ADIPONECTIN AND AMPK PATHWAY GENES AND PREGNANCY

Association between intrauterine mild hyperglycemia and post-natal high-fat diet with adiponectin and AMPK pathway genes

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Abstract
To investigate the mechanisms of maternal–fetal interactions in the setting of gestational diabetes mellitus. We investigated the long-term effects of intrauterine mild hyperglycemia and a postnatal high-fat diet on the glucose metabolism of adult offspring, and explored the role of adiponectin on hepatic gluconeogenesis. Twenty-one pregnant Wistar rats were randomly divided into an intrauterine hyperglycemia group (group D, n = 14) and a control group (group C, n = 7). Offspring were divided into four groups according to intrauterine blood glucose level and post-weaning dietary patterns (high-fat diet groups: DF and CF or normal diet groups: DN and CN, n = 8 per group). The average birth weights of group D offspring were higher than for group C. In the DF rats, low adiponectin mRNA expression in the nucleus and its level was significantly increased. Our study shows that the dietary structure of offspring has a large influence on the incidence of abnormal glucose tolerance.

Keywords
Diabetes, insulin resistance, pregnancy

Introduction
Recent socioeconomic developments and a general improvement in the standard of living have led to large alterations in dietary structure and way of life for many people in China. Globally, the incidence of type 2 diabetes mellitus (T2DM) has seen a large increase and causes great harm to human health [1]. Explorations into the pathogenesis of T2DM is ongoing, as is research into the theory of the Developmental Origins of Health and Disease, which includes the influence of the intrauterine environment on child growth and development, and the subsequent long-term effects on adult metabolism and health outcomes [2].

Gestational diabetes mellitus (GDM) is a common medical complication in pregnancy. GDM brings long-term health issues for offspring, such as obesity, impaired glucose tolerance (IGT), T2DM and other chronic diseases [3]. Since the recent introduction of new criteria for GDM diagnosis in China, a greater number of pregnancies require careful glucose control, as the incidence of mild hyperglycemia (17.5%) [4] and macrosomia in newborns is increasing. Consequently, an intrauterine mild hyperglycemia animal model is needed to investigate the mechanisms of maternal–fetal GDM complications.

Adiponectin is a protein produced by adipose cells and secreted into the blood stream, it is involved in the regulation of glucose metabolism. Gluconeogenesis maintains the balance of glucose metabolism and increases glycogen output, and impairments of this system are considered to be major causes of T2DM [5]. However, the specific signal transduction mechanisms by which adiponectin affects gluconeogenesis via the AMP-activated protein kinase (AMPK) pathway are unclear. Therefore, we established an intrauterine mild hyperglycemia rat model to investigate the long-term effects of exposure to intrauterine mild hyperglycemia, followed by a high-fat postnatal diet, on the glucose metabolism of the offspring; the role of adiponectin in hepatic gluconeogenesis in the adult offspring was also investigated.

Methods
Animals
All procedures involving animals were performed at the Peking University Health Science Center and were approved by the Peking University Experimental Animal Welfare Ethics section. Twenty-one pregnant SPF Wistar rats were randomly divided into either the intrauterine hyperglycemia group (group D, n = 14) or the control group (group C, n = 7). After a 12-h fast, streptozotocin (STZ; 25 mg/kg, Sigma Company, St. Louis, MO) was given to group D by a single intraperitoneal injection to induce intrauterine mild hyperglycemia (blood glucose of 10–20 mmol/L). Group C rats received an equal volume of citric acid–sodium citrate buffer (Beijing Bo Aigang Trade Center, Beijing, China). Following this procedure, there were nine rats in group D; group C maintained seven rats. By pregnancy day 20, all rats were on single cage feeding and observed daily in case of delivery. After delivery, the number of offspring per cage was limited to eight, half male and half female. All the offspring were weaned after 3 weeks. Offspring were divided into four groups according to intrauterine blood glucose level and post-weaning dietary patterns (high-fat diet groups: DF and CF or normal diet groups: DN and CN). Each group consisted of eight randomly selected male rats (Supplementary data S1).
Before tissue collection from rat offspring, blood glucose of pregnant rats, offspring birth weights and the fasting plasma glucose (FPG) levels of the rat offspring were measured (Supplementary data S2).

**Measurement of fasting insulin and serum adiponectin levels**

The fasting insulin (FINS) and serum adiponectin levels were measured using an enzyme-linked immunosorbent serologic assay (ELISA) (Supplementary data S3). The insulin resistance index was calculated using the homeostasis model assessment insulin resistance (HOMA-IR) index: HOMA-IR = FPG × FINS/22.5.

