Maternal protein restriction in rats leads to reduced PGC-1α expression via altered DNA methylation in skeletal muscle

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Received May 24, 2012; Accepted October 9, 2012

DOI: 10.3892/mmr.2012.1134

Abstract. Intrauterine growth retardation (IUGR) is thought to program insulin resistance, type 2 diabetes and other metabolic diseases in later life. Skeletal muscle is an important tissue involved in regulating the metabolism. We therefore hypothesized that the regulation of glucose- and lipid-related genes in skeletal muscle may contribute to metabolic changes in rats with IUGR. In this study, IUGR rats were bred from pregnant rats fed a protein-restricted (PR) diet. Insulin resistance (IR)-related metabolic parameters and the expression of key regulatory IR genes such as peroxisome proliferator-activated receptor γ (PPARγ) coactivator-1α (PGC-1α) and glucose transporter 4 (GLUT4) were measured in skeletal muscle from 18-month-old female IUGR rats. The methylation status of promoters of PGC-1α and GLUT4 were assessed in the same tissues. During the aging process, IUGR rats exhibited catch-up growth and obesity. In old age, they showed impaired glucose tolerance demonstrated by high glucose and insulin AUC (area under the curve) values. The expression of glucose transporter 4 (GLUT4) and PGC-1α in skeletal muscle was significantly reduced in IUGR rats. Mean CpG island methylation in the PGC-1α promoter sequence was significantly increased. These results suggest that a PR diet during gestation may induce epigenetic changes, such as DNA methylation, in the promoters of specific genes. The changes may affect gene expression and account for the metabolic alterations in female rats with IUGR.

Introduction

Human and animal studies have demonstrated a strong association between intrauterine growth retardation (IUGR) and increased incidence of insulin resistance, obesity and type 2 diabetes during adult life (1,2). This association has been conceptualized by a developmental programming hypothesis, which proposes that disease risk begins during fetal life as a result of 'programming' or long-term alterations in gene expression and function resulting from a suboptimal intrauterine milieu (3). Maternal undernutrition or abnormal utero-placental function are capable of limiting availability of substrates to the fetus and may induce secondary adaptations in the metabolism and gene expression that may be beneficial during intrauterine life but contribute to disease risk in later life.

Several IUGR rat models have been established in order to investigate the mechanisms underlying the intrauterine events and eventual adult phenotype. These animal models include maternal protein-restricted (PR) diets (4), maternal semi-nutrient restriction (5), maternal anemia (6), maternal hypoxia (7) and bilateral uterine artery ligation (8) in rats. Among these IUGR models, maternal PR diet is one of the most extensively studied and the outcomes for offspring bear striking similarities to human diabetes, both at the whole body and molecular level (9).

In order to understand the mechanisms responsible for glucose intolerance that develop in later life, several *in vivo* and *in vitro* studies in animals have focused on skeletal muscle as an important target tissue of glucose disposal. These studies suggest that major changes in the genes that regulate glucose metabolism are associated with the development of type 2 diabetes mellitus. These changes have been shown to affect the insulin receptor and its signaling system (10,11), the insulin-responsive glucose uptake and transporter system (12,13) and oxidative phosphorylation and ATP production (14). The roles played by other elements of the signaling system in IUGR rats are more controversial. These include the insulin-responsive glucose transporter 4 (GLUT4) (5,10-12), transcriptional coactivator peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α), which regulates the expression of genes for oxidative phosphorylation and ATP production (15), and the insulin receptor (IR) and insulin receptor substrate 1 (IRS-1), which are involved in insulin receptor signaling.

It has recently been proposed that epigenetic regulation of genes, particularly the methylation of clusters of CpG dinucleotides (islands) in promoter regions of certain genes, may contribute to metabolic reprogramming (16).

