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Original Article

High-fat diet induced insulin resistance in pregnant rats through pancreatic pax6 signaling pathway

Hao Wu, Yunyun Liu, Hongkun Wang, Xianming Xu

Department of Obstetrics and Gyencology, Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China

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Abstract: Objective: To explore the changes in pancreas islet function of pregnant rats after consumption of high-fat diet and the underlying mechanism. Methods: Thirty pregnant Wistar rats were randomly divided into two groups: high-fat diet group and normal control group. Twenty days after gestation, fasting blood glucose concentration (FBG) and fasting serum insulin concentration (FINS) were measured. Then, oral glucose tolerance test (OGTT) and insulin release test (IRT) were performed. Finally, all the rats were sacrificed and pancreas were harvested. Insulin sensitivity index (ISI) and insulin resistance index (HOMA-IR) were calculated according to FBG and FINS. RT-PCR and Real-time PCR were performed to study the expression of paired box 6 transcription factor (Pax6) and its target genes in pancreatic tissues. Results: The body weight was significantly increased in the high-fat diet group compared with that of normal control rats ($P<0.05$). The fasting plasma glucose of rats in high-fat diet group was significantly increased compared with that of normal control rats (6.62 mmol/L vs. 4.96 mmol/L, $P<0.05$), however there was no significant difference in fasting serum insulin concentration between the two groups. OGTT and IRT were abnormal in the high-fat diet group. The high-fat diet rats were more prone to impaired glucose tolerance and insulin resistance. The level of the expression of Pax6 transcription factor and its target genes in pancreas, such as pancreatic and duodenal homeobox factor-1 (Pdx1), v-maf musculoaponeurotic fibrosarcoma oncogene homolog A (MafA) and glucose transporter 2 (Glut2) were decreased significantly compared with those of normal control group. Conclusion: High-fat diet feeding during pregnancy may induce insulin resistance in maternal rats by inhibiting pancreatic Pax6 and its target genes expression.

Keywords: Nutrition, gestation, rats, insulin resistance, pax6, oral glucose tolerance test, insulin sensitivity index, insulin resistance index

Introduction

With the publication of studies about the correlation of gestational hyperglycemia and pregnancy prognosis, the threshold for diagnosis of gestational diabetes mellitus (GDM) has been decreased, so the incidence of GDM is rising [1]. If the glucose level in GDM women is poorly controlled, not only the growth and development of fetus will be affected immediately, but there will also be a variety of long-term effects on the offspring, such as a high incidence rate of adult type 2 diabetes [2]. GDM is associated with a number of factors such as genetics, lifestyle and so on [3]. In recent years, an unhealthy lifestyle is the main reason for the increased incidence of GDM, such as excessive and rapid weight gain during pregnancy due to too much calorie intake and too little physical activity; however the effect and underlying mechanism of over-nutrition on blood glucose and insulin levels (especially insufficient insulin synthesis) in pregnant women remain unclear. To understand this mechanism, it is critical to study the functional changes and the molecular mechanism of pancreatic islet β cells induced by hyperglycemia in pregnant women. Paired box transcription factor 6 (paired box 6, Pax6) has been found to be involved in the development of embryonic eye, central nervous system and pancreas [4]. In mouse pancreas, Pax6 was only expressed in the endocrine cells. It has been reported that Pax6 expression plays important role in the secretion of glucagon in mouse pancreatic α cells [5]. Recent studies have proved that Pax6 is critical in the regula-

Table 1. PCR primer sequences and product size

Primer name	Sequence (5'-3')	Product size (bp)
Pax6	F: GGACAGGGAGAAAACACCAA	127
	R: CCTCAATCTGCTCCTGGGTA	
Pdx1	F: AAACCGTCGCATGAAGTGGAA	109
	R: CGAGGTTACGGCACAATCCTG	
MafA	F: CTCAGAGTCCGAACCGAGG	135
	R: CGCACCCGACTTCTTTCTGT	
GLUT2	F: GGAGCCTCCAGTAAGAAGTCTG	97
	R: TGGCAGGTAGAATTAGTCTCAGG	
GAPDH	F: AGAACATCATCCCTGCATCC	114
	R:TGGATACATTGGGGGTAGGA	

