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Protein restriction during gestation alters histone modifications at the glucose transporter 4 (GLUT4) promoter region and induces GLUT4 expression in skeletal muscle of female rat offspring☆

Shasha Zheng, Michelle Rollet, Yuan-Xiang Pan*

Department of Food Science and Human Nutrition, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA

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Abstract

Maternal nutrition during pregnancy is an intrauterine factor that results in alteration of the offspring genome and associates with disease risk in the offspring. We investigated the impact of a maternal low-protein (LP) diet on the expression of glucose transporter 4 (GLUT4) in offspring skeletal muscle. GLUT4 is an insulin-regulated glucose transporter involved in insulin sensitivity and carbohydrate metabolism in muscle cells. We observed sex-dependent GLUT4 mRNA expression and increased GLUT4 protein content in female pup skeletal muscle with maternal LP. Analysis of transcriptional and epigenetic regulation of increased skeletal muscle GLUT4 expression in offspring rats revealed the regulatory mechanisms involved. The protein level of myocyte enhancer factor 2A (MEF2A), which has been known as an activator of GLUT4 transcription via the ability to carry out specific binding to the GLUT4 MEF2 binding sequence, increased in female pups whose mothers were fed a LP diet. Modifications of chromatin structure, including acetylated histone H3, acetylated histone H4 and di-methylated histone H3 at lysine 4, were detected at a significantly increased level at the GLUT4 promoter region in female pup muscle following a maternal LP diet. Glycogen content was also detected as up-regulated, accompanied by increased glycogen synthase in LP female offspring muscle. These results document that maternal protein restriction during pregnancy induces GLUT4 expression in female offspring skeletal muscle but not in males, which may indicate sex-dependent adaptation of glucose metabolism to a maternal LP diet.

Keywords: Gestational nutrition; Epigenetics; Developmental programming; Thrifty phenotype hypothesis

1. Introduction

Maternal nutrition deviation in pre- and postnatal periods is an important determinant of disease risk in adult life [1]. Nutrition restriction during fetal development can result in low birth weight, which has long-term consequences in postnatal life, including predisposition of offspring to obesity and type II diabetes [2]. About 13% of US births involve teen mothers, who are less likely to gain adequate weight during pregnancy, leading to low birth weight (CDC National Vital Statistics Report; 2009. Vol. 57, No. 7). Through the use of animal models, nutritional deficiency research has demonstrated the development of insulin resistance in the skeletal muscle of offspring that suffered from intrauterine growth restriction (IUGR) established by dietary calorie restriction [3]. Epidemiology and experimental studies have indicated that a maternal low-protein (LP) diet during fetal and neonatal development in both humans and experimental animals impacts an offspring's susceptibility to later development of altered carbohydrate metabolism [4].

In a recent study, a maternal LP diet during gestation and lactation affects postnatal growth, appetite, triglyceride and cholesterol concentrations, as well as insulin resistance in male but not in female offspring [5]. However, the molecular mechanism underlying the sex-differentiated carbohydrate metabolism and insulin sensitivity is rarely explained. The predominant adaptive mechanism could be aberrant glucose uptake into insulin-sensitive tissue, either skeletal muscle or adipose tissue [6]. The major insulin responsive protein involved in glucose absorption is glucose transporter 4 (GLUT4). Insulin controls glucose homeostasis by regulating the translocation of GLUT4 from the intracellular pool to the plasma membrane [7]. The disruption of GLUT4 results in glucose intolerance and insulin resistance. Macronutrient content and composition of the diet have proven to influence the expression of the glucose transporter genes and insulin sensitivity. A recent study examined mechanisms involved in the reduction of skeletal muscle GLUT4 mRNA in IUGR female rat offspring from dams that received 50% daily food intake during pregnancy and lactation [3]. In contrast, Charron and Kahn [8] found that calorie restriction in fasting adult rats down-regulates

Abbreviations: AAR, amino acid response; ATF, activating transcription factor; C/EBP, CCAAT/enhancer-binding protein; ChIP, chromatin immunoprecipitation; GLUT4, glucose transporter 4; IUGR, intrauterine growth restriction; LP, low protein; Pol II, RNA polymerase II.

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* Corresponding author. Tel.: +1 217 333 3466; fax: +1 217 265 0925.
E-mail address: yxpan@illinois.edu (Y.-X. Pan).

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GLUT4 expression in adipose cells but up-regulates it in skeletal muscle. Tissue-specific regulation of glucose transporter expression was proposed to be an adaptive response to altered nutrient availability [9], although the molecular mechanism is less understood. Moreover, associations of maternal nutrition restriction with gene expression in the offspring of carbohydrate metabolism-related genes create more complications.

There is growing evidence that maternal nutritional status can alter the epigenetic state of the offspring. The insulin resistance phenomena in IUGR female offspring has been shown to be transgenerationally transmitted [10]. Histone modifications at the GLUT4 promoter have been observed, which result in the transcriptional repression in female offspring when adult pregnant rats were exposed to a low calorie diet [3]. Therefore, the present study investigated the consequence of maternal protein restriction during teenage pregnancy on GLUT4 transcriptional regulation and epigenetic modifications in offspring skeletal muscle, particularly histone modifications and DNA methylation. Raychaudhuri et al. [3] showed that, in female IUGR pups, skeletal muscle GLUT4 transcription was repressed, which is accompanied by de-acetylation and di-methylation of specific amino-acid residues in the N-tail of histone H3. Such histone modifications contribute to co-repressor complex formation and destruction of co-activator complex, decreasing GLUT4 transcription at both birth and through later life, which increases the offspring's risk for pre-diabetes [3]. In contrast, the present data demonstrate that maternal LP intake (but not calorie restriction) up-regulates GLUT4 gene expression through chromatin modification programming specifically in females and alters glycogen content in offspring skeletal muscle.