**Real-time fluorescence quantitative RT-PCR**

For RNA extraction, individual tissues were homogenized in 1 ml of Trizol reagent and the RNA was purified according to the manufacturer’s instructions (Supplementary data S4). Gene expression levels were calculated using the comparative threshold method: the relative expression value of the target gene = 2 −ΔΔCT, where ΔΔCT = (ΔCTTarget − ΔCTActin), and CT is the fluorescence threshold value.

**Immunohistochemistry**

Immunohistochemical techniques were used to determine the hepatic expression of P-AMPK and CRTC2. The results were analyzed by Media Cybernetics (Bethesda, MD) Image-Pro Plus 6.0 image analysis software, with the average optical density value as the relative content of expression. The average optical density was calculated as the IOD SUM / area SUM (Supplementary data S5).

**Statistical analysis**

Results are presented as mean ± standard deviation. Any differences between two groups were determined using the t-test, and differences between four groups were determined using ANOVA and LSD tests. Pearson tests were used for correlation analyses between groups. Statistical significance was accepted at p < 0.05.

**Results**

**Blood glucose levels of pregnant rats**

The average blood glucose level of the rats with the group D was significantly higher than that of group C (p < 0.01; Table 1).

**Birth weights**

The birth weights of the offspring exposed to intrauterine mild hyperglycemia were increased compared with the offspring of the controls (group DN and group DF versus group CN p < 0.05; Table 2); the birth weight of group DF was increased compared with group CF (p < 0.05; Table 2).

**Glucose metabolism in offspring**

At the age of 28 weeks, there were no statistical differences in FPG, FINS or HOMA-IR between the four groups (p > 0.05, Table 2).

**Serum adiponectin levels and adiponectin mRNA expression in epididymal and perirenal fat tissue**

At the age of 28 weeks, the serum adiponectin levels of the group DF were decreased compared with group CF (p < 0.05, Table 2). The adiponectin mRNA expression of groups DN, DF and CF were significantly decreased in the perirenal fat compared with group CN (p < 0.05; Supplementary data S6. Table S2 and Figure 1A). In the epididymal fat of group DF rats, adiponectin mRNA expression was 3.51-fold lower than the expression in group CN rats (p < 0.05, Supplementary data S6. Table SII and Figure 1A). In both perirenal and epididymal fat tissue, the adiponectin mRNA expression of the group DF was much lower than the expression levels observed in the group DN or group CF (p < 0.05, Supplementary data S6. Table SII and Figure 1A).

**The hepatic mRNA expression of AdipoR1, PEPCK, G-6-Pase and PGC-1α**

At the age of 28 weeks, the hepatic AdipoR1 mRNA expression in the group DF was decreased compared with the group DN or group CF (p < 0.05, Supplementary data S6. Table SIII and Figure 1A). Hepatic PEPCK, G-6-Pase, and PGC-1α mRNA levels in the group DF were significantly elevated compared with the other three groups (p < 0.05, Supplementary data S6. Table SIII and Figure 1B). The level of adiponectin mRNA expression in both perirenal fat and epididymal fat was significantly positively correlated with the level of hepatic Adipo-R1 mRNA expression (r = 0.736 and r = 0.718, respectively; p < 0.05). Significant negative correlations were found between the adiponectin mRNA levels of perirenal fat and epididymal fat and hepatic PEPCK (r = −0.808, r = −0.815, p < 0.05), G-6-Pase (r = −0.744, r = −0.752, p < 0.05), and PGC-1α (r = −0.652, r = −0.674).

### Table 1. Blood glucose levels of pregnant rat (mmol/L).

| Group | Number | 5 d | 8 d | 11 d | 14 d | 17 d | 20 d | Mean |
|-------|--------|-----|-----|------|------|------|------|------|
| D     | 9      | 16.8 ± 5.4* | 15.4 ± 4.7* | 15.3 ± 4.7* | 18.3 ± 4.7* | 17.2 ± 3.8* | 16.7 ± 5.7* | 16.6 ± 3.4* |
| C     | 7      | 7.0 ± 1.4 | 5.5 ± 1.6 | 6.7 ± 1.4 | 5.3 ± 1.1 | 5.5 ± 0.7 | 4.9 ± 0.8 | 5.8 ± 1.1 |

Data are expressed as the means ± SD. Differences between the two groups were estimated with the t-test. *p < 0.01 versus C.

