ORIGINAL ARTICLE

Maternal protein restriction induces early-onset glucose intolerance and alters hepatic genes expression in the peroxisome proliferator-activated receptor pathway in offspring

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ABSTRACT

Aims/Introduction: Maternal undernutrition during pregnancy and/or lactation can alter the offspring's response to environmental challenges, and thus increases the risk of the development of metabolic diseases at a later age. However, whether maternal protein restriction can modulate glucose metabolism in the early life of offspring is less understood. Furthermore, we explored the potential underlying mechanisms that illustrate this phenotype.

Materials and Methods: To test this hypothesis, we examined the offspring of C57BL/6J mice at weaning to determine the effects of feeding their mothers a low-protein diet or normal chow diet throughout pregnancy and lactation. Gene array experiments and quantitative real-time polymerase chain reaction were utilized to explore the altered hepatic genes expression.

Results: The offspring of dams fed a low-protein diet had a lower birthweight and bodyweight, impaired glucose tolerance, decreased insulin sensitivity, and decreased serum cholesterol at weaning. Using gene array experiments, 253 differentially expressed genes were identified in the liver tissues of the offspring between the two groups. Bioinformatic analyses showed that all differentially expressed genes were mapped to 11 pathways. We focused on the 'peroxisome proliferator-activated receptor signaling pathway,' because peroxisome proliferator-activated receptors have emerged as central regulators of glucose and lipid homeostasis. Quantitative real-time polymerase chain reaction was utilized for the validation of genes in the pathway.

Conclusions: A maternal low-protein diet during pregnancy and lactation promotes early-onset glucose intolerance in the offspring mice, and the altered hepatic genes expression in peroxisome proliferator-activated receptor signaling pathway could play role in regulating this phenomenon.

INTRODUCTION

It is widely acknowledged that gestation is a critical time window, and maternal nutrition is a key determinant for the health of the offspring. The relationship between exposures during the critical periods of early life and lifetime consequences is well

established in developmental biology. This concept was first proposed by Barker and Hales in the 1990s[1], and is known as the 'fetal programming hypothesis,' which states that 'early adaptations to a nutritional stress/stimulus can permanently change the physiology and metabolism of the organism and continue to be expressed even in the absence of the stimulus/stress that initiated them'[2]. This hypothesis has been authenticated by a substantial number of epidemiological studies and

large-scale clinical cohorts, which have shown that malnutrition during fetal life can significantly impact the predisposition to the development of cardiovascular diseases and metabolic syndrome in adulthood[3,4]. In our previous study, lower birthweight was an independent risk factor for later diabetes, impaired glucose regulation and an increased presentation of metabolic syndrome components[5,6].

In addition to epidemiological studies and clinical cohorts, experimental support for this hypothesis predominately originated from animal models. Abundant animal model studies have shown that a maternal low-protein diet (LPD) increased the risk for intrauterine growth restriction and led to metabolic diseases in the offspring during adulthood, such as hypertriglyceridemia and insulin resistance, impaired glucose tolerance and diabetes. However, these studies were confined to long-term modifications (adulthood or aging) in the offspring of a maternal LPD with varying durations from 1 to 18 months[7–9]. A recent study showed a maternal LPD had an impact on glucose and lipid metabolism in adult offspring. More precisely, the diet patterns included a maternal LPD throughout gestation and lactation, and the offspring were inversely given a high-fat diet until the age of 22–30 weeks[10,11]. Thus, the effects of a maternal LPD on the offspring would be interfered by a post-weaning high-fat diet in offspring.

Therefore, whether maternal low-protein feeding can impact glucose metabolism in the early life of offspring is less understood. We hypothesized that a maternal LPD only during pregnancy and lactation might promote an early-onset glucose intolerance and insulin resistance of the offspring at weaning (3 weeks-of-age). Furthermore, the mechanistic link between a maternal LPD and the metabolic diseases of their offspring is not completely understood. Therefore, we explored the underlying mechanisms to illustrate the phenotype without the interference of a long-term effect and the offspring's diet condition post-weaning.