2. Methods and materials

2.1. Animals and treatments

The animal protocol for this study was approved by the International Animal Care and Use Committee (IACUC) at the University of Illinois. Timed-pregnant Sprague-Dawley rats (Charles River Laboratories) were obtained on Day 2 of gestation and weight matched into one of two isocaloric diets, control (180 g/kg casein) or LP diet (LP, 90 g/kg casein), which were modified according to a well-established diet formula [11] throughout gestation (Table 1). Each group contained four dams. Both groups had free access to rat chow and drinking water. Food intake was daily monitored by recording food left over. Animals were individually housed in standard polycarbonate cages with corncob bedding and maintained in a humidity- and temperature-controlled colony room on a 12-h light–dark cycle. Twenty-four hours after newborn, six pups (three female and three male) were selected and used in per litter to minimize variation in pups' nutrition status during suckling. Pups body weight was measured once a week after birth. Both groups of mothers were switched to standard diet through lactation [11]. At Day 24, in both experimental groups, the pups were weaned from the mother to a standard rat chow. To get enough sizes of tissues for sample analysis, pups were sacrificed when they were 38 days old. The gastrocnemius muscle of the right hind leg, visceral adipose tissue (mesenteric) and liver were then collected, snap-frozen in liquid nitrogen and stored at −70°C.

2.2. RNA isolation and cDNA synthesis

Frozen skeletal muscle samples were ground in a mortar and pestle with liquid nitrogen prior to total RNA isolation using TRI reagent (Sigma, St. Louis, MO, USA).

Table 1
Offspring body weight

Age (days)	Body weight (g)		P
	C (4)	LP (4)	
Birth (Day 0)	6.32±0.19	5.92±0.09	.11
7	16.55±0.69	14.89±0.46	.10
15	35.06±1.14	30.73±0.70	.02
22	59.37±1.40	49.98±1.63	.006
35	138.56±2.58	125.21±3.54	.006

Number of litters shown in parentheses. Body weight is shown as the average value at the specific day. Data are means±S.E.M. Significance testing was set at $P<.05$ derived from two-tailed t test.

Following isopropanol (Fisher Scientific, Fair Lawn, NJ, USA) precipitation, RNA was re-suspended and quantified by spectrophotometry (BIO-RAD Smart Spec Plus, Hercules, CA, USA) at A260/A280. High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) was used for reverse transcription of 2 μg of total RNA. All samples that were to be compared were reverse transcribed from the same master reaction mixture at the same time with a 'no RNA template' tube as negative control. The whole procedure was performed in a DNA 2720 Thermal Cycler (Applied Biosystems). The samples at the 20-μl reaction scale were heated at 37°C for 2 h for reverse transcription procedure following 85°C for 5 s to inactive reverse transcriptase and terminate the reaction. The final 20 μl of cDNA was diluted up to 400 μl by nuclease free water and stored at −20°C.

2.3. Real-time quantitative RT-PCR (qPCR)

To measure the relative amount of mRNA, qPCR was performed with a 96-well plate in a 7300 Real-Time PCR System (Applied Biosystems). The reactions were activated at 95°C for 10 min followed by 35 cycles of 95°C for 15 s and 60°C for 1 min. The mRNA level of ribosomal protein L7a was measured at the same time as the internal control. After PCR, a dissociation curve was generated by stepwise increase of the temperature from 55°C to 95°C to ensure that a unique product was amplified in the reaction. Primer efficiency was determined by a standard curve from a dilution series. Primers used for PCR are shown in Table 2.

2.4. Protein isolation and Western blotting

Twenty-five milligrams of frozen muscle sample was ground in liquid nitrogen and put into a 500-μl protein sample buffer [0.125 M Tris-HCl (pH 6.8), 5% 2-mercaptoethanol, 1% SDS, 20% glycerol, 0.4% bromophenol blue, protease inhibitor]. Protein was sonicated (Fisher Scientific model 100 Sonic Dismembrator, Pittsburgh, PA, USA) on ice with 25 pulses at power setting 2. Lowry assay was used to determine protein content. Briefly, 1 ml ice-cold TCA was used to precipitate protein from 10 μl of sample buffer, and the supernatant was completely pouring out followed by 30 min speed dry. One hundred microliters of 0.2% SDS/0.2 M NaOH was used to resolve protein pellet; 650 μl of Lowry copper reagent and 60 μl of 1 M Folin-Ciocalteu Reagent were then added for absorbance test. Samples containing 20 μg of protein were resolved by SDS-PAGE. After electrotransfer to a polyvinylidene fluoride membrane (0.2 μm, Bio-Rad) using a wet transfer protocol, 5% milk in TBS/T [30 mM Tris base (pH 7.6), 200 mM NaCl and 0.1% Tween 20] was used to block the membrane for 1 h at room temperature. The membrane was then incubated at 4°C overnight with a 1:1000 dilution in 5% BSA of rabbit polyclonal antibody against GLUT4 or glycogen synthase, or a 1:500 dilution in 5% BSA for antibody against MEF2 (Table 3). The next day, the membrane was washed five times for 5 min with TBS/T on a shaker and then incubated with peroxidase-conjugated goat anti-rabbit secondary antibody (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) in 1% milk at a 1:10,000 dilution for 1 h at room temperature. The membrane was then washed five times for 5 min each wash. The bound secondary antibody was detected using a SuperSignal West Dura Extended Duration Substrate kit (Thermo Scientific, Rockford, IL, USA), and Western blot images were captured and analyzed by a Chemi Doc system (Bio-Rad). An anti-actin antibody was used as an internal control to show the equality of protein levels loaded.

2.5. Glycogen content assay

Glycogen content in female offspring muscle was tested by using a Glycogen Assay Kit (BioVision #K646-100, Mountain View, CA, USA). Frozen muscle tissue (8 mg) was ground in liquid nitrogen and homogenized with 200 μl dH_2O on ice. The homogenates were boiled for 5 min to inactivate the enzymes. The boiled samples were spun at 13,000 rpm for 5 min to remove insoluble material. Hydrolysis enzyme mix was added into the supernatant and the standard that comes with the kit, and then the samples were incubated for 30 min at room temperature. Development reaction mix (50 μl) was added to each well containing glycogen standard or samples. The mixtures were incubated at room temperature for another 30 min, protected from light. The samples and glycogen standard were measured with the colorimetrical OD value at 570 nm. The sample's readings were applied to the standard curve to get the amount of glycogen.