tion of pancreatic β-cell function, insulin biosynthesis and glucose stimulated insulin secretion [6]. Therefore, by establishing a pregnant rat model of impaired glucose tolerance and insulin resistance through giving pregnant rats a high-fat and high calorie diet, the objectives of this study are 1) to explore the effect of such diet on maternal islet β-cell function, 2) to study the expression of Pax6 and its target genes, 3) to preliminarily probe into the mechanism underlying the β-cell function changes, 4) to provide experimental evidence for the increased incidence rate of gestational hyperglycemia (or GDM) induced by high-fat diet during pregnancy.

Materials and methods

Experimental animals and environment

Thirty 8-week old, clean and healthy virgin female Wistar rats with body weight 150~180 g; and ten 8-week old male Wistar rats with body weight 160~200 g, were purchased from the Chinese Academy of Sciences affiliated Shanghai Slack Experimental Animal Company (license number: SCXK Shanghai 2007-0005). Rats were housed in Clean Animal Laboratory at the First People's Hospital of Shanghai Jiaotong University Experimental Animal Center (license number: SYXK Shanghai 2009-0086), with controlled room temperature (22.0±1.0) °C, humidity 40%~60%, day and night alternating for 12 h, and free access to water.

Animal grouping and feeding

After adaptive feeding for one week, the female and male rats were mixed in one cage with a 3:1 ratio. The next morning, female rats were observed for the presence or absence of vaginal suppositories, which were taken with a cotton swab and observed further under the microscope. If sperms were found in three different fields, the female rate was marked as positive for pregnancy [7], and the date was marked as day one in the pregnancy diary (d1). Non-pregnant females continue to mate with male rats for two weeks after which non-pregnant ones will be discarded. Fortunately, all thirty female rates were found pregnant. The body weights of all pregnant rats were measured on d1 and then the rats were randomly divided into different groups: high-fat diet group (HF group, n=15) and normal diet group (NC group, n=15). All rats have free access to water. Normal diet and high-fat diet were purchased from the Chinese Academy of Sciences affiliated Shanghai Slack Experimental Animal Company. In normal diet, the total calories are 352 kcal/100 g, calorie ratio is 60.5% of the total carbohydrate calories, fat content is 13.8% of the total calories, protein content is 25.7% of the total calories; In high-fat diet, the total calories are 379 kcal/100 g, calorie ratio is 40% of the total carbohydrate calories, fat (rich in saturated fatty acids, lard-based) content is 40% of total calories, protein content is 20% of total calories. Normal diet was stored at room temperature, and high-fat diet was stored in -20°C refrigerator.

Feeding and weighting

The situations of fur color, activities, feeding and drinking were observed daily. The vaginal suppository situation of the female rats was observed daily during the mating periods. For pregnant rats, the amount of food intake was recorded daily, and body weight was recorded every other day throughout the pregnancy.

Measurement of fasting blood glucose and serum insulin

After fasting for 12 hours overnight, blood samples (about 1 ml) were collected from the orbital venous plexus of the rats with a capillary tube. Fasting blood glucose concentration (FBG) was measured immediately using a blood glucose meter and strips (Roche Accu-Check). The blood samples were then centrifuged at low speed (4°C, 5000 rpm, 15 min) within I hour; the supernatant was harvested and stored at -80°C for measuring fasting serum insulin level.

