Decreased expression of GLUT4 in male CG-IUGR rats may play a vital role in their increased susceptibility to diabetes mellitus in adulthood

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Abstract

Rats with intrauterine growth retardation and catch-up growth (CG-IUGR) after birth show increased susceptibility to diabetes mellitus in adulthood. The expression of glucose transporter type 4 (GLUT4) decreases in female IUGR offspring rats with seminutrient restriction during pregnancy. However, the male CG-IUGR rats also display an increased susceptibility to diabetes mellitus in adulthood. Whether there is another factor, besides GLUT4, in male CG-IUGR rat that mediates their susceptibility to diabetes mellitus? The male IUGR rats with catch-up growth were selected as the research objects. CG-IUGR rats had an increased fasting blood glucose level, and increased serum total cholesterol, triglyceride and free fatty acid levels. Glucose tolerance test and insulin tolerance test showed higher glucose levels and much higher insulin levels after a glucose load in CG-IUGR. The mRNA and protein expressions of IRS-2 in liver tissue, and IRS-1 and GLUT4 in skeletal muscle in CG-IUGR rats were down-regulated, but only the GLUT4 down-regulation displayed strong negative correlations with the decreased glucose tolerance capability by Pearson’s analysis. The methylation patterns of CpG islands in the promoter regions of IRS-1, IRS-2 and GLUT4 in CG-IUGR rats varied, which was not significantly correlated with their expressions. The male CG-IUGR rats showed decreased glucose tolerant capability, suggesting increased susceptibility to diabetes mellitus in adulthood. The GLUT4 down-regulation may play a vital role in the development of decreased glucose tolerance in male CG-IUGR rats. The methylation modification of the promoter region of GLUT4 does not appear to be involved in its expression.

Key words: CG-IUGR, diabetes mellitus, GLUT4, DNA methylation, metabolism

Introduction

Intrauterine growth retardation (IUGR) is one of the most important causes of increased perinatal mortality and morbidity, occurring in 16.4% of births in the developing world and 7%–11% in developed countries [1,2]. IUGR is commonly defined as fetal growth below that characterizing a commonly healthy population; thus, most IUGR individuals are small for gestational age (SGA) [3,4]. The term SGA has been used as a synonym for IUGR, although IUGR is not necessary for SGA. Most SGA individuals have been found to be a catch-up growth (CG-IUGR) at the early postnatal period. Numerous studies have demonstrated that early improvement in growth is beneficial for a number of important outcomes; hence, the personnel of neonatal follow-up clinics have been encouraged to promote early catch-up growth of SGA subjects [5]. However, since 2003 several epidemiology and related studies have shown that IUGR individuals, especially CG-IUGR, have an obviously increased
Susceptibility to metabolic syndromes (MSs) such as dyslipidemia, insulin resistance, diabetes mellitus, cardiovascular diseases, and cognitive dysfunction in adulthood [6–11]. These studies have proposed the hypothesis that restricting postnatal catch-up after prematurity could prevent later metabolic abnormalities. In 2001, Hales et al. [12] proposed the ‘thrifty phenotype hypothesis’ about diabetes mellitus, which suggests that the gene itself does not change according to the mother’s nutritional environment, but the expressions of genes can be changed in the critical period [12]. Under a malnutrition intruterine environment, the fetus will incur a series of adaptive changes to survive. These changes include lower physical size, metabolic rate, growth rate and secretion of insulin, which are all for energy saving and survival. After birth, if the environment is better than expected, it will cause adult diseases such as lipid accumulation, insulin resistance, and Type II diabetes mellitus. Accordingly, the thrifty phenotype hypothesis explains why adult MSs are very often seen in individuals with growth restriction at birth and rapid catch-up growth after birth.

However, there are still significant gaps in the knowledge of these double-edged effects and outcomes in IUGR individuals, and the mechanisms behind this phenomenon remain poorly understood. Park et al. [13] have found that the Pdx-1 gene was modified by methylating, including DNA methylation and histone methylation in IUGR rats. The Pdx-1 expression was also inhibited, leading to pancreas hypoplasia and increased susceptibility to Type II diabetes mellitus. Therefore, changes in the phenotype of rat offspring (namely epigenetic changes) caused by environmental factors at the embryo stage may be one of the important reasons for increased susceptibility to diabetes mellitus. The epigenetic regulation of gene expression does not change the gene sequence but modifies the structure by DNA methylation, histone modifications, and chromatin remodeling.