2.6. ChIP-qPCR

Chromatin immunoprecipitation (ChIP) analysis was performed according to a modified protocol [12]. Briefly, 200 mg of frozen skeletal muscle samples was ground in liquid nitrogen and resuspended in PBS and cross-linked in 1% formaldehyde for 10 min at room temperature. The tissue pellet was re-suspended in nuclei swelling buffer [5 mM Pipes (NaOH; pH 8.0), 85 mM KCl, 0.5 % NP40] containing protease inhibitor. The separated nuclei were lysed in SDS lysis buffer [50 M Tris-HCl (pH 8.1), 10 mM EDTA, 1% SDS] containing protease inhibitors. The resultant chromatin was sonicated (Fisher Scientific model 100 Sonic Dismembrator) on ice with six bursts for 40 s at power setting 5, with 2-min cooling interval between each burst. The average length of sonicated chromatin was determined by resolving on a 1.6% agarose gel and found to be around 500 bp. The sample was then centrifuged at 13,000 rpm for 10 min at 4°C to remove cell debris from the crude chromatin lysate. One milliliter of sheared chromatin was diluted in ChIP dilution buffer to total 10 ml. Ten percent of the diluted lysate was

Table 2
Primer sequence used in qPCR and ChIP assay

Gene (ensemble ID)	Forward primer (5′→3′)	Reversed primer (5′→3′)	Purpose
GLUT4 (ENSRNOG00000017226)	CCATAGGAGCTGGTGTGGTCAATAC	TCGCCCAGCTCGCTCTACTAAG	mRNA expression
ATF3 (ENSRNOG00000003745)	GGTGTCTGCACTGTGGGATGTTAAC	GCTGAAATACTCTGGACCGCATCTC	
ATF4 (ENSRNOG00000017801)	GTTCCCCAGGGTTTCTGTCTTC	CAACCTCACTTCCCAGCTCTAAAC	
C/EBPβ (ENSRNOG00000025114)	AGAACGAGCGGCTGCAGAAGA	GAACAAGTTCCGCAGCGTGC	
L7a (ENSRNOG00000005071)	GAGGCCAAAAAGGTGGTCAATCC	CCTGCCCAATGCCGAAGTTCT	
GYS1 (ENSRNOG00000020812)	TTGGGACACCTGCAACATC	ACAGGACGGCATCATTGG	
ATF3 (ENSRNOG00000003745)	GGCCAGTTCTCCCTGGAAGCTAT	CCCGCTTAACTCCTGGTTACCAAT	Gene promoter
GLUT4 (ENSRNOG00000017226)	CTTCAGCTCTCCGCATCTTTCC	CACTGCCACAACCCAAGGAAC	Gene promoter
GLUT4 (ENSRNOG00000017226)	CTTCAGCTCTCCGCATCTTTCC	CACTGCCACAACCCAAGGAAC	Histone modification
GLUT4 (ENSRNOG00000017226)	GGGTGCCTTGGGAACACTCAAC	CAACACCTGGGCAACCAGAATG	Transcription rate

subsequently incubated overnight on a hematology mixer (346, Fisher Scientific) with 2 μg of primary antibodies at 4°C (see Table 3 for antibody information). Pre-blocked salmon sperm DNA/protein A agarose beads (60 μl, 50% slurry; Upstate Biotechnology, Lake Placid, NY, USA) were then added to the chromatin for 2 h, followed by centrifugation at 2000 rpm for 1 min at 4°C. Supernatant of normal rabbit IgG was saved as the input control to reflect the total chromatin content for each sample. Chromatin pull down with antibodies for each sample was normalized as a ratio to its total chromatin content after real-time qPCR analysis. The pellets containing immunoprecipitated complexes were washed sequentially with 1 ml of low salt solution [0.1% SDS, 1% triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.0), 150 mM NaCl], high salt solution [0.1% SDS, 1% triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.0), 500 mM NaCl] and LiCl solution [0.25 M LiCl, 1% NP40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl (pH 8.0)] and TE (pH 8.0), twice. Antibody/protein/DNA complexes were eluted from Protein A agarose beads by twice adding 250 μl of the elution buffer (50 mM $NaHCO_3$ and 1% SDS) followed by shaking at 37°C at 300 rpm for 15 min and a flash spin down at room temperature. The combined supernatants were incubated at 65°C for 4–5 h after addition of 20 μl of 5 M NaCl and 1 μg of RNase A to reverse the formaldehyde cross-linking and release the DNA fragments. Samples were then treated with proteinase K at 37°C for 1 h to remove protein and purified with a Wizard SV Gel and PCR Clean-Up System (Promega).

Quantitative real-time PCR was then performed to study modified histones and transcription factor binding to DNA. Enrichment of ChIP-ed DNA, which was pulled down by specific antibodies, then reflected the binding status of protein of interest within the GLUT4 promoter. Five percent of immunoprecipitated DNA was used for each real-time PCR reaction. The standards and the ChIP-ed DNA samples were simultaneously amplified using the same reaction master mixture at 25 μl of scale. Real-time qPCR data were then optimized for the amount of input material (total protein) and expressed as the ratio to input. Primers used to amplify genomic sequences at the promoter region of GLUT4 are shown in Table 2. Normal rabbit IgG antibody was used as the negative control, which indicates non-specific binding. Other antibodies were determined to be 'no binding' if their level was equal to or less than IgG (ratio to input). All antibodies were from Upstate, except C/EBPβ (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Table 3
Antibodies used in Western blot and ChIP assay