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Key words: intrauterine growth retardation, insulin receptor, peroxisome proliferator-activated receptor γ coactivator-1α, methylation

Lillycrop *et al* demonstrated that feeding a PR diet to pregnant rats increased glucocorticoid receptor (GR) and peroxisome proliferator-activated receptor α (PPARα) expression in the liver of the offspring by inducing hypomethylation of respective promoters (17). These findings suggest that an epigenetic mechanism induced by prenatal nutrition may produce an altered phenotype in the offspring.

Given the metabolic phenotype in IUGR humans and the importance of IUGR as a risk factor for type 2 diabetes, we developed an IUGR experimental model using a maternal PR diet during gestation. To avoid the confounding factors of gender and hormones, only 18-month-old female offspring were selected for investigation in the present study. Our objectives were to evaluate the metabolic phenotype and insulin resistance status and to determine expression changes in genes involved in key insulin signaling, glucose metabolism and oxidative phosphorylation in the skeletal muscles. We also investigated DNA methylation of candidate genes that may contribute to metabolic phenotypes in IUGR offspring.

Materials and methods

Animal procedures. Virgin, 7- to 8-week-old Sprague-Dawley (SD) rats weighing 180±20 g were purchased from Shanghai Laboratory Animal Center (Chinese Academy of Science, Shanghai, China). All the animals were housed at 21-23˚C, 65-69% humidity with a 12-h light/dark cycle and had free access to food and tap water. Following 10 days of habituation, female rats were mated overnight with a male and copulation was verified the next morning by the presence of spermatozoa in vaginal smears.

At conception, pregnant dams were housed individually and fed isocaloric diets containing either normal (20%) protein (control) or a PR diet containing 8% protein until delivery. The composition of the diets has been described previously (4). After delivery, each mother rat fed eight pups (any extra pups were removed at random). All mother rats were fed with normal rat chow during the 21-day lactation period. Following weaning, three or four rats from the same group were housed in one cage. In order to avoid gender and hormonal influence, only female offspring were selected.

Ten female pups born from mothers who received the PR diet formed the IUGR group and 10 female pups from mothers fed a normal diet formed the control group. The rats were weighed weekly. All experiments were approved by the Animal Care and Use Committee of Southeast University (Nanjing, China).

Intraperitoneal glucose tolerance test (IGTT). The rats were subjected to an IGTT as described previously (8). Briefly, 18-month-old awake female control and IUGR rats received an intraperitoneal injection of 2 g/kg glucose after fasting for 12 h. Blood was collected from the tail veins 0, 15, 30, 60 and 120 min after glucose administration. The EDTA tubes containing the blood were gently mixed 10 times and centrifuged at 1500 x g for 10 min at 4˚C. The plasma was immediately transferred to a new tube and stored at -20˚C until assay. Plasma glucose was measured using the glucose oxidase method (Sigma Diagnostics, St. Louis, MO, USA). Insulin was quantified using a commercially available enzyme linked immunoabsorbent assay (ELISA) kit (Cayman Chemical Co., Ann Arbor, MI, USA). All measurements were performed in duplicate using a microplate reader (Bio-Tek Instruments, Inc., Winooski, VT, USA).

Skeletal muscle collection. At the end of the experimental period, 18-month-old female rats were sacrificed by decapitation. The gastrocnemius muscles of the right posterior limb were rapidly removed, frozen in liquid nitrogen and stored at -80˚C.

RNA isolation and quantitative real-time PCR. Total RNA was extracted from the skeletal muscles using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. DNA contamination was removed using an Amibion DNA-free kit (Applied Biosystems, Foster City, CA, USA). Aliquots (2 μg) of total RNA were reverse-transcribed using an iScript™ cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) at a final volume of 40 μl according to the manufacturer's instructions. The reaction was terminated by heating for 5 min at 25˚C, for 30 min at 42˚C and for 5 min at 85˚C and quickly cooling on ice.

