating the abundance and function of adipose tissue, to examine the potential mechanisms that drive the physiological changes brought on by prenatal hypoxia. The hypoxic animal model [1-5, 8, 9] is the only noninvasive technique available currently, therefore, it is not surprising that it has recently been used extensively to investigate the role of early programming of adipose tissue.

To determine whether maternal hypoxia during pregnancy, with an oxygen concentration of 10 ± 1% 3 h/day during 7-21 days of gestation induces a reduction in oxygen supply, we measured the arterial blood gas as an index of maternal hypoxemia. Our data demonstrated that chronic maternal hypoxia significantly decreased maternal PaO_2 and SaO_2 values compared with controls, suggesting that chronic maternal hypoxia around gestational Day 9 to Day 13 induced significant maternal hypoxemia and resulted in reduced neonatal size; perturbation of neonatal organ weight and proportion, but did not lead to reduced food intake, which was consistent with previous results [21, 22] (**Figure 1**). Regazzetti et al. [23] demon-

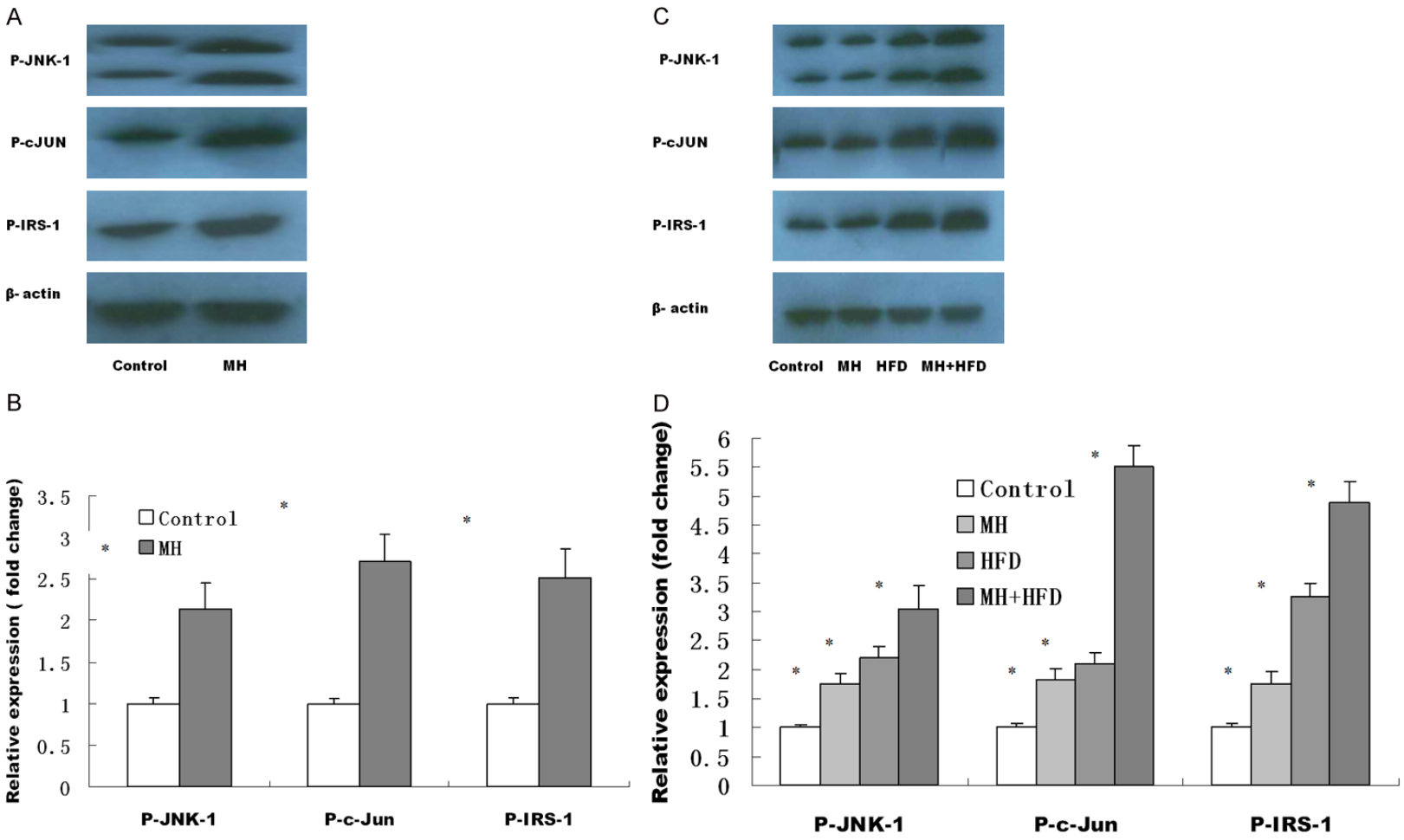

Figure 4. Effect of MH and HF diet on Phosphorylation of JNK (Thr183/Tyr185), C-jun (Ser63) and IRS-1 (Ser307) in the adipose tissue of offspring at Week 3 (A) and Week 15 (C). With summarized data (B and D). All data are expressed as means ± SEM (n=6-8 in each group). *P<0.05 compared with control group.

strated that hypoxia could create a state of IR in adipocytes, establishing it as a new etiology of insulin resistance in adipose tissue. The results of metabolism parameters measured in our study are consistent with these findings, showing that maternal hypoxia resulted in significantly elevated FINS and HOMA-IR levels, as well as increased P-IRS protein expression in the offspring. These findings indicate that conditions of sustained moderate hypoxia during gestational development have the potential to cause a state of IR in offspring. However, the processes and biological mechanisms that link intrauterine insults and manifestation of IR in adulthood were still not fully understood. So, the cellular mechanisms underlying these effects were also explored in this study.