Antibody	Introduction and company	Purpose
GLUT4	Cat no. 2299, Cell Signaling Technology (Beverly, MA, USA)	Protein analysis by Western blot
Glycogen synthase	Cat no. 3893, Cell Signaling Technology	
MEF2	Cat no. sc-313, Santa Cruz Biotechnology	
C/EBPβ	sc-150, Santa Cruz Biotechnology	C/EBPβ binding at gene promoter
H3Ac	Anti-acetylated histone 3 at lysine 9, 14 residues; 06-599, Upstate	Analysis of histone modifications
H4Ac	Anti-acetylated histone 3 at lysine 5, 8, 12 and 16 residues; 06-866, Upstate	
H3K4Me2	Anti-di-methylated histone 3 at lysine 4 residues; 07-030, Upstate	
H3K9Me3	Anti-tri-methylated histone 3 at lysine 9 residues; cs200604, Upstate	
IgG	sc-2027, Santa Cruz Biotechnology	
Pol II	sc-899, Santa Cruz Biotechnology	Analysis of RNA polymerase II

H3Ac: Acetylated histone H3; H4Ac: acetylated histone H4; H3K4Me2: di-methylated histone H3 at lysine 4 residues; H3K9Me3: tri-methylated histone H3 at lysine 9 residues.

2.7. Transcriptional activity

To measure the transcriptional activity from the GLUT4 gene, the method of Sandoval et al. [13] was used, which relies on ChIP analysis to examine RNA polymerase II (Pol II) binding at the coding region distal to the promoter. For this purpose, primers were designed within the protein coding region of the GLUT4 gene (Table 2). qPCR analysis was then performed using a 7300 real-time PCR system (Applied Biosystems), and the product was detected with SYBR Green. Serial dilutions of input chromatin were used to generate a standard curve, and the results were expressed as the ratio to input DNA.

2.8. Statistical analysis

Results are calculated as mean±S.E.M. Comparisons of mRNA/protein expressions and histone modification between control and treated groups were performed by two-tailed t test. All of the data in the LP groups were expressed as the ratio to control. Significance testing was set at the $P<.05$ level.

3. Results

3.1. Birth and body weight of offspring

At birth, the control pups weighed 6.32±0.19 g, while the LP pups weighed slightly less, 5.92±0.09 g. As the pups grew to 15 days, average body weight was significantly lower in the LP pups than in the control ($P=.02$). Table 1 compares the average of body weight at birth and at Days 7, 15, 22 and 35 of postnatal life, showing that the average weights of the LP offspring were reduced by 12% at Day 15, 16% at Day 22 and 13% at Day 35 ($P<.05$) as compared to the control pups. There were no differences in the daily food intake and the body weight of the dams during pregnancy (not shown). Litter size and litter sex distribution did not differ between the control and LP mothers (not shown).

3.2. GLUT4 mRNA expression followed sex-dependent manner in offspring skeletal muscle under maternal LP intake

GLUT4 mRNA expression has been reported to be decreased in female skeletal muscle by semi-restricted maternal food intake [3]. However, whether regulation of GLUT4 gene expression by maternal protein restriction is sex-dependent in skeletal muscle has not yet been reported. We investigated GLUT4 mRNA levels in 38-day-old female and male offspring skeletal muscle and adipose tissue by qPCR (Fig. 1A). In female pups, a 1.59-fold increase ($P=.001$) in GLUT4 mRNA content in skeletal muscle was observed in pups from litters of mothers with a restricted protein diet when compared to those of control mothers. However, in male offspring skeletal muscle, there was no change of GLUT4 mRNA level related to maternal LP. Also, in female offspring adipose tissue, a 1.45-fold increase ($P=.03$) of GLUT4 mRNA was detected by maternal protein restriction, but it did not alter in male offspring. Since the amount of visceral adipose in a 38-day-old rat is not enough to do all of the assays involved in this study, we focused on skeletal muscle to examine the molecular mechanisms of GLUT4 regulation by maternal protein restriction.

3.3. The effect of maternal LP on the transcription rate of GLUT4

To confirm the real transcriptional activity for the GLUT4 gene, ChIP DNA samples were used to monitor RNA polymerase II binding within the coding region. This analysis showed that the transcription rate was increased approximately 2.89-fold in female pup muscle as a result of the maternal LP diet (Fig. 1B). Therefore, the increased transcription appears to account for most of the induction in GLUT4 mRNA following maternal LP diet.

3.4. Maternal LP alters GLUT4 protein level in female offspring

To detect GLUT4 protein content in pup skeletal muscle, protein samples were subjected to Western blot analysis using an anti-GLUT4 antibody. In all muscle samples, GLUT4 was detected as one major band with a molecular mass of about 45 kDa (Fig. 2). The ratio of GLUT4 protein level between female control and LP groups was determined to be 1:1.78 (Fig. 2A; $P=.008$). A significantly higher GLUT4 protein content in the skeletal muscle of female LP pups was consistent with the trend observed in its mRNA expression. Moreover, GLUT4 protein levels in male pup muscle were unchanged, which also corresponds to the unchanged male offspring mRNA expression levels (Fig. 2B).

3.5. Glycogen synthase expression in rat offspring muscle

Glycogen synthase is an enzyme that incorporates glucose molecules into the glycogen particle. Recent studies suggested that glucose transport is the rate-limiting step for muscle glycogen synthesis [14]. Therefore, the expression level of glycogen synthase was investigated in the offspring of maternal LP and control diets. Glycogen synthase levels were enhanced in the muscles of female offspring in the LP group, with mRNA and protein levels increasing by 1.29- and 7.56-fold, respectively (Fig. 3A and B). In male pups, no significant variation was observed. Since glucose-6-phosphate is the pre-substrate used for glycogen synthesis and is converted from glucose by the enzyme hexokinase, the hexokinase expression level was checked as well (not shown). However, there was no difference in hexokinase expression between LP and control groups in female offspring.

3.6. Glycogen content increased in female offspring muscle by maternal protein restriction

Skeletal muscle is the main tissue for glycogen storage and contains two to four times more glycogen than the liver. The amount of glycogen was tested using a Glycogen Assay Kit. The results show that the glycogen content in female offspring skeletal muscle was increased by 35% (Fig. 3C; $P=.018$) in the maternal LP group. Thus, the potential physiological impact was demonstrated as corresponding to the altered GLUT4 expression in the offspring muscle.