The expression of IR, IRS-1, GLUT4, PGC-1α and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were assessed simultaneously in individual samples. Quantitative real-time PCR analysis was performed using SYBR-Green Master mix (Bio-Rad) and a CFX96™ Real-Time PCR Detection System instrument (Bio-Rad). The cycling consisted of 2 min at 50˚C and 2 min at 95˚C, followed by 40 cycles of 15 sec at 95˚C and 45 sec at 60˚C. Following completion of the final cycle, a melting curve analysis was performed to monitor the purity of the PCR products. Each sample was analyzed in duplicate.

RNA levels in the IUGR group were calculated relative to the control group, for which values were arbitrary set to 1 to obtain estimates of relative abundance. All primers were synthesized by Shengneng Bicolor Biotech (Shanghai, China) and were designed according to published sequences in GenBank as listed in Table I.

DNA methylation detection. DNA methylation in promoters was detected using bisulphate sequencing PCR (BSP-PCR). Briefly, genomic DNA from rat skeletal muscle was extracted using DNeasy Mini kits (Qiagen) according to the manufacturer's instructions. The genomic DNA (1 μg) was subjected to bisulphate modification using a CpGenome DNA Modification kit (Chemicon, Temecula, CA, USA) according to the manufacturer's instructions. The chemically modified DNA was then used as a template for methylation-specific PCR in 2 target genes (PGC-1α and GLUT4) in skeletal muscle. All primers (Table I) were designed according to the NCBI genome database using Methyl Primer Express v1.0 (ABI) and were synthesized by Shengneng Bicolor Biotech.

The PCR products were separated on 1% agarose gel, and the bands were purified with an agarose gel DNA purification kit (Promega, , Madison, WI, USA). The purified DNA was subcloned onto the pGEM-T Easy Vector (Promega). Positive clones were sequenced using M13 primer from Shanghai Invitrogen Biotech Co., Ltd. (Shanghai, China). The final

Table I. Sequence of DNA oligonucleotide primers used in quantitative real-time RT-PCR (qRT-PCR) and bisulphate sequencing PCR (BSP-PCR) experiments.

Method	Target gene	Forward primer	Reverse primer
qRT-PCR	IR	5'-TTCGAGGAGAGACCTTGGAA-3'	5'-TCGTGAGGTTGTGCTTGTTC-3'
	IRS-1	5'-TGGATGCAAGTGGATGACTC-3'	5'-CGGAGGATTGTTGAGATGGT-3'
	GLUT4	5'-ACAATGTCTTGGCTGTGCTG-3'	5'-TCCCACATACATAGGCACCA-3'
	PGC1α	5'-TCTGGAACTGCAGGCCTAACTC-3'	5'-GCAAGAGGGCTTCAGCTTTG-3'
	GAPDH	5'-CATGACAACTTTGGCATCGT-3'	5'-GGATGCAGGGATGATGTTCT-3'
BSP-PCR	PGC1α	5'-TTAGAGATTTAGGGGTGAAGTAA-3'	5'-CTAATCTTCAAAACCCCAAAAT-3'
	GLUT4	5'-TTTAGGAATTAATGTAGAGAAATG-3'	5'-AATAACTATTTTTAACTCCCAC-3'

Table II. Characteristics at birth in normal and protein restriction diet pregnant rats.

	Control rats (n=7)	PR rats (n=12)	P-value
Litter size	9.57±0.53	9.67±0.43	0.89
Litter gender distribution (M/F)	1.11±0.07	1.15±0.04	0.60
Incidence of IUGR (%)	4.48	66.38	<0.001

The results are expressed as the means ± SEM. P-values of litter size and gender distribution were obtained using two-tailed Student's t-test. Incidences of IUGR are calculated by χ^2 test. The level of significance was set at P<0.05. IUGR, intrauterine growth retardation; PR, protein restriction.

Table III. Body weights of the female rats at different times in the control and IUGR group.