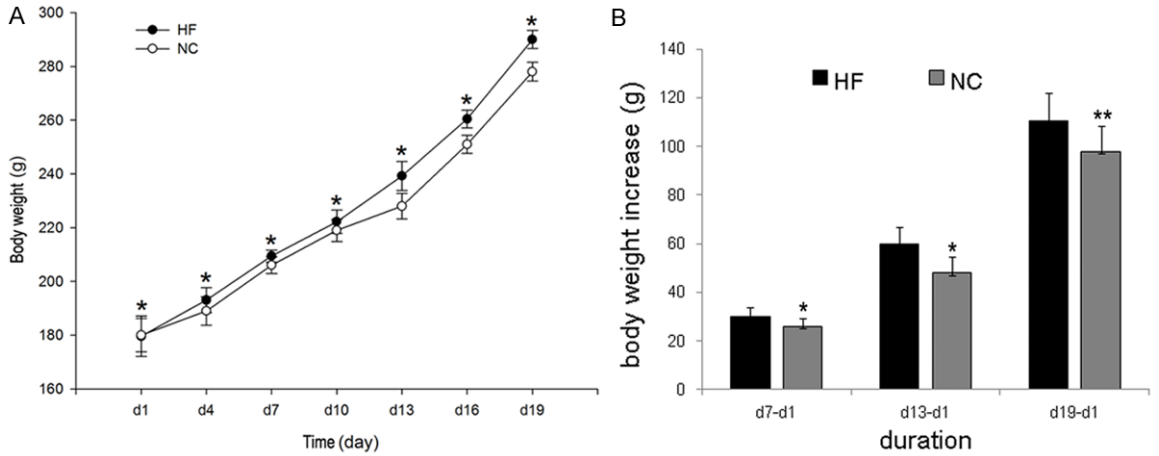

Figure 1. Impacts of high-fat diet on body weight of maternal rats during pregnancy. A. Curves of body weight change for pregnant rats in HF and NC groups. B. Comparison of body weight gain during pregnancy for rats in HF and NC groups. N = 15 for each group. *: $P<0.05$ vs. NC group, **: $P<0.01$ vs. NC group.

Serum insulin level was measured by double antibody sandwich ELISA. Blood samples were collected as mentioned above from pregnant rats on 2 days (d2) and day 20 (d20) of gestation [8]. FBG and FINS were determined respectively, insulin sensitivity index (ISI) and HOMA insulin resistance index (HOMA-IR) were calculated accordingly. Insulin sensitivity index (ISI)=1/(FBG×FINS), where insulin unit was converted as (1 ng/mL=21.2 mIU/L); HOMA insulin resistance index (HOMA-IR)=(FBG×FINS)/22.5.

Measurement of oral glucose tolerance test (OGTT) and insulin release test (IRT)

OGTT and IRT were measured on day 20 (d20) of gestation. After fasting for 12 hours overnight, blood samples (about 1 ml) were collected from the orbital venous plexus of the rats with a capillary tube. Fasting blood glucose concentration (FBG) was measured immediately. Then rats were fed with 50% glucose at the dose of 2.0 g/kg body weight through a gastric tube, 15 min, 30 min, 60 min and 120 min after glucose load, 0.5 ml blood was drawn from the tail vein, then blood glucose levels and serum insulin levels were measured instantly using methods as mentioned above.

Specimen harvesting

Pregnant rats were sacrificed by cervical dislocation after blood sample collection on day 20 (d20) of gestation, pancreatic tissue was harvested quickly after laparotomy, cut into small pieces with a diameter of 3~5 mm and were placed in RNAse free Eppendorf tubes and then stored at -80°C after frozen in liquid nitrogen for RNA isolation. The specimen harvesting were performed on ice, and all surgical devices were RNAse free.

RT-PCR and real-time RT-PCR

RNA was extracted from the stored pancreatic tissues by Trizol method. RNA concentration was then measured and purity was confirmed. A reverse transcription kit (iScript cDNA Synthesis Kit, Bio-Rad) was used to convert RNA into cDNA, and then RT-PCR (kit: PrimeScript RT-PCR Kit, Takara), and Real-time RT-PCR (Kit: SYBR® Premix Ex Taq™, TIi RNaseH Plus, Takara) were performed. The differential expression of pancreas Pax6 and its target genes Pdx1, MafA and Glut2 between HF group and NC groups was compared. Each sample was quadruplicated. Fold changes ($2^{\Delta Ct}$) were used to calculate the relative quantitation of each gene expression level. Then the difference of gene expression between HF and NC groups was compared as relative ratio. The primers for the PCR were synthesized by Shanghai Biological Engineering Company, and the primer sequences are shown in **Table 1**.