In this study, we prepared IUGR models with a 40% calorie diet during the entire pregnancy period, and the male rats with catch-up growth were selected to explore the potential mechanisms of the increased susceptibility to diabetes mellitus. We focused on the signaling molecules, such as insulin, insulin receptor substrate (IRS)-1, IRS-2, and glucose transporter type 4 (GLUT4), which play essential roles in the uptake of glucose by the target cells. Insulin combined with insulin receptor transfer insulin signals into the cell and causes changes to IRS conformation, which activates the IRS proteins, IRS-1 and IRS-2. Then, it induces the removal of GLUT (primarily GLUT4) molecules from the intracellular pool to the cell membrane, thus promoting the uptake and use of glucose. A comprehensive investigation of the expression patterns of these signaling molecules, their relationship with CG-IUGR-induced increased susceptibility to diabetes mellitus, and the potential epigenetic mechanisms may elucidate the pathogenesis of diabetes mellitus in male CG-IUGR rats.

Materials and Methods

Materials

Trizol reagents and M-MLV retrovirus reagent were from Invitrogen (Carlsbad, USA). Rabbit anti-IRS-1, anti-IRS-2, anti-GLUT4, and anti-β-actin polyclonal antibodies were purchased from Abcam (Cambridge, UK). HRP-conjugated secondary antibodies were from Sigma (St Louis, USA). RIPA buffer was obtained from Shenneng Bocai (Shanghai, China). SYBR Green II was from TaKeRa (Dalian, China). TA cloning kit was purchased from Life Technologies (Carlsbad, USA). Sulfite processing kit was from Qiagen (Hilden, Germany). All primers were synthesized by Shanghai General Biotechnology Company (Shanghai, China).

Preparation of animal models

Twenty-five 8-week-old healthy SD rats (20 females and 5 males; body weight 190–210 g; animal certificate No. 114007000563721) were provided by Vital River Laboratory Animal Center (Beijing, China). The rats were raised in a clean, quiet, and ventilated animal room at 25°C with 60% humidity. After 1 week of adaptive feeding, male and female rats were put in the same cage at 20:00. At 8:00–9:00 of the next morning, female rats with evidence of sperm plug in the vagina were recorded as pregnancy 0 days. Four female rats were fed routinely for 10 weeks with normal diet (309.6 kJ); Laboratory Animal Center of the Third Military Medical University, Chongqing, China) and then mated with a 10-week-old male rat without any blood relationship. Female rats were raised in single cages during pregnancy and were divided into control group and IUGR group. The control group was fed with a normal diet (309.6 kJ), and the IUGR group was fed with a reduced-calorie diet (40% of normal calorie, 123.8 kJ). Newborn rats in the IUGR group whose body weight at birth was < 2 SD below the average body weight of the control group were selected for the IUGR group. From the control group, eight newborn rats that met the diagnostic criteria were taken. But in order to ensure the catch-up growth of the offspring, only five newborn rats were taken in IUGR group. The offspring rats were weaned 3 weeks after birth and fed routine diet till 8 weeks of age. We only selected male offspring rats with catch-up growth as the research subjects to avoid confounding factors of gender and hormones. All animal experimental procedures were approved by the Animal Ethics Committee and conducted according to the Animal Protection Law of the People’s Republic of China.

Measurement of body length and body weight

The number of newborn rats was counted within 2 h after birth in each group. Offspring rats were gently straightened and fixed to measure the length from nose to tail (±0.01 cm). Changes in offspring body weight were monitored weekly by an electronic balance, recorded, and analyzed. Body mass index (BMI) was calculated as follows: BMI = body weight (kg)/body length squared (m²).

Glucose tolerance test and insulin tolerance test

Eight-week-old male offspring rats were selected for glucose tolerance test and insulin tolerance test (GTT and ITT). The rats were fasted for 12 h but were given free access to water. Fasting glucose was measured at 9:00 AM, then GTT was measured via intraperitoneal injection with 20% glucose solution (2 g/kg) and ITT was measured via intraperitoneal injection with insulin (0.1 U/kg). Blood was taken by cutting the tail at 15, 30, 60, 90, and 120 min after injection, and a blood glucose monitor system (One Touch Ultra Test Strips; LifeScan, Milpitas, USA) was used to determine the corresponding blood glucose level. Then blood glucose area under curve (AUC) during GTT was calculated.