3.7. No interaction of C/EBPβ protein at the GLUT4 gene promoter

According to the data that we previously published, amino acid response (AAR) pathway was not activated in female offspring muscle by this maternal protein-restricted animal model [15]. But as a cofactor for ATF3 and ATF4 in the AAR pathway, we did observe significantly increased expression of C/EBPβ mRNA in

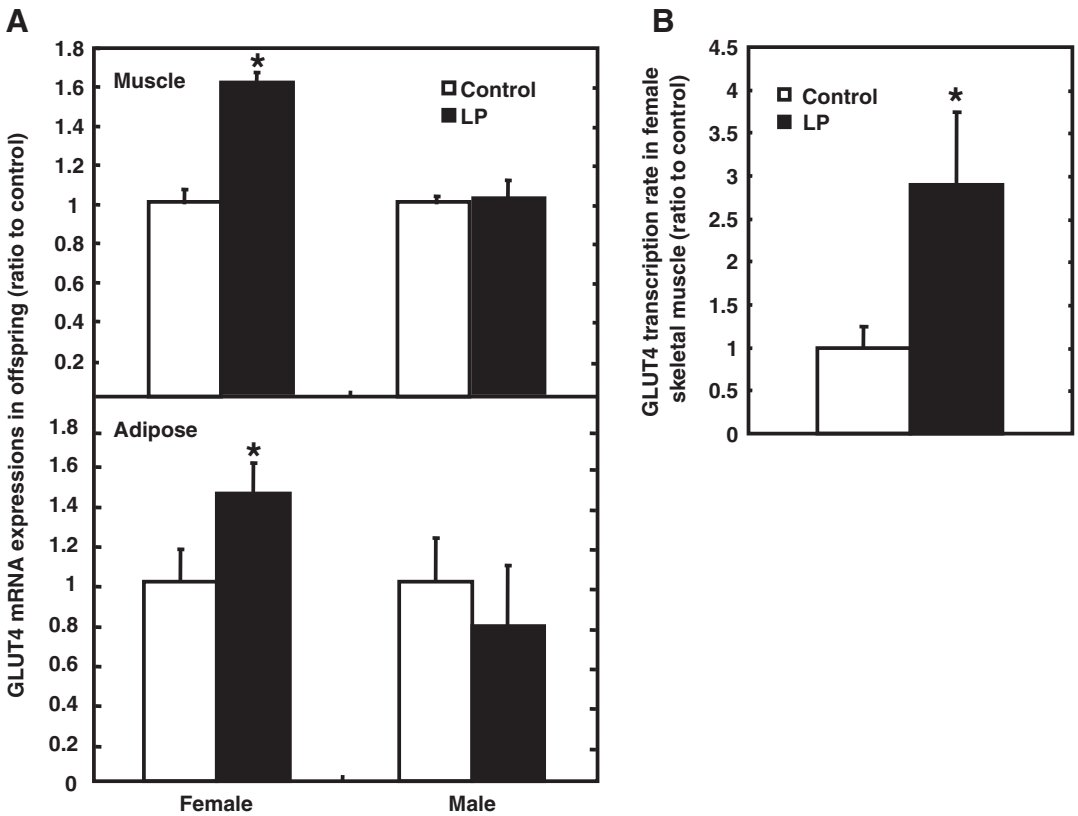

Fig. 1. GLUT4 mRNA expression and transcription rate. (A) Expression of GLUT4 mRNA in offspring skeletal muscle and adipose tissue from LP mothers vs. control ($n=5$). mRNA levels are expressed as mean ratio to control after normalization by L7a. (B) The transcription rate of the GLUT4 gene was analyzed by testing the Pol II binding at the coding region. Data shown as a ratio to the input DNA. The values represent the mean±S.E.M., *$P<.05$.

female offspring muscle from the maternal LP group. To identify whether or not the increased C/EBPβ expression could contribute to the altered GLUT4 transcription in female offspring muscle, because C/EBPβ also acts as a mediator in regulating carbohydrate metabolism in skeletal muscle independent of the AAR pathway, ChIP assay was performed using rabbit polyclonal antibody against C/EBPβ (see Table 3 for antibody information). In Fig. 4, the ATF3 gene was used as a positive control as it has been known to be regulated through direct binding by C/EBPβ. The binding of C/EBPβ at the GLUT4 promoter was found to be not present (<IgG of 0.02 ratio to input), suggesting that C/EBPβ does not bind to the GLUT4 promoter in our case.

3.8. Transcriptional enhancer — MEF2A was increased in female offspring muscle

To further investigate the potential mechanism that contributed to the up-regulated GLUT4 expression, myocyte enhancer factor 2A (MEF2A), which is known as a transcriptional enhancer to carry out specific binding to the GLUT4 MEF2 binding sequence [16], was tested by Western blot. Fig. 5 shows that MEF2 protein content was also increased 1.7-fold in female offspring of protein-restricted mothers. Since the binding of MEF2 is required for normal GLUT4 expression, the increased MEF2 protein could contribute to the up-regulated GLUT4 expression. We also tried to test the exact binding of MEF2 at GLUT4 promoter by using CHIP assay. There was, however, no commercial working antibody for us.

3.9. Histone modifications on GLUT4 promoter in rat offspring muscle

ChIP assays were performed to investigate the *in vivo* Pol II binding status at the GLUT4 promoter. Pol II binding at the GLUT4 promoter region in female offspring skeletal muscle with maternal LP was increased 1.46-fold compared to control (Fig. 6A; $P=.04$). Data were consistent with the increased GLUT4 mRNA expression. Since Pol II binding status at a promoter is often regulated by chromatin structure, methylated-histone or acetylated-histone antibodies were used in the ChIP assay at the GLUT4 promoter from maternal LP and control diet offspring.