### Table 2. Offspring birth weight, glucose metabolism and adiponectin.

| Group | Number | Birth weight (g) | FPG (mmol/L) | FINS (mIU/L) | HOMA-IR | Adiponectin (mg/L) |
|-------|--------|-----------------|--------------|--------------|---------|-------------------|
| CN    | 8      | 6.6 ± 0.5       | 5.22 ± 0.40  | 9.25 ± 1.39  | 2.17 ± 0.37 | 11.79 ± 1.82     |
| DN    | 8      | 7.4 ± 0.6*      | 4.94 ± 0.50  | 9.00 ± 1.01  | 2.02 ± 0.32 | 11.75 ± 1.22     |
| CF    | 8      | 6.7 ± 0.5       | 5.11 ± 0.46  | 8.83 ± 0.76  | 2.01 ± 0.26 | 12.18 ± 2.20     |
| DF    | 8      | 7.4 ± 0.2†      | 5.30 ± 0.35  | 8.49 ± 0.85  | 2.01 ± 0.23 | 10.91 ± 2.02†    |

Data are expressed as the means ± SD. FPG, FINS, HOMA-IR and adiponectin were all measured at 28 weeks of age. Differences between the four groups were determined by ANOVA and the LSD test. *p < 0.05 versus CN; †p < 0.05 versus CF.
p < 0.05) mRNA levels. Hepatic PEPCK (r = 0.798, p < 0.05), G-6-Pase (r = 0.763, p < 0.05) mRNA expression and the PGC-1α mRNA expression were positively correlated (Figure 2).

The hepatic expression of P-AMPK and CRTC2

At the age of 28 weeks, hepatic P-AMPK was located in the cytoplasm of these male offspring. Hepatic P-AMPK expression in the group DF was significantly decreased compared with the other three groups (p < 0.05, Table 3 and Figure 3A).

Hepatic CRTC2 was expressed in the nucleus, hepatic CRTC2 expression in the group DF was significantly increased compared with other three groups (p < 0.05, Table 3 and Figure 3B).

Discussion

The average blood glucose levels of the mild hyperglycemic rats, and the average birth weight of their offspring, were all significantly higher than those of the control rats; this is equivalent to the situation in humans, where pregnant women with mild and moderate hyperglycemia have a higher risk of giving birth to macrosomic offspring.

In rats, research on long-term outcomes has confirmed that hyperglycemia in pregnant rats is associated with obesity, IGT, hyperinsulinemia and insulin resistance in adolescent and adult offspring [3]. Experimental animal models and studies of human epidemiology have found that the metabolic programming caused by intrauterine hyperglycemia could be passed on to the next generation, resulting in a vicious cycle of T2DM [6–8]. Van et al. demonstrated that intrauterine mild hyperglycemia could cause IGT in female offspring and the future development of GDM [9]. However, in our study, a mildly hyperglycemic intrauterine environment had no effect on FPG and insulin levels, and hyperglycemia and insulin resistance in male offspring was not apparent at the age of 28 weeks. Earlier studies in the intrauterine mild hyperglycemia rat model (MDM) and the intrauterine severe hyperglycemia rat model (SDM) found that IGT susceptibility increased in adult offspring, but that the mechanisms underlying the IGT were different in each model. Further analysis found that glucose-stimulated insulin secretion was reduced in adult MDM offspring, but increased in adult SDM offspring. In the adult MDM offspring, IGT was caused by the low reactivity of insulin to glucose, possibly due to excessive stress on the islet beta cells induced by intrauterine mild hyperglycemia; while in the adult offspring of SDM, IGT was caused by the low uptake capacity of insulin into peripheral tissues (liver, skeletal muscle, etc.), possibly due to changes in the insulin receptor or other molecules involved in the post-receptor signaling pathways in peripheral tissues induced by intrauterine severe hyperglycemia [10]. Therefore, abnormal glucose metabolism in adult offspring can be due to several different factors, such as intrauterine blood glucose level and gender.

Adiponectin is a protein produced by adipose cells and secreted into the blood stream. Adiponectin concentrations exhibit strong cross-sectional relationships with obesity, inflammation, and diabetes, and have been extensively evaluated as epidemiological markers of diabetes and cardiovascular disease risk [11]. Patients with visceral obesity or T2DM have reduced serum adiponectin levels, and the expression of adiponectin mRNA in

Figure 1. (A) The adiponectin mRNA and adipon1 mRNA expression (2^ΔΔCT value). a p < 0.05 versus CN; b p < 0.05 versus DN; c p < 0.05 versus CF. The value of CN was 1. A negative value indicated that the multiple relationship was reduced. (B) The hepatic mRNA expression of PEPCK, G-6-Pase and PGC-1α (2^ΔACT value). a p < 0.05 versus CN; b p < 0.05 versus DN; c p < 0.05 versus CF. The value of CN was 1. A positive value indicated that the multiple relationship was increased.
the visceral fat is decreased [12,13]; low serum adiponectin levels are associated with high insulin resistance. Increased glycogen output induced by disorders of gluconeogenesis is an important cause of insulin resistance in liver, and effective inhibition of gluconeogenesis reduces fasting blood glucose. Adiponectin participates in the regulation of glucose metabolism and insulin resistance might, in part, be caused by its inhibition of gluconeogenesis and glycogen output [14].