Determination of serum insulin

Radioimmunoassay was used to determine the fasting serum insulin and insulin level at 15 min after intraperitoneal injection of 20% glucose solution (2 g/kg). The kits were produced by Beijing North Institute of Biological Technology (Beijing, China). The tests were carried out in the Radiology Department of the First Affiliated Hospital of Zunyi Medical University (Zunyi, China).
Determination of serum total cholesterol, triglyceride and free fatty acid levels
At the end of the eighth week, the rats were anesthetized by intraperitoneal injection of 2% sodium pentobarbital (0.2 ml/100 g of body weight). Blood (2 ml) was taken from the rats’ common carotid artery and centrifuged immediately at 1200 g for 10 min. The serum was separated and preserved at −80°C. The samples were sent to the Clinical Laboratory Department of the First Affiliated Hospital of Zunyi Medical University, to determine the serum levels of total cholesterol (TC), triglyceride (TG), and free fatty acid (FFA) by automatic biochemical analyzer AU8281 (Beckman, Pasadena, USA).

Total RNA extraction and reverse transcription of total RNA
Eight 8-week-old male offspring rats were randomly selected from each group. The rats were administered with 2% sodium pentobarbital (0.2 ml/100 g of body weight) and 20% glucose solution (2 g/kg) via intraperitoneal injection. Then, 100 mg of muscle tissues and 100 mg of liver tissues were removed from each rat. Total RNA was extracted from the tissues using Trizol reagent, and an ultraviolet spectrophotometer was used to determine the optical density (OD) at 260 and 280 nm. OD260/OD280 value was calculated to check the purity of the prepared RNA. M-MLV reagent was used for reverse transcription according to the manufacturer’s instructions.

Real-time fluorescence quantitative polymerase chain reaction
A 96-well CONNECT PCR was used to determine the relative mRNA expression. β-Actin was used as the reference gene for relative content of target genes. Real-time fluorescence quantitative polymerase chain reaction (PCR) amplification was carried out on a CFX Connect™ Real-Time PCR Detection System (Bio-Rad, Hercules, USA). The cycling conditions (IRS-2, IRS-1, GLUT4, and β-actin) are as follows: denaturation at 95°C for 30 s, followed by 39 cycles of 95°C for 5 s and 56°C–77°C for 30 s. To verify that the amplified product is the only target gene product, we added a dissociation curve from 65°C to 95°C after the PCR. The amplification efficiency of the primers was calculated by a standard curve of seven diluted concentrations. 2−ΔΔCt was used to calculate the relative mRNA content of each gene. The lengths of sequences and products are shown in Table 1.

Western blot analysis
Total protein was extracted with RIPA buffer from 100 mg of muscle and 100 mg of liver tissues. The protein concentration was determined by the Bradford method. Protein samples (50 μg of total protein) were subject to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) (Bio-Rad), and then separated proteins were transferred onto PVDF membranes. The membranes were blocked with 6% skim milk powder and incubated with primary antibodies against IRS-1 (1:300), IRS-2 (1:200), GLUT4 (1:2000), and β-actin (1:2000) at 4°C overnight. After washing three times with 1% TBS-Tween solution at room temperature for 1 h, the membranes were incubated with the HRP-conjugated secondary antibody (1:2500) at room temperature for 2 h and visualized using an ECL kit. Semi-quantification of protein bands was evaluated by densitometry using a UVP BioSpectrum Imaging System (UVP, Upland, USA).

Bisulfite sequencing PCR for methylation analysis
By analyzing the CpG island in the promoter regions of genes through online web page (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi), two typical CpG islands in IRS-2 gene promoter regions and one typical CpG island in IRS-2 and GLUT4 gene promoter regions were found, respectively (Fig. 1). A DNA extraction kit was used to extract DNA from rat liver and skeletal muscle tissues. The extracted DNA was modified with sodium bisulfite, and then PCR was carried out using the following conditions (IRS-2, IRS-1, GLUT4): denaturation at 95°C for 30 s, followed by 40 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 30 s. Amplified bisulfate-treated PCR products were purified by gel extraction and inserted into a pMD18-T vector for sequencing. Bisulfite sequencing DNA methylation analysis was used for data analysis. Primer sequences for bisulfite sequencing PCR are shown in Table 2.

Statistical analysis
SPSS 19.0 was used for statistical analysis. All the data are presented as the mean ± standard deviation. Student’s t test was used to determine the statistical significance. Calculator information was determined by χ2 test. Pearson correlation analysis was used for correlation test. P < 0.05 was considered statistically significant difference.