MATERIALS AND METHODS
Animals
C57BL/6J mice (approximately 8 weeks-of-age) were obtained from the Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences and Peking Union Medical College (Beijing, China; SCXK-2013-0018). The animals were maintained under a 12-h light–dark cycle (lights on at 07.00 h) at a constant temperature of 22 ± 2°C, with standard pellet diet and water available *ad libitum*. All procedures were approved by the animal care and use committee of the Peking Union Medical College Hospital (Beijing, China; MC-07-6004), and were carried out in compliance with the *Guide for the Care and Use of Laboratory Animals*, 8th ed., 2011.

Experimental Procedures
All female mice received a normal chow diet (NCD) before mating, and they were allowed to acclimate to their new environment for 1 week. After the week, virgin females were time mated. Females were checked daily for postcopulatory plugs, and the presence of a plug in the morning after mating was considered day 0.5 of pregnancy. Then, the pregnant mice were randomly allocated to a NCD or a LPD. More specifically, the control group received a 200-g protein/kg diet, and the LPD group received an isoenergetic 80-g protein/kg diet throughout gestation and lactation, as described previously[12]. Nutrient composition of NCD is 20% casein, 20.3% sucrose, 41.5% maize starch, 12% corn oil and 4% cellulose, whereas the composition of LPD is 8% casein, 25.3% sucrose, 48.5 maize starch, 12% corn oil and 4% cellulose. All the food was purchased from Research Diets Inc, New Brunswick, NJ, USA. The mice were fed *ad libitum*, and their daily food consumption was calculated by weighing the remaining food twice a week. The litter size was standardized to six pups. The pups were weaned at postnatal day 21. At weaning, the mice were anesthetized with chloral hydrate and killed. Blood samples were taken from the intraorbital retrobulbar plexus after a 10-h fast in the anesthetized mice. After clotting, the serum was obtained by centrifugation at 4,000 *g* for 5 min in a microcentrifuge at room temperature and stored at −80°C. The liver samples were quickly removed, snap frozen in dry ice and stored at −80°C for further analyses. The samples for microscopic evaluation were fixed in paraformaldehyde for hematoxylin–eosin staining. In the experiments, 12 mice per group were analyzed, and the mice were derived from three different dams on average.

Measurement of Birthweight, Bodyweight and Bodylength
The mean litter weights were used to compare the birthweights between the NCD and LPD groups, because the actual number of newborn pups was very large and to minimize the effect of within-litter differences. The bodyweights and bodylengths of the pups were measured from birth to weaning twice per week. The bodylength was calculated by adding the crown–rump length and the tail length. After the mice were anesthetized, the crown–rump length was measured from the tip of the nose to the end of the fur line on the tail. The tail length was measured from the end of the fur growth to the tip of the tail, as described previously[13].

Intraperitoneal Glucose Tolerance Tests
Glucose tolerance tests were carried out on the low-protein and control offspring at 3 weeks-of-age. The mice were fasted for 10-h and were weighed. Then, a glucose (2 mg/g bodyweight) load was given intraperitoneally. Before (0 min) and at the indicated time-points (30, 60 and 120 min) after injection, the blood glucose (BG) concentrations were measured in the blood collected by tail tip bleeding with a Contour TS glucometer (Bayer, Beijing, China). The area under the curve (AUC) of the intraperitoneal glucose tolerance test (IPGTT) was calculated by the trapezoid formula: $AUC = 0.5 \times (BG0 + BG30)/2 + 0.5 \times (BG30 + BG60)/2 + 1 \times (BG60 + BG120)/2$, as previously described[14].

Serum and Hepatic Biochemistry Analyses

Serum insulin concentrations were determined using the Mouse Ultrasensitive Insulin ELISA kit from ALPCO Diagnostics (80-INSMSU-E01; Salem, NH, USA). Insulin sensitivity was assessed using the homeostasis model assessment of insulin resistance (HOMA-IR), which was calculated as the fasting insulin concentration (µU/mL) × fasting glucose concentration (mmol/L)/22.5[15]. The serum and hepatic lipid levels were measured using a colorimetric cholesterol quantitation kit (K603-100; Biovision Inc., Mountain View, CA, USA) and a colorimetric triacylglycerol quantitation kit (K622-100; Biovision Inc., Mountain View, CA, USA) according to the manufacturer's protocol[16,17].

Histological Examination of the Liver

The liver specimens were fixed in 10% formalin for at least 24 h, and then embedded in paraffin in preparation for sectioning. Serial sections of 7-µm thickness were obtained using a microtome. Deparaffinized and fixed sections were stained with hematoxylin–eosin. Microscopic examinations were carried out on each stained liver section in each group.