PEPCK is a key enzyme that catalyzes the first step reaction in gluconeogenesis, and G-6-Pase catalyzes the final reaction in gluconeogenesis and glycogenolysis; both enzymes have a role in glycogen generation. PGC-1α, discovered recently, is a key transcriptional gene in the complex signaling pathway that regulates gluconeogenesis; PGC-1α genes in humans and rats are highly homologous. PGC-1α determines the key link of gluconeogenesis startup by crosstalk with PEPCK gene and G-6-Pase gene, and alterations in its expression are related to obesity, diabetes and lipid metabolism disorders. In our study, at the age of 28 weeks, the male offspring in group DF had low serum adiponectin levels, low adiponectin mRNA expression in perirenal fat, low P-AMPK and CRTC2 expression, and significant differences in glycogen generation compared to the other groups. The mRNA expression correlations between adiponectin and PEPCK, G-6-Pase, PGC-1α (ΔCT value); The mRNA expression correlations between PEPCK, G-6-Pase and PGC-1α (ΔCT value).

Table 3. Average optical density values of hepatic P-AMPK and CRTC2.

| Group | Number | P-AMPK | CRTC2 |
|-------|--------|--------|-------|
| CN 5  | 0.36 ± 0.07 | 8.21 × 10^{-3} ± 0.02 × 10^{-1} |
| DN 5  | 0.29 ± 0.10 | 4.59 × 10^{-3} ± 0.01 |
| CF 5  | 0.29 ± 0.08 | 4.58 × 10^{-2} ± 0.02 |
| DF 5  | 9.62 × 10^{-2} ± 0.03* || 0.16 ± 0.03* || |

Data are expressed as the means ± SD. All indicators were measured at 28 weeks of age. Differences between the four groups were determined by ANOVA and the LSD test.

*<i>p</i> < 0.05 versus CN; †<i>p</i> < 0.05 versus DN; ‡<i>p</i> < 0.05 versus CF.

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Glucose production is impaired in obesity due to the overexpression of PGC-1α, PEPCK, and G-6-Pase mRNA. The expression of hepatic PEPCK and G-6-Pase mRNA and PGC-1α mRNA expression were highly positively correlated. Changes in expression of these glycogen output genes would lead to hepatic insulin resistance, and a high-fat diet could accelerate the emergence of insulin resistance in the offspring. A high-fat diet is a risk factor for obesity and insulin resistance, and in both animals and humans, long-term consumption of a high-fat diet can cause insulin resistance [15,16]. Obesity is closely related to T2DM and inducing insulin resistance increases the risk of T2DM [17]. Metabolic disorders and insulin resistance caused by overweight and obesity are associated with levels of adipocytokines. In female patients with obesity, short-term dietary intervention has been shown to significantly increase plasma adiponectin levels and increase the adiponectin gene expression in adipose tissue, suggesting that short-term dietary intervention could be used to improve the levels of adipocytokines in these patients [18,19].

The cAMP response element binding protein (CREB) is a nuclear transcription factor that plays a vital role in gluconeogenesis startup. The CREB-regulated transcription co-activator 2 (CRTC2) is a protein that regulates the activity of a transcription factor, which could be a molecular switch for glycogen output, whose role is similar to insulin and substrates of glucagon [20]. Yamauchi and Dzamko et al. found that in the liver, when combined with AdipoR1, adiponectin could activate AMPK to become phosphorylated-AMPK (P-AMPK), which would inhibit CRTC2 dephosphorylation, rendering CRTC2 unable to enter the nucleus and so prevent further binding to CREB, resulting in a reduction in the expression of PEPCK and G-6-Pase. This would cause an inhibition of gluconeogenesis and hepatic glucose generation and output, and would result in an improvement in insulin sensitivity [21,22]. The results of our study, described above, were consistent with the results of previous studies.

The management of GDM patients should not be terminated after childbirth to prevent the incidence of abnormal glucose tolerance in the offspring of GDM patients. As the diet of their offspring has a large influence on the incidence of abnormal glucose tolerance, clinical practitioners should promote a healthy lifestyle to the families of GDM patients and closely follow-up their children’s growth and development to prevent the occurrence of abnormal glucose tolerance.

Figure 3. (A) The hepatic expression of P-AMPK ×400. P-AMPK was located in the cytoplasm, brown stainings were positive expression. (B) The hepatic expression of CRTC2 × 400. CRTC2 was expressed in the nucleus, brown stainings were positive expression.
Declaration of interest
The authors report no declaration of interest. Huixia Yang is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Kai Zhang wrote the manuscript and analyzed data. Xin Li analyzed data. Li Zhang and Huixia Yang contributed to review and edit the manuscript.

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Supplementary data available online

DOI: 10.3109/09513590.2015.1092134

Adiponectin and AMPK