Results
General effects of low-calorie diet on offspring rats
Rats in the IUGR group showed catch-up growth from lactation. The average body weight of the CG-IUGR group at the first week was higher than that of the control group, and this difference was maintained until the eighth week. The differences were statistically significant (P < 0.05). The growth and development conditions of the offspring are shown in Table 3.

Table 1. Sequence of primers used in real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank accession No</th>
<th>Primers (5’-3’)</th>
<th>Product (bp)</th>
<th>Annealing temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLUT4</td>
<td>NM_012751</td>
<td>Forward: CTTGCTTCTATTTTGCCGTCTCC Reverse: CATTGGGCTCAGTCTTCTTCTCT</td>
<td>195</td>
<td>56</td>
</tr>
<tr>
<td>IRS-1</td>
<td>NM_012969</td>
<td>Forward: CGTCTTCTCTTCTGCTTACACCTC Reverse: ACGAACTACCTTTGGCGCTCTTG</td>
<td>149</td>
<td>56</td>
</tr>
<tr>
<td>IRS-2</td>
<td>NM_001168633</td>
<td>Forward: GTATTAGATAAAGGACCAAAGGGC Reverse: AAGTACAGGGAGAAATGACGCA</td>
<td>198</td>
<td>57</td>
</tr>
<tr>
<td>β-actin</td>
<td>NM_031144</td>
<td>Forward: CTGAACTAAGAAGGCAACC</td>
<td>112</td>
<td>56</td>
</tr>
</tbody>
</table>
Figure 1. The CpG islands in the promoter regions of three genes. The CpG islands in the promoter regions of IRS-2, IRS-1, and GLUT4 genes: the blue region represents the CpG island. (A) The first CpG island of IRS-2 gene. (B) The second CpG island of IRS-2 gene. (C) The CpG island of IRS-1 gene. (D) The CpG island of GLUT4 gene. CG-IUGR: intrauterine growth retardation with catch-up growth; IRS: insulin receptor substrate; GLUT4: glucose transporter type 4.

Table 2. Sequence of primers used in bisulfite sequencing PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer (5'-3')</th>
<th>Product (bp)</th>
<th>Annealing temperature (ºC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLUT4</td>
<td>Forward: GAAGTTTGGTTAGAATAGTAAGTTTGAGG</td>
<td>429</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>Reverse: CCAACACACACATAAAAAACTACAACC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IRS-1</td>
<td>Forward: TGTTTGTTTTATATTTYGGGT</td>
<td>207</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>Reverse: CCRATAACACCACAAAAACACRCCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IRS-2(1)</td>
<td>Forward: GGYGGYGGGAAAYGTAG</td>
<td>230</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>Reverse: CACACRTAACTCTACRRTAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IRS-2(2)</td>
<td>Forward: GTGGTGTAGTAGTAGTAG</td>
<td>181</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>Reverse: CCCRAAAAACTAAACTAAACCRAAAATC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Gluco-lipid metabolic patterns, GTT, and ITT in CG-IUGR rats vs. control

The CG-IUGR rats manifested a moderately elevated fasting blood glucose level (Fig. 2) and a decreased fasting insulin level compared with the control group (Table 4; P < 0.05). The GTT and ITT revealed a significant decrease in the glucose tolerant capacity of CG-IUGR rats; the blood glucose levels in CG-IUGR rats at 15, 30, 60, 90, and 120 min were all significantly higher than those in the control group (P < 0.05) resulting in a significantly higher AUC in GTT (P < 0.05; Figs. 2 and 3). The insulin level at 15 min after the glucose load clearly increased more than that in the control group. All the above results suggest decreased insulin effects and an increased susceptibility of CG-IUGR rats to diabetes mellitus (Figs. 2 and 3, and Table 4). In addition, the serum TC, TG, and FFA levels were all higher in the CG-IUGR group than those in the control group, which may promote a tendency to develop metabolic syndrome in later life (Table 5).