Statistical analysis

Statistical analysis was performed using SPSS 17.0 software. Quantitative data which are in line with the normal distribution were expressed

Table 2. FBG, FINS, ISI and HOMA-IR in pregnant rats (mean ± SD)

Groups	FBG (mmol/L)		FINS (mIU/L)		ISI		HOMA-IR	
	d2	d20	d2	d20	d2	d20	d2	d20
NC	4.93±0.59	4.96±0.67	5.94±0.72	6.15±0.42	0.03±0.010	0.03±0.009	1.30±0.031	1.36±0.23
HF	4.97±0.62	6.62±0.78*	5.72±0.62	5.57±0.54	0.04±0.011	0.03±0.012	1.26±0.027	1.64±0.29*

*: $P<0.05$ vs. NC groups.

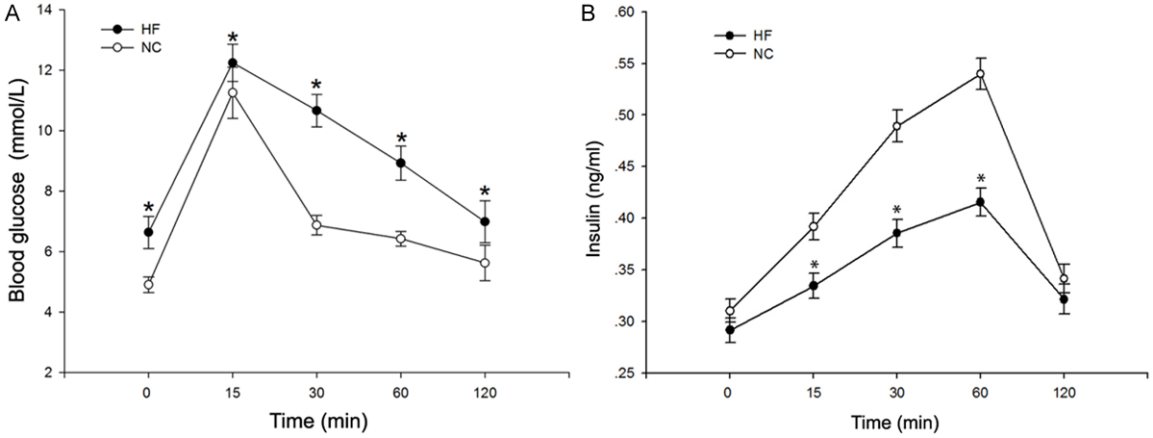

Figure 2. Impacts of high-fat diet on OGTT and IRT in maternal rats during pregnancy. A. Curves of OGTT change for pregnant rats in HF and NC groups. B. Curves of IRT change for pregnant rats in HF and NC groups. N=15 for each group. *: $P<0.05$ vs. NC group.

as mean ± SD. The insulin data were non-normal distribution and were analyzed after a logarithmic transformation. $P<0.05$ was considered statistically significant.

Results

Impact of high-fat diet on maternal body weight during pregnancy

The body weight of rats in HF group during the whole pregnancy was higher compared with that of rats during the same pregnancy time point in NC group (**Figure 1A**), and the difference was statistically significant on day 7, day 13, and day 19 of gestation ($P<0.05$). Meanwhile, the body weight gains from pre-pregnant for rats in HF group on day 7, day 13, and day 19 of gestation were also significantly higher compared with those in NC group at the same time points ($P<0.05$) (**Figure 1B**).

Impact of high-fat diet on FBG and FINS level in pregnant rats

The FGB levels in pregnant rats in HF group at day 20 were significantly higher than those in NC group ($P<0.05$), with high blood sugar symptoms (**Table 2**). There is no significant difference in terms of FINS levels and ISIs between the two groups at day 20, while HOMA-IR in HF group was significantly higher than that in NC group ($P<0.05$) as shown in **Table 2**.

Impact of high-fat diet on OGTT and the IRT in pregnant rats

The OGTTs of all time-points in pregnant rats in HF group at day 20 were significantly higher than those in NC group ($P<0.05$) (**Figure 2A**), while the fasting insulin level and insulin release at all time-points in HF group were lower than those in NC group at the same time point, and the difference was significant at 15 min, 30 min and 60 min ($P<0.05$) as shown in **Figure 2B**.

