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Original Article

Impaired expression of IRS-2 and GCK contribute to glucose dysregulation during catch-up growth in immature rats after intrauterine growth restriction

Jie Zhang[1,2], Ying Xin[1]

[1]*Department of Paediatrics, Shengjing Hospital of China Medical University, Shenyang 110004, Liaoning, China;*
[2]*Department of Paediatrics, Shanghai General Hospital, Shanghai Jiao Tong University, Shanghai 201620, China*

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Abstract: Objective: To study abnormal glucose metabolism during catch-up growth in immature mammals after intrauterine growth restriction (IUGR), and reveal the crucial changes of the insulin pathway which may contribute to glucose dysregulation. Method: The IUGR model was established by adopting a low protein diet during pregnancy of female Wistar rats. The IUGR rats and normal weight controls were investigated at newborn, 3-week-old and 8-week-old for the level of fasting plasma glucose (FPG) and fasting serum insulin (FINS). The expression of insulin receptor substrate 2 (IRS-2) and glucokinase (GCK) in the liver were also detected. Besides, an oral glucose tolerance test (OGTT) was performed on 8-week-old rats. Results: The low protein diet of mother rats tended to cause IUGR in newborn rats with 22.88% lower birth weight and 20.62% lower pancreas weight than the normal weight controls ($P<0.05$). IUGR rats caught up with controls in body weight and pancreas weight at 8-week-old ($P<0.05$). The insulin secretion in IUGR rats gradually surpass that of controls in catch-up growth, along with significantly lower β cell function indexes. On the other hand, the expression of IRS-2 and GCK were significantly lower in the livers of IUGR rats than that of controls at all-time points (all $P<0.05$). Conclusion: Abnormal glucose metabolism was suggested in immature rats with IUGR by gradual loss of insulin responsiveness, and the mechanism underlying may be related to the persistently impaired expression of IRS-2 and GCK, as well as gradually exceeded secretion of insulin during catch-up growth after IUGR.

Keywords: Intrauterine growth restriction, catch-up growth, glucose dysregulation, insulin receptor substrate 2, glucokinase, insulin responsiveness

Introduction

Intrauterine growth restriction/retardation (IUGR) refers to poor growth of a fetus compared with the normal level for a particular gestational age [1]. It can be diagnosed by direct assessment of intrauterine growth with ultrasonography when the fetal length/height is less than two standard deviations below the mean [2] or the estimated fetal weight is under 10th percentile [3]. As one of the leading causes of perinatal mortality and morbidity, IUGR has immense implications for the short term and long term growth of children. It is an important public health concern in the developing countries, and the mean IUGR rate in China is 9.4% [4]. The causes of IUGR are multiple, involving many different factors such as genetic anomalies, intrauterine infection, deficient maternal diet, poor maternal nutrition storage, inadequate uterine blood flow, or insufficient nutrients passing through the placenta [5].

Apart from low birth weight, the influence of IUGR on the development of some organs is also evident. This might be due to the adaptive compensation in response to deficient nutritional intake during pregnancy, where the nutrition supply for vital organs such as brain are primarily guaranteed at the expense of reduced nutrition supply for non-vital organs such as the pancreas, liver or muscle [6]. Petrik et al. [7] established an IUGR animal model by feeding pregnant mice with low protein calorific diet

(LP), and found that IUGR caused the number of pancreatic β-cells to decrease and impaired their ability to secrete insulin in late fetal and neonatal mice. On the other hand, many IUGR infants show postnatal catch-up growth after birth where the infants show rapid weight gain [8]. However, this rapid growth has been associated with problems later in life, such as high body fat deposition [9], increased blood pressure [10], and diabetes [11].

Insulin, which is mainly secreted by pancreatic β-cells, plays a key role in lowering blood glucose level. If the number or functions of β-cells are impaired, insulin secretion could be disturbed, which may result in glucose metabolic disorders [12]. IUGR animal models are susceptible to impaired glucose tolerance (IGT) when they grow to adulthood [13], and IUGR rats have higher risks for obesity or other metabolic diseases, such as dyslipidemia, insulin resistance, type 2 diabetes, and cardiovascular disease [14]. Clinical research also confirmed that, IUGR is significantly associated with IGT and diabetes in adulthood [15-17], and it may cause cardiovascular diseases, resulting in permanent changes in many organs [18-20].