ChIP assays were performed with antibodies specific for histone modifications associated with either enhanced gene transcription (acetylated histone H3, acetylated histone H4, and di-methylated histone H3 at lysine 4 residues) or transcriptional repression through diminished methylation (tri-methylated histone H3 at lysine 9 residues). Increases in both acetylated histone H3 and acetylated histone H4 were observed in the muscle of female pups from dams fed a LP diet vs. the control, by 2.11- and 1.81-fold ($P<.05$), respectively (Fig. 6A). Methylation of histone H3 lysine 4 was also increased with maternal LP (1.66-fold; $P<.05$), but no difference was detected at lysine 9 of histone H3. In male offspring, histone code modifications did not differ at the GLUT4 promoter region (Fig. 6B). Comparison of histone modifications between female and male offspring from control mothers also revealed that the basal acetylations of histone H3 and H4 were lower in males, while the methylation levels of histone H3 were similar between sex.

4. Discussion

Skeletal muscle is the most important tissue responsible for whole-body glucose uptake in humans [17] and rodents [18]. Insulin resistance is associated with decreased glucose uptake in insulin-sensitive tissues, i.e., skeletal muscle [19]. In the present study, we investigated GLUT4 gene expression and modifications of histones within the promoter in female and male pups with maternal protein restriction. This study demonstrates for the first time that GLUT4 gene expression is significantly higher in the skeletal muscle of female offspring subjected to *in utero* maternal protein restriction. We also showed that there are sex-dependent modifications to the GLUT4 promoter region and that this epigenetic change significantly increases the expression of GLUT4 mRNA only in female pups with maternal LP. This new observation suggests that skeletal muscle in female pups might have greater capability to make GLUT4 transporter when more glucose transporters are needed, reflecting an adaptation to protein restriction first and only encountered during gestation. It is interesting to think that an estrogen-involving signaling pathway may influence this response in a sex-specific manner. Estrogen has been reported to increase expression of plasma membrane GLUT4 and to boost carbohydrate metabolism [20]. Considering the critical role environment plays in fetus development, the observed sex difference

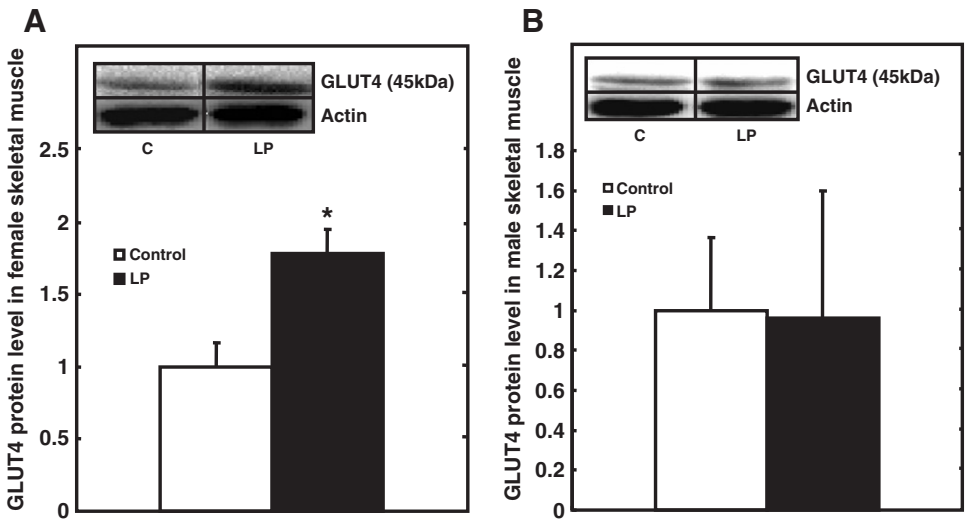

Fig. 2. GLUT4 protein content by Western blot. (A) Expression of GLUT4 protein level in female offspring skeletal muscle from LP mothers vs. control. The bands shown represent the average level of GLUT4 protein in control and LP groups ($n=6$). (B) Expression of GLUT4 protein level in male offspring skeletal muscle from LP mothers vs. control. The bands represent the average level of GLUT4 protein in control and LP groups ($n=6$). LP protein levels are expressed as mean ratio to control. The values represent the mean±S.E.M., *$P<.05$.