Iimpact of high-fat diet on expression of Pax6 and its target genes, Pdx1, MafA and GLUT2 in the pancreas of pregnant rats

RT-PCR and Real-time PCR test results showed that on day 20, the expression levels of Pax6 and its target genes Pdx1, MafA and Glut2 in HF group were all significantly lower than those in NC group ($P<0.05$) (**Figures 3** and **4**).

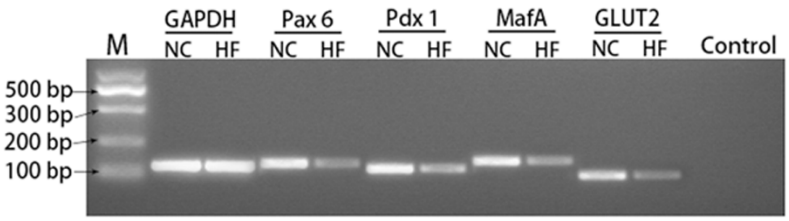

Figure 3. Impact of high-fat diet on expression of Pax6 and its target genes, Pdx1, MafA and GLUT2 in the pancreas of maternal mice during pregnancy shown by RT-PCR. RT-PCR results showed that on day 20, the expression levels of Pax6, Pdx1, MafA and Glut2 in HF group were all significantly lower than those in NC group. Shown are the representative images of experiments that were repeated at least three times.

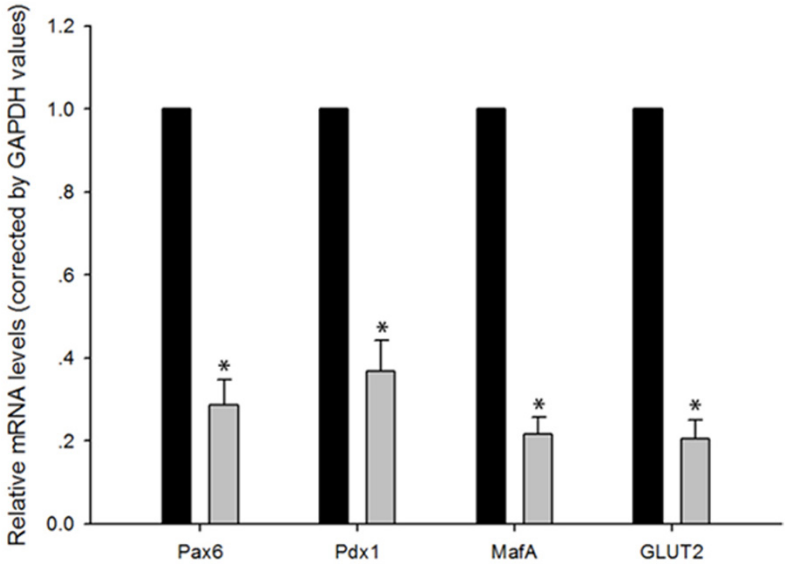

Figure 4. Impact of high-fat diet on expression of Pax6 and its target genes, Pdx1, MafA and GLUT2 in the pancreas of maternal mice during pregnancy shown by Real-time PCR. Real-time PCR test results showed that on day 20, the expression levels of Pax6 and its target genes Pdx1, MafA and Glut2 in HF group were all significantly lower than those in NC group (*: $P<0.05$). Shown are the representative data of experiments that were repeated at least three times.

Discussions

In recent years, a growing number of studies have shown that dietary factors play an important role in the pathogenesis of GDM. Many scholars are trying to find a better way to prevent the incidence, control the development and improve the outcome of GDM [9-12]. Current studies suggest that the incidence of GDM is related to impaired function of islet β cells [12]. The differentiation and functional status of β cell are controlled by a series of regulatory transcription factors, such as Pax4, Nkx2.2, Pdx1, MafA, Nkx6.1, Pax6 and Neuro D1/Beta2, etc., the importance and reciprocal link of which have been demonstrated in mice [13-17]. It was found that in Pax6 knockout mice, though there was no significant change in the number of endocrine cells, the endocrine function was seriously damaged. Pax6 has been shown to be important for maintenance of differentiation of islet cells, in particular α and β cells [5, 17].