Insulin regulates blood glucose mainly by binding with the insulin receptor (IR), which phosphorylates the insulin receptor substrate (IRS) to activate downstream molecules, for example, phosphatidylinositol 3 kinase (PI3K) and protein kinase B [21-24]. IRS-1 is mainly skeletal muscle based, while IRS-2 is in the liver, skeletal muscle and fat. As the liver is the major site for glucose metabolism the IRS-2 signaling pathway is likely to be involved in defective β cell secretion resulting in in diabetes mellitus [25, 26].

Glucokinase (GCK) promotes hepatic glycogen synthesis, stimulates insulin secretion and catalyzes conversion of glucose into 6- phosphoric acid glucose to affect glucose metabolism [27]. Therefore, abnormal GCK activation plays an important role in the disturbance of glucose metabolism as is demonstrated in animal models and clinical studies [28, 29]. In the liver GCK activity and expression is insulin dependent while in beta-cells it is glucose dependent [30].

In the present study, we established an IUGR model by adopting a low protein maternal diet, in order to detect blood glucose regulation in IUGR individuals at different postnatal periods (newborn (within 12 hours of birth), 3-week-old and 8-week-old), in comparison with contemporary normal individuals. In all the three postnatal periods, rats were weighed and the fasting plasma glucose (FPG) and the fasting serum insulin (FINS) were detected. The levels of IRS-2 and GCK were measured in the liver with western blot or immunohistochemical staining. At 8-weeks-old, the oral glucose tolerance test (OGTT) was performed.

Our study aimed to confirm the abnormal glucose metabolism in catch-up growth after IUGR in immature mammals, and to reveal the crucial changes in the insulin pathway that may contribute to glucose dysregulation. Thus, our study may provide theoretical basis for the prevention and treatment of IUGR-induced abnormal glucose metabolism, thereby helping to take remedial measures as early as the beginning of life.

Material and method

Establishment of the IUGR animal model

All animal experiments in the study were approved by the Animal Care and Use Committee in the China Medical University.

A total of 50 healthy Wistar rats of clean degrade (female:male = 4:1; weight 220-280 g) were provided by the Animal Laboratory of the Shengjing Hospital, China Medical University. Females were mated to males the evening before. The next morning, saline swabs were used to make vaginal smears, and a hair-like mass was observed under a microscope, which was deemed as conception and recorded as the first day of conception.

Mother rats were randomly divided into a low-protein diet group and a standard diet group under 24°C temperature, 60% humidity, and exposure to light for 12 hours conditions. The low-protein diet group was fed with low protein fodder (containing 1572 kJ/100 g calories, 6.7% fat, 63.4% carbohydrate, and 8.0% protein), while the standard diet group was fed with standard fodder (containing 1583 kJ/100 g calories, 4.5% fat, 57.8% carbohydrate, and 23% protein). The bedding was changed, and fodder and water were added each day. All pregnant rats experienced spontaneous labor,

and newborn rats were weighed within 12 hours of birth with 0.01 g accuracy.

Rats were confirmed as IUGR according to an established method [31]. IUGR rats born in the low-protein diet group were used as the experiment group (IUGR group), while rats of normal birth weight born in the standard diet group were used as the control group (CON group). Newborn rats in both groups were breast fed by their mothers and the mother rats were fed with standard fodder after delivery. After three weeks, immature rats in both groups were weaned and isolated, and fed with standard fodder.

At newborn, 3-week-old, and 8-week-old, each eight rats (four males and four females) from the IUGR group and the CON group respectively were weighed. Blood samples were taken for FPG and FINS detection (3-week-old rats and 8-week-old rats were fasted for 12 hours before blood collection). And then the rats were sacrificed with pancreas weighed and two samples of liver tissues collected, in which one sample was reserved in a -70°C refrigerator and later used for western blot, and another sample was put into 4% paraformaldehyde solution and later used for immunohistochemical staining. Besides, OGTT were detected in 8-week-old Rats before sacrifice.