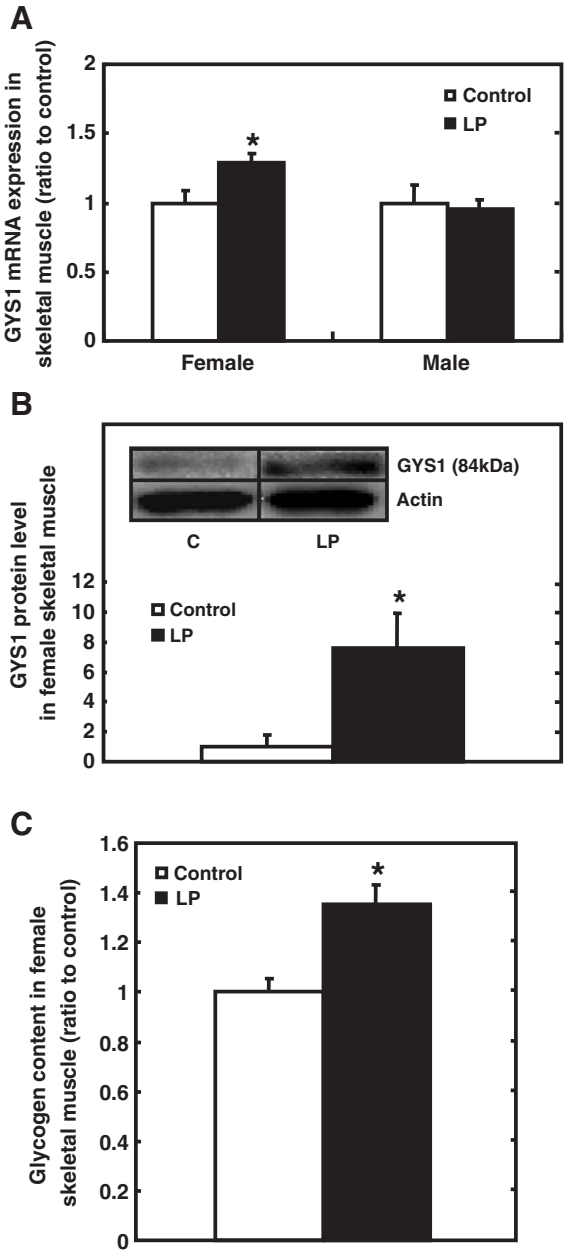

Fig. 3. Glycogen synthase expression and glycogen content. (A) Expression of glycogen synthase mRNA in offspring skeletal muscle from LP mothers vs. control (n=5). LP mRNA levels are expressed as mean ratio to control after normalization by L7a. (B) Expression of glycogen synthase protein level in female offspring skeletal muscle from LP mothers vs. control. The bands shown represent the average level of glycogen synthase protein in control and LP groups (n=6). (C) Glycogen content in female offspring skeletal muscle from LP mothers vs. control (n=5). The values represent the mean±S.E.M., *P<.05.

in gene expression in offspring may also be due to gender-specific perturbation of placental function, an idea supported by the reported alteration in gene expression patterns, including increased GLUT4 expression, that occurred in the placentas of male fetuses upon early pregnancy stress [21].

In this study, we investigated the potential involvement of a cofactor, C/EBPβ protein, in amino acid response pathway on altering GLUT4 expression as a consequence of maternal LP diet. C/EBP proteins are also involved in a wide range of cellular processes, such as cellular proliferation, carbohydrate metabolism, adipocyte differentiation and inflammation [22]. In our case, however, we previously

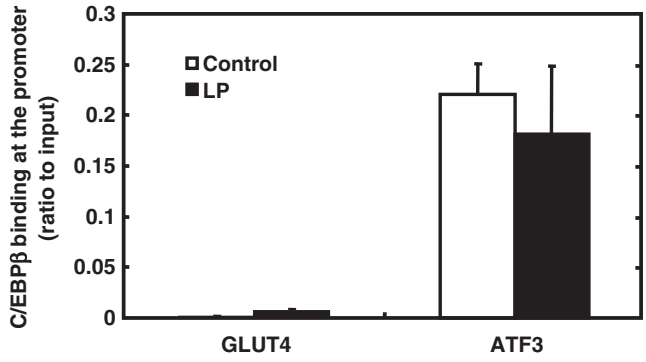

Fig. 4. Interaction of C/EBPβ protein at the GLUT4 gene promoter. Binding of C/EBPβ at the GLUT4 promoter in female offspring muscle. Data are shown as a ratio to the input DNA. The ATF3 gene was used as a positive control. All the values represent the mean±S.E.M.

found no change in ATF gene expression upon maternal protein restriction in offspring muscle. We did observe a significant increase of C/EBPβ in LP female offspring muscle. However, ChIP assays revealed that the C/EBPβ protein does not interact with the GLUT4 promoter. The ChIP assay we performed is designed to cross-link all the interacting factors, including those that are not directly bound with the DNA sequence. Therefore, the mechanism regulating the GLUT4 gene may not involve C/EBPβ as a transcription factor.

It was established through transgenic investigations that conserved GLUT4 promoter regions play a crucial role in its expression in skeletal muscle. Myocyte enhancer factor 2 (MEF2) is a required transcription factor for normal GLUT4 expression. In skeletal muscle, MEF2A has the ability to specifically bind to the GLUT4 MEF2 binding sequence to activate GLUT4 transcription in conjunction with the binding of GLUT4 enhancer factor to domain 1, located upstream of the MEF2 binding site [23]. Disruption of the MEF2-binding site turned off tissue-specific GLUT4 expression [24]. In our rat model, we observed that the total MEF2A protein level increased in LP offspring muscle. In another rat study, Holmes et al. [25] also observed roughly twofold increase in total MEF2 protein upon stimulation of AMP-activated protein kinase in muscle. Correspondingly, nuclear MEF2A protein increased fivefold and MEF2 binding to the GLUT4 consensus

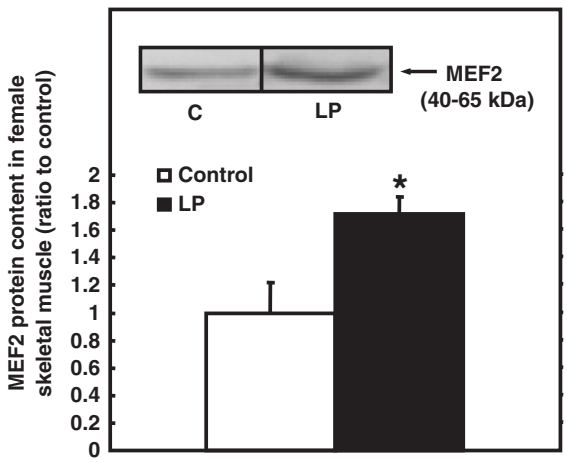

Fig. 5. MEF2 protein content by Western blot. Expression of MEF2 protein level in female offspring skeletal muscle from LP mothers vs. control. The bands shown represent the average level of MEF2 protein in control and LP groups (n=6). LP protein levels are expressed as mean ratio to control. The values represent the mean±S.E.M., *P<.05.