	Body weight (g)		
Age	CON (n=12)	IUGR (n=12)	P-value
At birth	6.65±0.20	4.93±0.16[b]	<0.001
1 week	12.19±0.44	9.67±0.51[b]	<0.01
4 weeks	88.81±4.66	85.72±4.76	0.65
8 weeks	176.72±6.91	189.90±7.96	0.22
12 weeks	214.18±7.94	244.14±8.31[a]	0.02
12 months	232.84±8.05	259.98±7.52[a]	0.02
18 months	234.70±8.15	261.81±9.32[a]	0.04

All data are the means ± SEM. P-values were obtained from the two-tailed Student's t-test. [a]P<0.05 vs. CON and [b]P<0.01 vs. CON. CON, control group; IUGR, intrauterine growth retardation.

sequence results were processed using an online computer program: http://biq-analyzer.bioinf.mpi-sb.mpg.de/ (18).

Statistical analysis. Statistical analyses were performed using SPSS version 15.0 statistical software. The data are presented as the means ± standard error (SEM). The differences between control and IUGR groups were determined by two-tailed Student's t-tests or χ^2 tests. Values of P<0.05 were considered to indicate a statistically significant difference.

Results

Body weight at birth and postnatally. The gestation period of pregnant rats fed both normal protein and PR diet was between 21 and 22 days. There were no significant differences in litter sizes or litter gender distribution (Table II) between normal and PR diet dams. The average birth weight of pups from normal diet pregnant dams was calculated. Pups whose birth weight was below the 10th percentile for the average birth weight were defined as having IUGR. The incidence of IUGR in pregnant rats on the PR diet (66.4%) was significantly higher than that of rats on the normal diet (4.48%, P<0.001) (Table II). These results confirmed that administration of an isocaloric PR diet to gestating rats did not affect fertility and provided a convincing IUGR model (4).

The average body weight at birth and at different periods of postnatal life in control and IUGR female rats is shown in Table III. Birth weights of IUGR rats (4.93±0.16 g) were markedly lower than those of control rats (6.65±0.20 g; P<0.05). At 4 weeks of age, the weights of IUGR rats began to approach those of rats in the control group. At 4-8 weeks of age, the growth of IUGR rats accelerated and surpassed that of control rats. The difference at this time point was not statistically significant. However, at 12 weeks of age, IUGR rats were significantly obese (244.14±8.31 g) compared with control rats (214.18±7.94 g; P<0.05). This difference persisted until the end of the experiment.

Plasma glucose and insulin concentrations. IGTT was performed in 18-month-old female IUGR and control rats to investigate whether older female IUGR rats develop insulin resistance. The analysis revealed that the fasting glucose in the IUGR rats was slightly higher than in control rats, but the difference was not statistically significant (Fig. 1A; P=0.09). However, plasma glucose concentrations at 15, 30 and 60 min were significantly higher in IUGR rats than in control rats (P<0.001 at all three time points) resulting in a significantly higher area under the curve (AUC) (Fig. 1B; P<0.001).