Determination of FPG and FINS

0.2 mL of blood was taken from the retrobulbar vein to detect FPG. Another 0.2 ml of blood sample was collected and left static for 15 min, and then centrifuged with high speed freezing centrifuge (sigma 31k 5C model, U.S.A.) at 3000 rpm for 10 min. The serum was reserved in a -70°C freezer and later used for FINS detection.

FPG was measured using the glucokinase assay in an automatic blood glucose analyzer (GA05A, Japan).

FINS was determined using a chemiluminescence assay. The test was performed according to the instructions of the insulin quantitative determination kit (Yuande Bio-Medical Engineering Co., Ltd., Beijing, China).

The insulin sensitivity index (ISI), homeostasis model assessment of insulin resistance (HOMA-IR) and homeostasis model assessment of β cell function index (HBCI) were calculated as follows:

$$ISI = 1/[FPG\ (mmol/L) \times FINS\ (mU/L)]$$

$$HOMA\text{-}IR = FINS\ (mU/L) \times FPG\ (mmol/L)/22.5$$

$$HBCI = 20 \times FINS\ (mU/L)/(FPG\ (mmol/L) - 3.5)$$

OGTT in 8-week-old rats

After the rats were fasted for 12 hours, 25% glucose (2 g/Kg) was used for intragastric administration, and 0.4 ml of blood was collected from the retrobulbar vein at 0 min, 30 min, and 120 min, respectively. The blood samples were later used for measuring plasma glucose and serum insulin, using the same test methods as FPG and FINS detection.

Western blot

100 mg liver tissues from each rat were collected. According to the instructions of the total protein extraction kit (KeyGEN Biotech Co., Ltd., Nanjing, China), six times the volume of precooled cell lysis buffer was added to the tissues after being cut into pieces, and then the tissues were pulverized by an ultrasound homogenizer for 20 s with an interval of 20 s, three times in total and then centrifuged at 15000 rpm for 20 min at 4°C. The supernatant was removed and represented the total protein from the tissue cells. The phenol reagent method was adopted to quantify the protein sample, and then addition of lysis buffer was used to adjust samples to the same concentration of protein.

10 μl of 5 × sample buffer solution were added to 40 μl of protein liquid, then, electrophoresis was performed in a 5% sodium dodecyl sulfate -polyacrylamide gel. After washing with tween Tris buffered saline (TTBS), GCK mouse anti rat IgG and IRS-2 rabbit anti rat IgG (Santa Cruz, U.S.A.) were added onto the membrane and hybridized overnight at 4°C. The membrane was transferred to rabbit anti rat or mouse anti rat horse radish peroxidase (HRP) conjugated IgG 2nd antibody solution for hybridization. Images were analyzed by microscopic image analysis system (MetaMorph/C-5050/BX41 UIC/OLYMPUS US/JP, U.S.A.).

Immunohistochemical staining

Fixed samples were taken out of a 4% paraformaldehyde solution, shaved, and dehydrated.

Table 1. Comparison of the growth and development between the CON group and the IUGR group

	CON			IUGR		
	0 d	3 w	8 w	0 d	3 w	8 w
Body weight (g)	6.25 ± 0.56	40.12 ± 5.59	140.56 ± 10.33	4.82 ± 0.51**	39.66 ± 5.46	156.87 ± 12.98*
Pancreas weight (mg)	33.17 ± 3.05	162.12 ± 7.12	441.27 ± 30.08	26.33 ± 3.17*	159.63 ± 6.72	456.01 ± 29.13

In each group, n = 8 for every measurement at a specific time. Data are expressed as mean ± SD. *P<0.05, **P<0.01.