Pax6 is a member of the paired box gene (Pax) family of transcription factors, which is expressed in the pancreas, eyes, ears, and the central nervous system and other tissues. Pax6 gene is mainly expressed in the early stage of development of mouse pancreas and in mature endocrine cells. Researchers found there was a lack of cells secreting glucagon in the pancreas of Pax6 mutation mice, suggesting that the expression of Pax6 gene is essential for pancreatic α cell differentiation [5]. Moreover, a most recent study showed that Pax6 could affect the biosynthesis and secretion of insulin and the effect of glucose on β cells through transcriptional regulation of key genes in β cells; moreover, it was also demonstrated by the same study that Pax6 controlled the expression of key genes participating in transcription of insulin gene and insulin secretion processes, such as the expression level of Pdx1, MafA, Glut2, PC1/3, insulin 1 and 2 mRNA [6]. Pancreatic and duodenal homeobox factor-1 (Pdx1) was involved in the early development of pancreas and the differentiation of late stage pancreatic β cells, and in the maintenance of the form and normal function of β cells through regulating the expression of a series of genes, especially through the regula-

tion of insulin and insulin-related genes, such as glucose transporter 2 (Glut2), to promote insulin secretion and maintain the normal function of islet β cells. So Pdx1 plays important roles in the development, proliferation, differentiation and maturation of β cells and regulation of their secretory function during embryonic stage [18, 19]. v-maf musculoaponeurotic fibrosarcoma oncogene homolog A (MafA) is a transcription factor specifically expressed in β cells, and involved in the regulation of expression of genes related to insulin synthesis, secretion and glucose metabolism. MafA plays an important role in blood sugar mediated regulation of insulin gene expression, and its expression and functional status were regulated by glucose at multiple levels. In diabetic state, the expression MafA and/or activity is reduced, which subsequently inhibits the biosynthesis and secretion of insulin [20]. Recent studies have found that in MafA gene-deficient mice, the expression levels of insulin, Pdx1, Beta2 and Glut-2 genes were also reduced, indicating that MafA is also a key regulatory factor for glucose-stimulated insulin secretion [19].

In this study, pregnant rats were fed with high-fat diet, which induced impaired glucose tolerance and insulin resistance during pregnancy [21, 22]. This method has been used in the study of type 2 diabetes in animals and it has become a classic model after confirmation that high-fat diet can induce type 2 diabetes [23]. Our results showed that in the late stage of pregnancy (d20), the fasting blood glucose level of rats in HF group was higher than that in NC group, insulin resistance index was higher than that in NC group, oral glucose tolerance test and insulin release test in HF group rats were also abnormal, Pax6 and its target genes, Pdx1, MafA and Glut2 expression were also decreased. It has been reported, the biosynthesis and secretion of glucose-stimulated insulin secretion (insulin release) is also regulated by Pax6, Pdx1 and MafA. Therefore, we hypothesized that in high blood sugar and high cholesterol status, Pax6 is the key factor regulating insulin biosynthesis and secretion at the gene transcription level. Dietary factors, especially high-fat diet, can cause insufficiency in insulin biosynthesis and secretion by reducing the expression of Pax6 and associated transcription factors so that blood glucose level cannot be properly regulated, and insulin-stimulated glucose uptake was also decreased, resulting in "islet cell insulin resistance (central resistance)".

In conclusion, pregnant rats fed on a high-fat and high calorie diet showed impaired glucose tolerance, insulin resistance, reduced insulin secretion and sensitivity in the late stage of pregnancy, which were likely caused by reduced expression of Pax6 and its target genes, such as Pdx1, MafA, Glut2. Our study provides an explanation, at least in part, for why over-nutritious pregnant women predispose to GDM. Moreover, our study can also provide useful references to make proper diet manual for pregnant women with high risk for GDM. Literature search reveals few related studies and further studies are needed to decipher the details of the underlying molecular mechanism and signaling pathways involved.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Xianming Xu, Department of Obstetrics and Gyencology, Shanghai General Hospital, Shanghai Jiao Tong University, 100 Haining Road, Shanghai 200080, Shanghai, China. Tel: +86 2163240090; Fax: +86 2163-240090; E-mail: xuxm11@163.com

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