Table 3. Comparison of body weight, body length, and BMI between control and CG-IUGR male offspring rats from birth to 8 weeks of age

<table>
<thead>
<tr>
<th>Age (week)</th>
<th>Body weight (g)</th>
<th>Body length (cm)</th>
<th>BMI (kg/m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (n = 24)</td>
<td>CG-IUGR (n = 23)</td>
<td>Control (n = 24)</td>
</tr>
<tr>
<td>0</td>
<td>7.10 ± 0.18</td>
<td>5.96 ± 0.27</td>
<td>6.22 ± 0.11</td>
</tr>
<tr>
<td>1</td>
<td>17.41 ± 2.04</td>
<td>24.45 ± 2.47</td>
<td>8.75 ± 0.21</td>
</tr>
<tr>
<td>2</td>
<td>30.32 ± 3.42</td>
<td>36.18 ± 3.33</td>
<td>9.65 ± 0.22</td>
</tr>
<tr>
<td>3</td>
<td>50.09 ± 7.13</td>
<td>56.35 ± 6.68</td>
<td>11.87 ± 0.37</td>
</tr>
<tr>
<td>4</td>
<td>73.78 ± 4.74</td>
<td>84.46 ± 8.31</td>
<td>14.87 ± 0.58</td>
</tr>
<tr>
<td>5</td>
<td>101.29 ± 7.21</td>
<td>120.47 ± 8.47</td>
<td>16.29 ± 0.61</td>
</tr>
<tr>
<td>6</td>
<td>123.90 ± 6.53</td>
<td>151.90 ± 13.87</td>
<td>17.28 ± 0.60</td>
</tr>
<tr>
<td>7</td>
<td>147.24 ± 4.57</td>
<td>178.34 ± 13.32</td>
<td>18.28 ± 0.65</td>
</tr>
<tr>
<td>8</td>
<td>163.43 ± 8.19</td>
<td>200.13 ± 9.20</td>
<td>19.43 ± 0.82</td>
</tr>
</tbody>
</table>

Data are presented as the mean ± standard deviation. * P < 0.05 vs. control rats at the same age.

Table 4. Serum insulin levels in 8-week-old male offspring rats during GTT

<table>
<thead>
<tr>
<th>Group</th>
<th>Serum insulin (μIU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 min</td>
</tr>
<tr>
<td>Control</td>
<td>25.83 ± 4.46</td>
</tr>
<tr>
<td>CG-IUGR</td>
<td>15.96 ± 3.06*</td>
</tr>
</tbody>
</table>

Data are presented as the mean ± standard deviation (n = 8). *P < 0.05 vs. control rats.

Figure 2. Glucose levels and AUC determined by GTT in 8-week-old male offspring rats. (A) GTT. (B) AUC of glucose in GTT. Data are presented as the mean ± standard deviation (control rats, n = 12; CG-IUGR rats, n = 13). CG-IUGR: intrauterine growth retardation with catch-up growth; GTT: glucose tolerance test; AUC, area under curve. *P < 0.05 vs. control rats.

Figure 3. Glucose levels determined by ITT in 8-week-old male offspring rats

Glucose levels were determined by ITT in control and CG-IUGR rats. Data are presented as the mean ± standard deviation (control rats, n = 12; CG-IUGR rats, n = 13). CG-IUGR: intrauterine growth retardation with catch-up growth; ITT: insulin tolerance test. *P < 0.05 vs. control rats.
mRNA and protein expressions of IRS-2, IRS-1, and GLUT4
IRS-1 mainly promotes glucose uptake and utilization in muscle and adipose tissue. IRS-2 mainly promotes liver glycogen synthesis and inhibits gluconeogenesis, while GLUT4 is mainly located in skeletal muscle tissue. In this study, the mRNA and protein expressions of IRS-2 in liver tissues, and IRS-1 and GLUT4 in skeletal muscle were examined. Compared with the control group, the mRNA expression of IRS-2 in liver tissue of the CG-IUGR group was decreased ($P < 0.05$), and the mRNA expressions of IRS-1 and GLUT4 in skeletal muscle of the CG-IUGR group were decreased (Fig. 4; $P < 0.05$).

Protein expression was examined by western blot analysis. The expression patterns of IRS-2 in liver tissue, and IRS-1 and GLUT4 in skeletal muscle were similar to those of the mRNA (Fig. 5).

Alteration in methylation patterns in promoter regions of IRS-2, IRS-1, and GLUT4
To investigate whether the methylation patterns of the IRS-2, IRS-1, and GLUT4 genes are modified in CG-IUGR rats, bisulfite sequencing PCR was used to elucidate the specific methylation site and number in the promoter of IRS-2 in rat liver and in the promoters of IRS-1 and GLUT4 in skeletal muscles. If a CpG site in the promoter is methylated, which is resistant to bisulfite treatment, it remains as CG after PCR. If the CpG site is not methylated, the C is replaced by U through the bisulfite treatment, and it appears as TG after PCR. All other C in non-CpG sites were replaced by T after bisulfite treatment, indicating that the results were reliable.