Table 2. Comparison of the β cell function indexes between the CON group and the IUGR group

	CON			IUGR		
	0 d	3 w	8 w	0 d	3 w	8 w
FPG (mmol/L)	4.36 ± 0.32	4.62 ± 0.36	4.69 ± 0.41	4.53 ± 0.37	4.85 ± 0.19	5.01 ± 0.45
FINS (mU/L)	15.76 ± 2.26	16.17 ± 3.15	19.56 ± 3.27	14.92 ± 2.63	17.06 ± 3.11	28.27 ± 4.32*
ISI	-4.15 ± 0.15	-4.31 ± 0.12	-5.10 ± 0.19	-4.10 ± 0.13	-4.42 ± 0.22	-6.13 ± 0.31*
HOMA-IR	0.56 ± 0.01	1.20 ± 0.06	1.46 ± 0.02	0.39 ± 0.02	1.25 ± 0.04	2.97 ± 0.76**
HBCI	5.40 ± 0.52	5.67 ± 0.46	6.36 ± 0.37	5.59 ± 0.28	5.53 ± 0.36	5.44 ± 0.35*

In each group, n = 8 for every measurement at a specific time. Data are expressed as mean ± SD. *P<0.05, **P<0.01.

Samples were paraffin-embedded in a machine (LS-100, Shenyang, China), and continuously sectioned into 5 μm slices with LKB8600, an ultra-thin paraffin sectioning machine (Leica JUNG RM 2050).

For immunohistochemical staining, the slices were routinely dewaxed and hydrated. Slices were incubated respectively with mouse-anti-rat GCK primary antibody or rabbit-anti-rat IRS-2 primary antibody (ZSGB-BIO, Beijing, China) at room temperature for 1 h or 4°C overnight, followed by incubation with biotinylated secondary antibody at 37°C. Then slices were incubated with HRP-labeled SABC at 37°C for 20 min and colored with 3,3'-diaminobenzidine (DAB) using the DAB developing kit (ZLI-9032, Beijing Zhongshan, China) and re-stained by hematoxylin and eosin (HE).

Five different fields of view were taken for each section, and then the data were scanned by a computer image analyzer (image-Pro Plus6.0, Media Cybernetics, USA).

Statistical analysis

Statistical analyses were performed using the SPSS 11.5 software (SPSS Inc., Chicago, IL, USA). All data are expressed as mean ± SD. The incidences of IUGR in groups with different diet were compared by the x^2 test. Comparisons between the CON group and the IUGR group at the same time frame were made by Student's test. For either group, each specific type of measurements taken at different time points were compared with analysis of variance (ANOVA). $P<0.05$ indicated significant difference.

Results

The IUGR rats had developmental retardation followed by postnatal catch-up growth

The gestation period of all the mother rats was 21 d. In the low-protein diet group, the incidence of IUGR was 61.32%, which is significantly higher than that (9.28%) in the standard diet group ($x^2 = 58.36$, $P<0.001$).

The average birth weight of newborns from the IUGR group was 22.88% lower than the CON group, and the difference was significant ($P<0.001$) (**Table 1**). After 3 weeks of growth, the weight of rats in two groups was not significantly different. But after 8 weeks, the IUGR rats had a significantly higher weight than the CON rats ($P<0.05$).

We also compare the development of the organ associated with glucose metabolism between two groups. For rats in the IUGR group, their pancreas was 20.62% lighter than rats in the CON group ($P<0.05$), but at 3 weeks and 8 weeks later, the difference of pancreas weight was no longer significant (**Table 1**).

The IUGR rats gradually lost insulin responsiveness, indicative of impaired β cell function

We turned to evaluate the insulin responsiveness in both groups. We found that the FPG of

Table 3. Comparison of OGTT at 8 week postnatally between the CON group and the IUGR group

	CON			IUGR		
	0 min	30 min	120 min	0 min	30 min	120 min
Blood glucose (mmol/L)	5.02 ± 0.83	6.59 ± 0.81	8.86 ± 1.55	5.93 ± 0.72	7.38 ± 0.94	11.06 ± 1.52
Serum insulin (mU/L)	35.63 ± 5.25	46.55 ± 5.13	56.36 ± 6.92	73.78 ± 3.36*	86.65 ± 4.56*	78.81 ± 5.02*

In each group, n = 8 for every measurement at a specific time. Data are expressed as mean ± SD. *P<0.05.