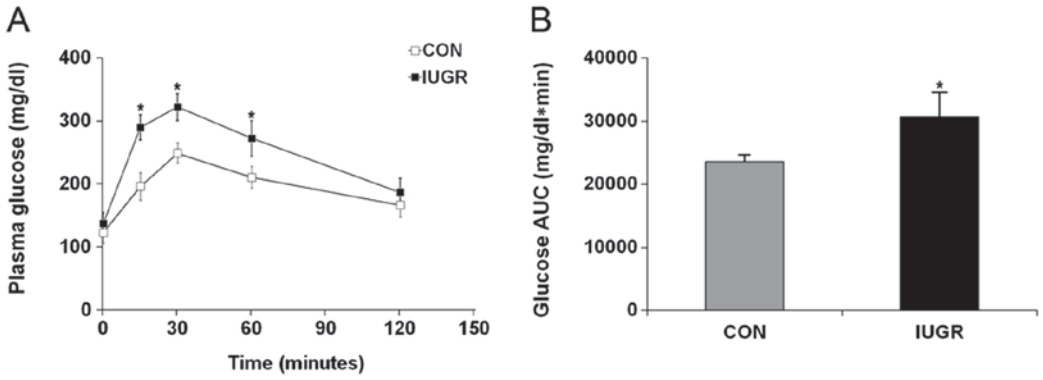

Figure 1. Plasma glucose during intraperitoneal glucose tolerance test (IGTT) performed in 18-month-old female control group (CON) and intrauterine growth retardation (IUGR) rats. (A) Plasma glucose concentrations during IGTT at 15, 30 and 60 min in IUGR rats (black bar) were significantly higher than in CON rats (white bar). (B) Plasma glucose area under the curve (AUC) during IGTT was significantly lower in CON rats (gray bar) than in IUGR rats (black bar). All results are expressed as the means ± SEM (*P<0.05, n=6).

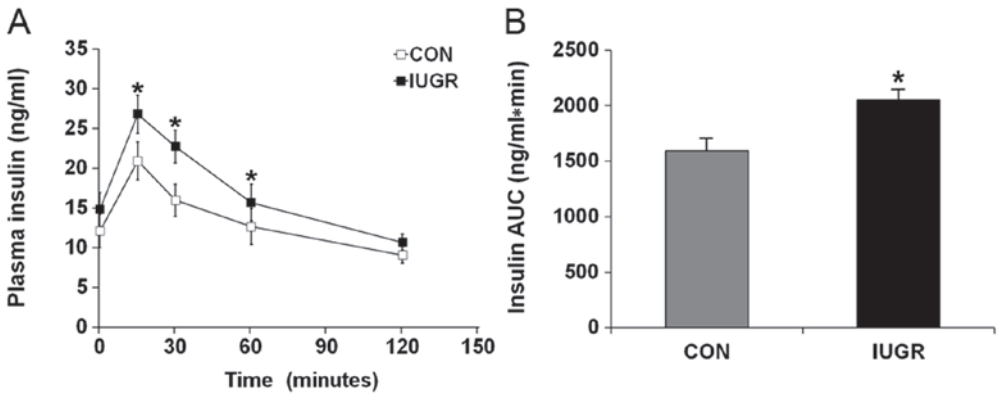

Figure 2. Plasma insulin during intraperitoneal glucose tolerance test (IGTT) performed in 18-month-old female control group (CON) and intrauterine growth retardation (IUGR) rats. (A) Plasma insulin concentrations during IGTT at 15, 30 and 60 min in IUGR rats (black bar) was significantly higher than those in CON rats (white bar). (B) Plasma insulin area under the curve (AUC) during IGTT was significantly lower in CON rats (grey bar) than in IUGR rats (black bar). All results are expressed as the means ± SEM (*P<0.05, n=6).

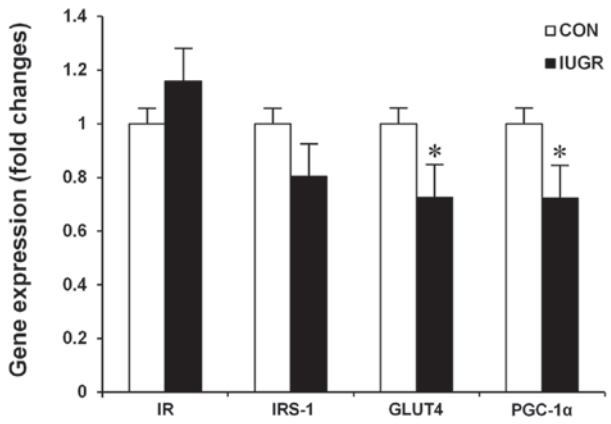

Figure 3. Relative abundance of gene mRNA assessed in skeletal muscle from 18-month-old female control group (CON) and intrauterine growth retardation (IUGR) rats.. IR, IRS-1, GLUT4 and PGC-1α mRNA expression were measured by quantitative real-time PCR. GAPDH was used as a reference gene and the $2^{-\Delta\Delta Ct}$ method was used to analyze gene expression. IUGR results were expressed as fold of change as compared to CON (CON set at unity). The mRNA expression of GLUT4 and PGC-1α were significantly lower in IUGR rats (black bar) than those in CON rats (white bar). All values are presented as the means ± SEM (*P<0.05, n=10).