Figure 6 shows the methylation profiles of the three genes. Red represents the methylated site and blue represents the non-methylated site. The methylation rates of the CpG islands in the
Figure 6. Methylation patterns of CpG islands in the promoter regions of three genes. Red represents the methylated site, and blue represents the non-methylated site. Note: This is one typical result. The red methylated CpG dinucleotides in the second CpG island of the IRS-2 gene promoter in liver tissues was much higher in the CG-IUGR group than that in the control group. (A) The first CpG island of IRS-2 gene. (B) The second CpG island of IRS-2 gene. (C) The CpG island of IRS-1 gene. (D) The first CpG island of GLUT4 gene.
promoter regions of the three genes show the methylation patterns of these CpG islands. The methylation profile of 25 CpG sites in the first CpG island of IRS-2 gene promoter showed no difference (6.74% vs. 4.48%; \( P > 0.05 \)). The methylation profile of 23 CpG sites in the second CpG island of the IRS-2 gene promoter showed that the methylation rate in the CG-IUGR group was 19.18%, which was much higher than that in the control group (6.42%; \( \chi^2 = 16.35, P < 0.05 \)). The methylation profiles of 26 CpG sites in the CpG island of the IRS-1 promoter showed that the methylation rate in the CG-IUGR group was 5.10%, which was higher than that of the control group (1.22%; \( \chi^2 = 6.45, P < 0.05 \)). The methylation rate in 10 CpG sites of the GLUT4 gene was 0% in both the CG-IUGR group and the control group (\( P > 0.05 \)). There are six results as shown in Table 6, and only one typical result is shown in Fig. 6.

### Table 6. Methylation rates of CpG islands in the promoter regions of three genes

<table>
<thead>
<tr>
<th>Group</th>
<th>IRS-1</th>
<th>IRS-2</th>
<th>GLUT4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1st CpG island</td>
<td>2nd CpG island</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.22%</td>
<td>4.48%</td>
<td>6.42%</td>
</tr>
<tr>
<td>CG-IUGR</td>
<td>5.10%*</td>
<td>6.74%</td>
<td>19.18%*</td>
</tr>
</tbody>
</table>

*\( P < 0.05 \) vs. control rats. \( n = 6 \).

**Correlation analysis between glucose tolerance and the expression of IRS-1, IRS-2, and GLUT4 mRNA**

The above-mentioned GTT results demonstrated a decreased glucose tolerance in the CG-IUGR rats, indicating an increased susceptibility to diabetes mellitus. Furthermore, the expressions of the IRS-2, IRS-1, and GLUT4 genes were similarly decreased. To determine whether these expression patterns have any intrinsic correlation with the decreased glucose tolerance and increased susceptibility to diabetes mellitus in CG-IUGR rats, their relationship was analyzed by the Pearson’s correlation test. The blood glucose concentration reached its peak at 15 min after the glucose load, so we analyzed the Pearson’s correlation coefficient of the blood glucose level vs. the expression levels of IRS-2, IRS-1, and GLUT4 mRNA, and the Pearson’s correlation coefficient of the blood insulin level vs. the expression levels of IRS-1, IRS-2, and GLUT4 mRNA at 15 min after glucose load in the CG-IUGR rats.

The results showed that only the GLUT4 expression level displayed a strong negative correlation with both the glucose and insulin levels (Pearson’s correlation coefficients: \(-0.971\) and \(-0.939\), respectively; \( P < 0.05 \); Fig. 7) after the glucose load. The IRS-2 expression level also displayed a negative correlation with the glucose level (Pearson’s correlation coefficient \(-0.720, P < 0.05 \); Fig. 7) but did not show a negative correlation with the insulin level. The IRS-1 expression displayed no correlation with the glucose level or the insulin level, although their expression levels showed a similar decrease to that of GLUT4. Thus, the Pearson’s analysis suggests a potential involvement of GLUT4 in the pathogenesis of the decreased glucose tolerance and increased susceptibility to diabetes mellitus of CG-IUGR rats.