Table 4. Comparison of the hepatic expression of IRS-2 or GCK between the CON group and the IUGR group

	CON			IUGR		
	0 d	3 w	8 w	0 d	3 w	8 w
IRS-2 (Western Blot)	0.49 ± 0.03	0.55 ± 0.07	0.57 ± 0.05	0.34 ± 0.03*	0.41 ± 0.03*	0.41 ± 0.04*
IRS-2 (Immunohistochemical Staining)	0.72 ± 0.07	0.75 ± 0.08	0.89 ± 0.08	0.61 ± 0.04*	0.55 ± 0.02*	0.63 ± 0.05*
GCK (Western Blot)	0.47 ± 0.03	0.49 ± 0.02	0.54 ± 0.04	0.32 ± 0.03*	0.37 ± 0.02**	0.35 ± 0.02*
GCK (Immunohistochemical Staining)	0.63 ± 0.07	0.77 ± 0.06	0.67 ± 0.08	0.43 ± 0.05*	0.55 ± 0.05*	0.41 ± 0.04*

In each group, n = 8 for every measurement at a specific time. Data are expressed as mean ± SD. *P<0.05, **P<0.01.

rats from the IUGR group and the CON group were not significantly different at all the detection time after birth (**Table 2**). Likewise, at 0 d and 3 weeks after birth, the FINS were also not significantly different between two groups (**Table 2**). However, at 8 weeks, the FINS in IUGR rats was significantly higher than that of the CON rats ($P<0.01$).

We then calculated the β cell function indexes based on FPG and FINS. At 0 d and 3 weeks after birth, the IUGR and CON rats showed no significantly different ISI, but 8 weeks later, the ISI of IUGR rats was significantly lower than that of the control ($P<0.05$) (**Table 2**). As for the HOMA-IR, no significant difference was found at 0 d and 3 weeks after birth, but IUGR rats displayed significantly increased HOMA-IR when they grew to 8 weeks old, with $P<0.01$ compared with the CON (**Table 2**). Similarly, the HBCI in rats was not significantly different at first (0 d and 3 weeks), but then it increased significantly in the IUGR group after 8 weeks of growth, with $P<0.05$ when compared with the CON group (**Table 2**).

Next we made a comparison of OGTT between rats from the IUGR group and the CON group. There was no significant difference of the level of plasma glucose detected between two groups at three time points (**Table 3**). However, the levels of insulin in the IUGR group were all significantly higher than the CON group throughout the experiment ($P<0.05$).

Expression of IRS-2 and GCK in the liver of IUGR rat were both significantly downregulated

As detected by western blot, the expression of IRS-2 and the expression of GCK in the liver of the IUGR group were significantly lower than that in the CON group. No significant difference was found between the different time points in the IUGR group, and no significant difference was found in the CON group (**Table 4**).

As detected by immunohistochemical staining, the IRS-2 expression and the GCK expression in hepatic cells quantified by image analyzer (**Table 4**) showed a significant difference between the IUGR rats and the CON rats at newborn (P<0.05), 3-week-old (P<0.05) and 8-week-old time points (P<0.05) (**Figure 1**).

Discussion

There are many ways to establish an IUGR model, such as passive smoking [32], restricting food intake with drugs [33], uterine artery ligation [34], or low-protein calorific diet feeding [35]. Animal studies have shown that malnutrition might affect the critical period of fetal growth, thereby impacting the structure and function of certain tissues or organs for a very long time [36]. In this study, the IUGR model was created by feeding the maternal rats with a low-protein diet, and in their offspring, the incidence of IUGR was 61.32%, while that in the standard diet group was only 9.28%. The average birth weight of IUGR rats (4.82 g) was