Hyperglycemia during IGTT in the IUGR group was associated with a significant increase in the insulin response. Plasma insulin concentrations in the IUGR group 15, 30 and 60 min after the glucose challenge were significantly higher than in the control group (Fig. 2A; P<0.001). This resulted in a higher insulin AUC than in the control group (Fig. 2B; P<0.001).

Quantitative real-time PCR. In comparison with age-matched control rats, 18-month-old IUGR rats exhibited a significant decrease in expression of GLUT4 (P=0.0308) and PGC-1α mRNA (P=0.0416) (Fig. 3). No statistically significant between-group differences were found for IR (P=0.2589) or IRS-1 (P=0.2265) genes.

DNA methylation. As shown in Fig. 4, the 11 CpG sites in the examined promoter sequence of GLUT4 were rarely methylated but did include some sporadic methylated sites. Despite this, methylation of the PGC-1α gene was significantly higher in the IUGR group (average of 16.18% of all the 17 CpG sites) than in the control group (9.31%; P<0.05) (Fig. 5).

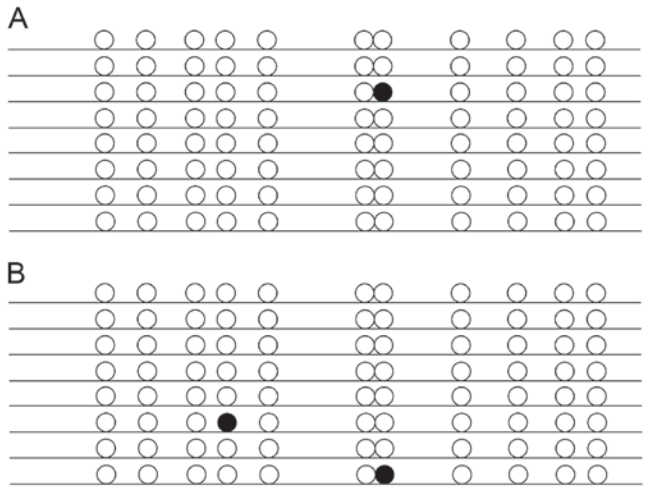

Figure 4. Methylation profiles in the promoter region of glucose transporter 4 (GLUT4). (A) Methylation patterns of CpG islands in 8 positive clones of the CON group. (B) Methylation patterns of CpG islands in 8 positive clones of the IUGR group. Each line represents a separate clone. A filled (black) circle indicates a methylated CpG, whereas open circles indicate an unmethylated CpG. All the 11 CpG sites in the two groups were sparsely methylated. CON, control group; IUGR, intrauterine growth retardation.