**Discussion**

In this study, we set up an IUGR rat model by giving a low-calorie diet (40% of normal diet) to pregnant rats. The male F1 rats with catch-up growth were selected for exploring the susceptibility to diabetes mellitus and the potential mechanisms. The male CG-IUGR rats showed increased fasting blood glucose, TG, TG, FFA, and insulin levels 15 min after the glucose load, and decreased glucose tolerance capability, which demonstrated decreased insulin effects and may have an increased susceptibility to diabetes mellitus. Three important molecules of the insulin signaling pathway, i.e. IRS-2, IRS-1, and GLUT4, had a similarly decreased mRNA and protein expressions, suggesting that they were involved in the pathogenesis of the decreased glucose tolerance capability. However, only the expression of GLUT4 displayed a strong negative correlation with the decreased glucose tolerance capability by the Pearson’s correlation analysis. To elucidate the potential mechanism of the down-regulation of these genes, we examined the methylation patterns in the promoter regions of the IRS-2, IRS-1, and GLUT4 genes. These promoters showed different methylation patterns in the CpG islands. The methylation profile of the IRS-2 gene displayed relatively moderate hypermethylation, the IRS-1 gene showed mild hypermethylation, while no differences in the methylation pattern were observed in the GLUT4 gene in male CG-IUGR rats vs. the control group. The methylation profiles of these three genes did not show a unanimous relationship with their expression patterns, which may suggest that they have different epigenetic regulation.

The fasting insulin level in CG-IUGR rats was found to be decreased in this study. In clinics, however, hyperinsulinemia is often considered to be an important indicator for assessing insulin resistance, which seems to be inconsistent with our results. Park et al. [13] found pancreas hypoplasia in IUGR rats. Fewer beta cells and less insulin secretion in male offspring at 3 months of age with a maternal low-calorie diet have also been reported [14]. Tripathy et al. [15] found that the lack of insulin secretion existed when glucose tolerance was decreased. Thus, the decreased fasting insulin level in male CG-IUGR rats found in this study may suggest a potential aberrant pancreas function or a decrease of beta cells. However, in this study, the male CG-IUGR rats had a much higher insulin level after glucose load and decreased expressions of IRS-2, IRS-1, and GLUT4 than those of the control group, which may indicate significant decreased insulin effects and result in compensatory insulin secretion. All these results suggest that pancreas dysfunction and decreased insulin effects may exist. We only selected the male offspring to avoid confounding factors of gender and hormones. Raychaudhuri et al. [16] reported a different expression of GLUT4 between male and female IUGR rats; the female IUGR rats showed a decreased expression of GLUT4 in skeletal muscle, but the males did not. In our pilot experiment, both the male and female IUGR rats showed similar glucose intolerance, hence we wondered what the different mechanism underlying the glucose intolerance in male IUGR rats is, which was another reason for selecting male rats.

GLUT4 has been reported to play a vital role in mediating insulin sensitivity and in the pathogenesis of glucose intolerance in IUGR rats; therefore, we selected GLUT4 and its upstream signaling molecules, IRS-1 and IRS-2, as the target molecules in the pathogenic investigation of male rats. We originally thought that if the expression of GLUT4 does not change in male IUGR rats as reported by Raychaudhuri et al. [16], there must be some other molecules that play a vital role in the pathogenesis of the increased susceptibility to diabetes mellitus in male IUGR rats. Therefore, we selected the signaling molecules GLUT4, IRS-1, and IRS-2, which play essential roles in transferring insulin signals into the cell and initiate the GLUT4 function. However, unexpectedly we found that all three molecules displayed decreased mRNA and protein...
Figure 7. Pearson’s correlation analysis between glucose tolerance and the mRNA expressions of IRS-1, IRS-2, and GLUT4.

(A) Positive correlation between blood glucose and insulin levels after glucose load. (B) and (C) Strong negative correlation of blood glucose and insulin levels with the expression of GLUT4 mRNA. (D) Negative correlation between blood glucose level and the expression of IRS-2 mRNA. (E) No correlation between insulin level and the expression of IRS-2. (F) and (G) No correlation of blood glucose and insulin levels with the expression of IRS-1 mRNA. CG-IUGR: intrauterine growth retardation with catch-up growth; IRS: insulin receptor substrate; GLUT4: glucose transporter type 4.
expressions in male CG-IUGR rats, which is inconsistent with what was reported by Raychaudhuri et al. about the expression of GLUT4 [16]. A possible explanation is that different animal models were used. In our animal model, rats were fed with 40% of the normal calorie diet for pregnant rats during the entire pregnancy, and the nutrient restriction was stopped after birth for offspring to develop the catch-up growth. Only the male offspring with catch-up growth (CG-IUGR) were enrolled in the experiment group. The animal model used by Raychaudhuri et al. [16] was seminutrient-restricted, where the pregnant rats received 50% of the normal calorie diet from Days 11–21 of gestation, and the seminutrient restriction continued in the lactation period for the same mothers who reared newborn pups. Thus, their IUGR model was somewhat different from our CG-IUGR model.