Ouchi et al. [17] reported a new role for secreted frizzled-related protein 5 (Sfrp5), a protein previously linked to the Wnt signaling pathway, as an anti-inflammatory adipokine. More recently, several reports [17-20] have shed light on the potential role of the adipokine Sfrp5 secreted by adipose tissue in insulin signaling pathways.

Since the emerging role of adipose tissue as a dynamic endocrine organ with an extent of anatomical and physiological plasticity, linking early-life event with long-term alterations in adipose tissue structure and function. Thus, in the present study, we hypothesize that the changes in insulin signaling observed in the MH group were related to the changes in Sfrp5 expression and noncanonical Wnt/JNK signaling. Of particular interest, the results of our study demonstrated lower levels of Sfrp5 mRNA and protein in the adipose tissue of male offspring of the maternal hypoxia group compared control adipose tissue, while Wnt5a protein expression was found to be increased when compared to control counterparts. In addition, an increase in phosphorylated C-jun N-terminal kinase (P-Cjun) and C-jun N-terminal kinase 1 (P-JNK-1) protein were found in adipose tissue, suggesting a down-regulation of insulin signaling in MH group compared to normoxic counterparts, showing that Sfrp5 can act as a decoy receptor, binding Wnt5a and sequestering it in the extracellular environment, preventing the activation of Fz and attenuating Wnt/JNK signaling to inhibit chronic inflammation and consequently improve insulin sensitivity, revealing a new underlying molecular mechanism for the development of IR.

Thus, the changes observed in the expression of Sfrp5 and key genes involved in Wnt/JNK signaling in the adipose tissue of offspring were likely dependent, at least initially, on the maternal hypoxic environment and these alterations may have long-term functional consequences. Therefore, the regulatory axis of Sfrp5-Wnt in the adipose tissue may provide an underlying mechanism via which maternal hypoxia during pregnancy alters the noncanonical Wnt/JNK signaling pathway in adipose tissue and participates in development of IR in offspring and could be exploited as a potential target to control maternal hypoxia-linked abnormalities.

Furthermore, consistent with the findings of Ouchi et al. [17, 23], our study showed that offspring in the MH group fed on a high-fat diet for 12 weeks after weaning had an increase in the ratio of Wnt5a to Sfrp5 and showed higher insulin and HOMA-IR levels, indicating greater IR. Moreover, elevated phosphorylation of key genes of the noncanonical Wnt signaling were observed in offspring in the MH group fed on a high-fat diet, which makes a strong argument that the balance between Sfrp5 and Wnt5a expression could serve as a rheostat to control the degree of noncanonical Wnt/JNK signaling pathway in adipose tissue, which in turn modulates the state of insulin sensitivity. These results may be due to an *in utero* programming effect induced by hypoxia exposure that reduces the total adipocyte number, increasing the likelihood of altered fetal development of adiposity or increased visceral adiposity, which may alter the secretion of adipokines and increase the risk of developing decreased sensitivity to insulin, creating a feed-forward IR state.

In this study, maternal hypoxia during pregnancy was found to alter the secretion of adipokine Sfrp5 in adipose tissue of the offspring, which may contribute to the development of IR via Wnt/JNK signaling. This effect was enhanced in offspring with hyperlipidemia induced by a postnatal high-fat diet. Collectively, these findings highlight the importance of exposure to hypoxia in the developmental period and how it may contribute to development of IR in adulthood and provide considerable insight into the possible underlying molecular mechanism, which may lead to the development of strategies to prevent and monitor MetS in individuals exposed to hypoxia *in utero*.

At this point, it is not possible to define the specific changes in Sfrp5 expression and noncanonical Wnt/JNK signaling pathway in the offspring of the MH group that led to this programmed priming of IR. However, the results of this study indicate that hypoxia during the developmental period may serve as one of the regulators of this process, leading to persistent modulations in gene expression and increased susceptibility to the MetS induced by a high-fat diet in adulthood. Therefore, the potential effects of maternal hypoxic stress exerted on the fetuses should be considered, as well as the effect of oxygen deprivation and reperfusion injury on fetuses. Further research is needed to answer questions raised by the results of this study, including whether sex-related differences exist, whether disturbance of Sfrp5 expression in adipose tissues could be altered during fetal period, and if noncanonical-Wnt/JNK signaling in muscle and/or liver could interact with the insulin signaling pathway.

Conclusion

To our knowledge, the present study is the first to determine the impact of chronic maternal hypoxia on adipokineSfrp5 gene expression in offspring. The study indicated that maternal hypoxia during pregnancy has an effect on postnatal Sfrp5 expression in the adipose tissue of offspring and that the balance between Sfrp5 and Wnt5a expression may serve as a rheostat to control the degree of Wnt/JNK signaling and modulates the state of insulin sensitivity. Thus, prenatal hypoxia may provide an underlying mechanism for the development of IR and may therefore serve as a novel risk factor for MetS. Furthermore, the effects of prenatal hypoxia were augmented by hyperlipidemia induced by a high-fat diet in adulthood.

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Disclosure of conflict of interest

None.

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