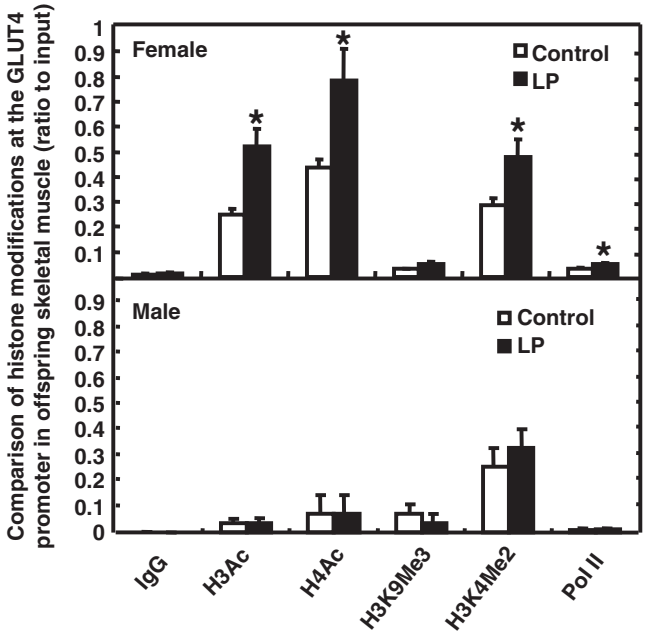

Fig. 6. Chromatin modifications within the GLUT4 gene promoter. ChIP assay demonstrating histone modifications and binding of Pol II at GLUT4 promoter region in offspring skeletal muscle. Data are shown as a ratio to the input DNA. Data are plotted as the increase relative to control. H4Ac: Acetylated histone H4; H3Ac: acetylated histone H3; H3K4Me2: di-methylated histone H3 at lysine 4; H3K9Me3: tri-methylated histone H3 at lysine 9; Pol II: RNA polymerase II. All the values represent the mean±S.E.M., *P<.05 (n=5).

sequence increased twofold. In a transgenic mice model, the protein levels of MEF2, determined by Western blot using a MEF2A antibody, were decreased in the diabetic state and recovered following insulin treatment [24]. The binding activities of MEF2 to GLUT4 reflect its protein level changes. Thus, the increase of MEF2A total protein level we observed might suggest that MEF2A is an important transcription factor that activates GLUT4 in LP pup's skeleton muscle in response to maternal protein restriction.

The myogenic differentiation factor D (MyoD) was reported to bind MEF2 and thyroid hormone receptor α1 (TRα1), synergistically driving gene expression for myogenesis, which would include skeletal muscle GLUT4 transcription [26]. There is also a MyoD binding site in the GLUT4 promoter region, proximal to the MEF2-binding site. However, we only observed marginally enhanced binding of MyoD protein to the GLUT4 promoter region in LP offspring with results close to significant (not shown).

Sex-specific histone modifications at the GLUT4 promoter, as observed in our study, may prove to be the key changes in nuclear protein–GLUT4 promoter interaction. Such modifications include increased levels of acetylated histone H3, acetylated histone H4 and di-methylated histone H3 at lysine 4 in female offspring rats following a maternal LP diet. Most importantly, the histone status perturbations may be memorized in the fetus and remain in the adult female offspring. The trans-generation transmitted insulin-resistant phenotype of the IUGR adult female offspring has suggested similar epigenetic alternations on histones. In contrast to our study, diminished skeletal muscle GLUT4 mRNA in IUGR female rat offspring was related to H3K14 de-acetylation and di-methylation of H3K9. It is worth noting that histone hypermethylation has both stimulatory and inhibitory effects on gene transcriptions, dependent on methylation sites on different lysines in histone H3. H3K4 methylation will generally induce gene transcription, whereas H3K9 methylation will silence gene transcription. Nevertheless, both studies showed that histone modification caused by maternal diet could alter offspring GLUT4 expression and potentially disturb carbohydrate metabolism in skeleton muscle. However, the epigenetic consequences could differ substantially based on diet constitutions and diet quantities. On the other hand, the basal acetylations of histone H3 and H4 were low in male offspring of control mothers with comparison to female offspring. This might suggest that, within GLUT4 gene promoter, females have relatively more opened chromatin structure than males to recruit other transcription factors.

The rate-limiting reaction for the formation of muscle glycogen has been generally considered to be the transfer of glucose from UDP-glucose to an amylase chain, a reaction mediated by glycogen synthase [27]. However, it has been suggested that glucose transport rather than glycogen synthase activity is rate limiting for muscle glycogen synthesis. For example, total GLUT4 protein content in muscle has been investigated in numerous studies to understand the long-term metabolic and functional consequences on intracellular substrate utilization, glycogen synthesis and glycogen content for example. In transgenic mice, overexpression of GLUT4 resulted in an increase in muscle glycogen concentration 20% above the level of the non-transgenic mice [28]. Glycogen synthesis rate was 50% faster in the muscle of transgenic mice that overexpressed GLUT4 than in that of control mice. In addition, up-regulation of GLUT4 in fast-twitch muscle resulted in a 2.5-fold increase in glycogen storage in muscle [29]. In our data, total expression of glycogen synthase and that of glycogen content were both up-regulated in female offspring by a maternal LP diet. This up-regulation in glycogen storage may be closely associated with the up-regulation of total GLUT4 content. Further increase in glycogen synthesis due to increased GLUT4 and glycogen synthase has a protective effect on metabolism syndrome. The results of this study seem to be discordant with the findings of other studies showing higher incidence of insulin resistance. However, there are several differences in our studies and other studies. Most of the other studies showing increased insulin resistance used either total nutrient restriction (calorie restriction) [3,10] or LP during both gestation and lactation [5]. According to our own study, only gestational protein restriction did not cause any difference on either dams' or pups' glucose or insulin level (unpublished data). Only lactation LP diet changed the insulin level in offspring. In this study, the LP diet was used for gestation period only and all of the dams were put onto a standard rat chow throughout the lactation. Thus, we studied total GLUT4 content and glycogen storage, which may reflect a sex-dependent adaptation in offspring to gestational protein restriction on long-term metabolic and functional consequences, which is also consistent with studies showing that male offspring had higher risk of developing type 2 diabetes later in life [30].

Overall, gestation LP intake (but not calorie restriction) up-regulates GLUT4 gene expression through chromatin modification programming specifically in female offspring skeletal muscle and induces an increase of glycogen synthase and glycogen content. Expressions of regulatory genes associated with carbohydrate metabolism, C/EBPβ and MEF2A, increased in LP female pup skeletal muscle. These results indicate that sex-dependent adaptation of glucose metabolism to a gestation LP diet may have a protective effect on metabolism syndrome in female offspring.

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