Although GLUT4, IRS-1, and IRS-2 displayed decreased expressions, the Pearson’s correlation analysis only revealed a strong negative correlation of GLUT4 with the decreased glucose tolerance capability, which indicates that GLUT4 plays a role in mediating the pathogenesis of glucose intolerance in both genders of IUGR rats. IRS-1 and IRS-2 displayed a similar decreased expression pattern with GLUT4, but the Pearson’s correlation analysis did not show any significant correlation between their expressions and the decreased glucose tolerance capability. Therefore, GLUT4 probably plays a key role in the pathogenesis of glucose intolerance in male IUGR rats.

GLUT4, the major insulin-responsive isoform of the GLUT family (GLUT; Slc2 family of transport proteins), is expressed in insulin-sensitive tissues such as skeletal muscle, adipose tissue, and cardiac muscle, and mediates glucose transport across the cell membrane, a rate-limiting step in glucose metabolism under normal physiological circumstances [17]. Targeted disruption of skeletal muscle GLUT4 resulting in severe insulin resistance and glucose intolerance confirmed the vital role of this isoform in mediating insulin sensitivity. This study further demonstrated the vital role of GLUT4 in mediating the pathogenesis of glucose intolerance, not only in female IUGR rats but also in male CG-IUGR rats. IRS-1 and IRS-2 displayed decreased expressions; however, the function of this decrease in the glucose intolerance of the IUGR rats is still unclear and warrants further study.

The epigenetic modification of DNA with 5-methylcytosine is an important regulatory event involved in normal development, as well as in diseases. A growing amount of evidence has shown that aberrant DNA methylation in some genes may play an important role in IUGR. Previous studies have revealed the relative hypomethylation of the glucocorticoid receptor 1q13 promoter and the peroxisome proliferator-activated receptor alpha promoter with enhanced expression of the corresponding genes in IUGR rats [18, 19]. A classical human example is the Dutch Famine in the winter of 1944–1945; even after 60 years, the gene methylation level of insulin-like growth factor 2 is lower in offspring whose mothers had suffered famine during pregnancy than in offspring whose mothers had not suffered famine during pregnancy [20]. This study confirmed, for the first time, the hypothesis that nutrition conditions during pregnancy can cause epigenetic changes in offspring that will last for a lifetime.

This study examined the methylation patterns in the promoter regions of the IRS-1, IRS-2, and GLUT4 genes. The methylation profile of the IRS-2 gene displayed relatively moderate hypermethylation, the IRS-1 gene showed a mild hypermethylation, while no methylation changes were found in the GLUT4 gene promoter region. Although these three genes demonstrated a similar decreased expression, their methylation patterns were different. Whether the decreased expression of IRS-1 and IRS-2 correlates with their hypermethylation needs further exploration. The decreased expression of GLUT4 in male CG-IUGR rats, however, was not induced by methylation modification of the DNA sequence. Raychaudhuri et al. [16] reported the same finding in their seminutrient-restricted female IUGR rats, where no abnormal methylation of the DNA sequence of the GLUT4 gene promoter region was found. Their results suggested that DNA methylation of the GLUT4 promoter did not appear to be involved in decreased GLUT4 expression in female IUGR rats. However, they demonstrated an abnormal modification in histones, including methylation and deacetylation, which were the histone code modifications. This epigenetic mechanism was confirmed for the suppression of GLUT4 transcription in female IUGR rats. Liang et al. [21] found that knockdown of MEF2A, -2C, -2D, and Zac1 could significantly inhibit glucose uptake and GLUT4 expression in cardiomyocytes. Liao et al. [22] found that SOCS-1 and SOCS-3 knockdown ameliorated GLUT4 translocation under insulin-stimulated conditions in IUGR individuals. So, we believe that there may be other transcription factors participating in the regulation of GLUT4.

Here we demonstrated an obviously increased susceptibility to diabetes mellitus in male CG-IUGR rats whose decreased expression of GLUT4 may be an important reason for their glucose intolerance. Intrauterine calorie restriction does not cause aberrant methylation in the GLUT4 promoter, suggesting that diminished GLUT4 transcription in male IUGR rats is not caused by DNA methylation in the GLUT4 promoter. IRS-1 and IRS-2 display decreased expressions, but there is no significant correlation between their expressions and the glucose intolerance in male CG-IUGR rats.

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References