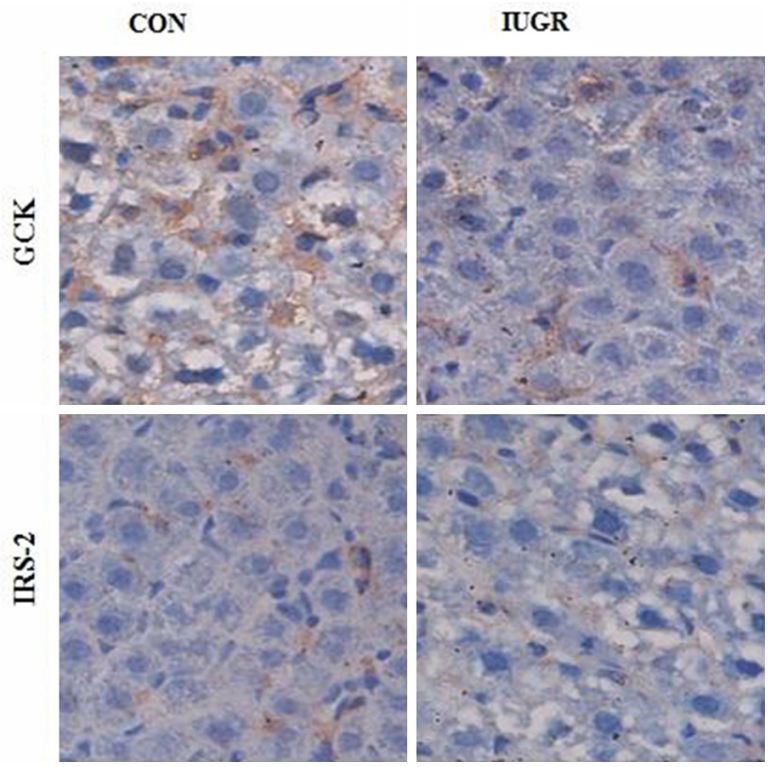

Figure 1. Representative immunohistochemical staining (brown) for GCK or IRS-2 in the hepatic cells of 8-week-old immature rats from CON group or IUGR group (n = 8, respectively). The nuclei appeared blue with HE re-staining. Magnification: 400 ×.

77.12% of that (6.25 g) of CON rats, which was a significant difference. And we found that the development of pancreatic tissue was influenced by IUGR too, as the average pancreas weight of newborn CON rats was 33.17 mg, while that of IUGR rats was 26.33 mg, only 79.38% of the CON group. This all indicated that low protein intake and amino acid deficiency are important factors causing IUGR.

In this study, we also observed that rats showed catch-up growth shortly after birth; 3 weeks later, the average body weight of IUGR rats was insignificantly lower than the control, while 8 weeks later, the average body weight of IUGR rats was significantly higher than the control (**Table 1**) ($P<0.05$). What's more, the pancreas weight of rats aged 3 weeks and 8 weeks showed no significant difference between two groups which also indicated a catch-up growth. This suggested IUGR rats had displayed obvious catch-up growth, as their body weight increased significantly.

Barker [37] suggested there are many causes for IGT after infant IUGR, but mostly they are related to insufficient supply of certain proteins and amino acids (especially cysteine) that are essential for β cell development and insulin secretion during late fetal growth. Yet we found that the serum insulin level in the IUGR group was nearly the same as the CON group in the newborn period, and even surpassed the controls as the rats grew. In the OGTT carried out after 8 weeks of growth, we also saw that the insulin levels of IUGR rats were significantly higher than the CON group (**Table 3**) ($P<0.05$). Thus we consider that abnormal glucose metabolism in immature rats should not be attributed to the decreased insulin secretion which in fact was excessive in the catch-up growth.