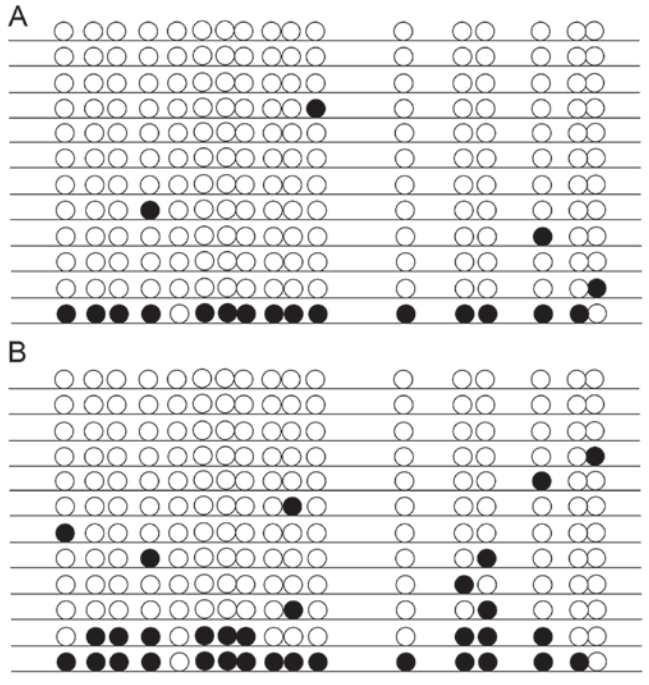

Figure 5. Methylation profiles in the promoter region of peroxisome proliferators-activated receptor γ coactivator-1α (PGC-1α). (A) Methylation patterns of CpG islands in 12 positive clones of the CON group. (B) Methylation patterns of CpG islands in 12 positive clones of the IUGR group. Each line represents a separate clone. A filled (black) circle indicates a methylated CpG, whereas an open circle indicates an unmethylated CpG. Differences of methylation rate in 17 CpG sites between the IUGR group and CON group were statistically significant ($P<0.05$). CON, control group; IUGR, intrauterine growth retardation.

Discussion

The nutritional environment at the fetal and neonatal stages has been suggested to be a critical factor in development (1). In the current study, we focused primarily on the female offspring in order to avoid gender and hormone effects on metabolism. Our data showed that a maternal PR diet during gestation had a profound and long-term impact on the offspring. The offspring initially exhibited low birth weight and IUGR but went on to develop obesity and peripheral insulin resistance in older age. We also showed that GLUT4 and PGC-1α mRNA expression were reduced in skeletal muscles from the older female IUGR rats, and demonstrated that epigenetic mechanisms are likely to be operative in the pathogenesis of insulin resistance and metabolic phenotype, since DNA methylation of the PGC-1α promoter was found to be increased.

Data from IUGR animal models support the opinion that poor fetal growth has permanent consequences in adulthood. Birth weights of IUGR induced by PR diet (19) and bilateral uterine artery ligation (8) during gestation have been reported by others to be significantly lower than those of controls. Our finding that administration of a PR diet during pregnancy also interfered with the general growth of the pups, initially resulting in a lower birth weight but subsequently resulting in obesity, also supports the previously reported findings (8,19). We also found that IUGR rats exhibited peripheral insulin resistance and displayed hyperglycemia and hyperinsulinemia during IGTT. Such observations suggest that animals with IUGR secrete more insulin than control rats, but are unable to sustain normal glycemia. This finding agrees with earlier studies in malnourished animals during gestation (20). The findings from our animal model support the hypothesis that intrauterine protein restriction results in a phenotype that mirrors the epidemiological association between low birth weight and subsequent development of impaired glucose tolerance and type 2 diabetes in humans (21).

Skeletal muscle is the major tissue presenting insulin-responsive glucose uptake. In our study, hyperinsulinemia was associated with a decrease in skeletal muscle GLUT4 expression. Previous *in vitro* investigations have demonstrated variable results regarding IUGR-induced changes in skeletal muscle GLUT4 mRNA expression. Different rat models of IUGR adult offspring have also shown conflicting results. No change in expression was reported with a utero-placental insufficiency model (22), whereas a total calorie restriction model resulted in a significant decrease in expression (23). Our investigation using PR diet-induced IUGR also demonstrated significantly decreased skeletal muscle GLUT4 mRNA concentration in mature animals. This observation is consistent with the decline in total GLUT4 concentration reported previously in IUGR (24) and replicated in the young adult IUGR human skeletal muscle (25). Other investigators have demonstrated that insulin resistance is associated with an impaired regulation of insulin-induced GLUT4 gene expression in skeletal muscle and adipose tissue in human IUGR subjects (26).

Transcriptional coactivator PGC-1α is a key metabolic factor regulating the expression of genes for oxidative phosphorylation in several tissues including skeletal muscle, liver and adipose tissue and is an important factor in the development of type 2 diabetes (15). Previous studies suggest that reduced expression of PGC-1α in the islets (27) and skeletal muscle (28) is associated with insulin resistance in patients with type 2 diabetes. However, the level of PGC-1α in skeletal

muscles from mature IUGR offspring has been unknown to date. Our results indicate that the expression of the PGC-1α gene is reduced in skeletal muscle from 18-month-old female IUGR offspring. However, we found no difference in IR or IRS-1 gene expression between the IUGR and control groups. The lack of statistical significance in IR and IRS-1 mRNA expression may suggest that the molecular defect lies downstream of the insulin receptor. Together, these data suggest that whole body glucose intolerance in our model may be due to dysregulation of GLUT4 and PGC-1α expression.

There is growing evidence that gene promoter-specific DNA methylation changes (17) are involved in nutritional aberrations. Since decreased GLUT4 and PGC-1α gene expression both progress in old age, we hypothesized that such reductions may be mediated in part by altered DNA methylation. However, our study did not find any changes in DNA methylation in the GLUT4 promoter. In other studies, genes such as the insulin-like growth factor 2 were shown to be differentially methylated in regions far upstream of the entire gene and were found to modify downstream gene transcription (29). Whether a similar situation exists in the case of GLUT4 expression cannot be ruled out by our studies, as we primarily focused on the gene promoter region.

Other workers have reported that histone code modifications repress skeletal muscle GLUT4 transcription in the postnatal period and that these changes persist in adult female IUGR offspring (23). Upstream of the GLUT4 promoter, there are several binding sites for various transcriptional factors that could potentially regulate GLUT4 expression under different situations (30). Thus, we speculate that reduced expression of GLUT4 in skeletal muscle from 18-month-old female IUGR rats may be due to altered methylation of other genomic region(s), altered histone modification, or changes in binding/expression of other transcription factors regulating GLUT4.

It has also been reported that DNA methylation of PGC-1α increased in human diabetic islets from T2D patients (27) and the umbilical cord of newborns from mothers with high pregestational BMI (31). Furthermore, PGC-1α promoters were found to be methylated to a higher extent in skeletal muscle biopsies from young and lean low birth weight (LBW) offspring compared with normal birth weight (NBW) subjects subjected to an isocaloric control diet (32). Our finding that 18-month-old female IUGR rats exhibited increased DNA methylation of PGC-1α in muscle tissues is in accordance with these reports, and suggests that IUGR may be involved in the reduced PGC-1α gene expression and subsequently in the development of insulin resistance in type 2 diabetes. It should be noted that the corresponding methylation pattern of the genes examined in our study was only undertaken at 18 months. However, Lillycrop *et al* (33) reported that the pattern of methylation in the hepatic PPARα promoter induced by maternal PR may persist into adulthood. Whether methylation changes of the PGC-1α promoter in skeletal muscle exhibit the same trend requires further investigation.

In conclusion, we have shown that a PR diet during pregnancy leads to epigenetic modulation of PGC-1α in the skeletal muscles of 18-month-old female offspring, which may be associated with downregulation of PGC-1α transcription. Perturbations in PGC-1α and GLUT4 expression in skeletal muscle may contribute to the insulin resistance in offspring with IUGR. These findings provide novel insights into the molecular mechanisms of skeletal dysfunction, indicating that transcription regulation of oxidative phosphorylation by PGC-1α may be involved in the pathological process of IUGR through epigenetic factors such as DNA methylation. This hypothesis requires confirmation by further elucidation of the signaling pathways leading to DNA methylation of PGC-1α and other potential genes.

Acknowledgements

This study was supported by a grant from the program (ZKX09013) of Nanjing Medical Science and Technique Development Foundation.

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