Since abnormal glucose metabolism may not be caused by decreased insulin secretion, we turned to focus on other mechanism such as impaired insulin sensitivity. After its first publication in 1985, the HOMA model has been revised and improved many times, and it is widely used for evaluating insulin sensitivity. It is fairly straightforward as it only requires the FINS and FPG levels to evaluate β-cell function (HOMAβ) and insulin resistance (HOMA-IR) [38]. In this study, we compared the insulin sensitivity of two groups by calculating the HOMA-IR, as well as the ISI and HBCI, which are also methods for insulin sensitivity and β-cell sensitivity evaluation [39, 40]. The results showed that the increase of insulin secretion does not necessarily represent a better β-cell function. On the contrary, the calculation of insulin sensitivity indicated that IUGR rats had significantly decreased insulin sensitivity, increased insulin resistance, and decreased β-cell function at 8 weeks after birth (**Table 2**) ($P<0.01$).

Our experiment also indicated that in the IUGR model established under a low protein diet, expression of IRS-2 or GCK in the livers of newborn rats, 3-week-old rats and 8-week-old rats

were remarkably reduced, and significant differences were observed between the IUGR group and the CON group (**Table 4**; **Figure 1**). While no marked changes in the expression were observed with increased age and weight. As a result, the insulin signal transduction would be affected with decreased biological effects, which may subsequently cause the glucose metabolism disorders. Expression of GCK in the liver was reduced, and this may cause obstruction of glucose phosphorylation, reduction of glucose conversion into 6- phosphoric acid glucose, suppression of all pathways of glycogen synthesis and glucose catabolism, and elevate blood glucose levels.

It has been reported that the catch-up growth in many IUGR infants is associated with the compensatory self-regulation in response to intrauterine nutrition deficiency, and the rapid postnatal catch-up growth following intrauterine growth restriction may be pathogenetic for the development of metabolic diseases such as type II diabetes [41]. When fetuses cannot gain sufficient nutrition from the placenta, they may increase the need for endogenous hepatic glucose production (HGP). This activated process is resistant to suppression by insulin and may contribute to postnatal abnormal glucose regulation [42]. Our study suggests a possible explanation for glucose dysregulation during catch-up growth after IUGR, which may echo the "activated HGP" theory. The expression of IRS-2 and GCK proteins in the liver were always downregulated during catch-up growth, which may be owing to the persistent suppression of insulin signaling pathway lasting from fetus period for the sake of HGP promotion. On the other hand, the serum insulin level whether fasting or not was gradually higher in IUGR individuals compared with normal controls, which may result from compensatory feedback regulation. Overall it is likely that the exceeded insulin secretion together with the lower response to the insulin signaling pathway in the liver led to the impaired blood glucose regulation reflected by ISI, HOMA-IR and HBCI. In another word, there may be a long-term lower insulin responsiveness in the liver from the fetal stage to postnatal periods in IUGR individuals.

As a conclusion, abnormal glucose metabolism was found in immature rats with IUGR, and the mechanism underlying may be related to the persistently impaired expression of IRS-2 and GCK, as well as gradually exceeded secretion of insulin during catch-up growth after IUGR. The results from our study imply pivotal roles for IRS-2 and GCK in blood glucose regulation which may be decreased as a consequence of IUGR. While the signals that downregulated IRS-2 or GCK as an important part of metabolic reprogramming in IUGR need further exploration.

This study has some limitations. According to previous studies, the susceptibility of IUGR individuals to metabolic disorders and related diseases in their adulthood is likely to be affected by gender. Male rats have been found to be more likely to develop IGT and hyperinsulinemia in adulthood, but female rats may also show significant diabetic changes and have a higher tendency for obesity [20]. Similar gender differences have also been seen in proteomic evaluation of IUGR sheep [21, 22]. We did not investigate the gender differences that recent study suggests should be expected [43], while as an alternative strategy the groups investigated were gender balanced.

Besides, we did not fully investigate the mechanisms involved in insulin regulation. Further experiments involving more signaling molecules would provide more details about the mechanisms involved.

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Disclosure of conflict of interest

None.

Address correspondence to: Ying Xin, Department of Paediatrics, Shengjing Hospital of China Medical University, No.36 of Sanhao Street, Heping District, Shenyang 110004, Liaoning, China. Tel: +86 24 96615-52111; Fax: +86 24 23892617; E-mail: xin.ying168@live.cn

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