Preparation of Total Ribonucleic Acid

Total ribonucleic acid (RNA) was extracted from frozen liver tissues with TRIzol reagent (Life Technologies Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. The quality and concentration of the RNA were determined on a Nanodrop (ND-1000; NanoDrop Products, Wilmington, DE, USA) with RNA concentration >100.0 ng/µL and the ratio of OD260/280 = ~2.0. The RNA samples were immediately frozen and stored at −80°C for further analyses.

Gene Array Experiments

Gene expression profiles in the liver tissues from the offspring mice were determined by whole genome-wide gene expression array analyses. Because of financial constraints, we could not perform a whole genome array for each biological replicate. Each group contained three biological replicates, which can provide a relatively reliable estimate of the mean gene expression for each group. Then, the samples were processed following Affymetrix recommendations, and the complementary deoxyribonucleid acid (cDNA) was hybridized to the Affymetrix Mouse Gene ST 1.0 array. Both the background correction and normalization were carried out using a probe logarithmic intensity error algorithm. The t-test was used for preprocessing and statistical analyses. The fold change and P-value were used to determine the probe sets that showed significant differential expression between the experimental conditions. Specifically, the probes with a fold change >1.5 and a significant difference ($P < 0.05$) were chosen for further analyses.

Bioinformatic Analyses of Gene Array Data

Subsequently, the signals of the liver tissues from the LPD and NCD offspring were averaged, and comparisons of the expression pattern changes were carried out between the two groups. The fold changes were calculated based on the average values from each group. Differentially expressed genes were hierarchically clustered using the gplots R package (Wirtschaftsuniversität Wien, Vienna, Austria). According to the instructions of the Affymetrix platform, we established the following two criteria to identify the differentially expressed genes: (i) detection P-value <0.05 in both of the two sample groups; and (ii) fold change >1.5. To identify the biological meaning of the group of genes with changed expression, the subset of genes that met the aforementioned criteria was analyzed with the Gene Ontology classification system and the Kyoto Encyclopedia of Genes and Genomes pathways using the web-based tool, WebGestalt: update 2013 (WEB-based GEne SeT Analyses Toolkit:update2013; http://bioinfo.vanderbilt.edu/webgestalt/). The overrepresentation of genes with altered expression within specific Gene Ontology categories was determined through a hypergeometric test.

Measurement of Messenger RNA Expression Using Real-Time Polymerase Chain Reaction

For validation of the gene array results, four genes from the differentially expressed gene list were selected for quantitative real-time polymerase chain reaction (qRT–PCR) analyses by expanding the quantity samples. Furthermore, the genes expression of the peroxisome proliferator-activated receptor (PPAR) isoforms α, δ and γ were also examined, because PPAR isoforms indeed play important roles in the PPAR pathway, and they are shown to be regulated by a maternal LPD in offspring at later time-points ($n = 12$ per group). Before PCR, each total RNA was processed with Rnase-free Dnase (Qiagen, New York, NY, USA). RNA was reverse transcribed by 1 µg of total RNA from each sample using the Power cDNA Synthesis kit (A3500; Promega BioSciences LLC, Sunnyvale, CA, USA). The cDNA (2 µL) was amplified with the SYBR® Green PCR Master Mix (RR420A; Takara Bio Inc., Otsu, Japan) in a final volume of 20 µL. We used OLIGO 7.0 software (Molecular Biology Insights Inc., Cascade, CO, USA) to design the sequences of the primers, which were purchased from Applied Biosystems (Grand Island, NY, USA). The reaction production was accurately measured in the exponential phase of amplification by the ABI prism Vii7 Sequence Detection System (ABI Prism® Vii7; Applied Biosystems, Life Technologies). The reaction conditions consisted of an initial activation step (30 s at 95°C) and a cycling step (denaturation for 5 s at 95°C and annealing for 34 s at 60°C for 40 cycles). All reactions were carried out with three biological replicates, and each analysis consisted of three technical replicates. A melting curve analyses was carried out after each run, and we used agarose gel electrophoresis to confirm the amplicon size and reaction specificity. The sequences of the primers used are listed in Table 1. The results were standardized against the reference (housekeeping) β-actin gene, and the relative expression levels were quantified by the $2^{-\Delta\Delta C_t}$ method, with the relative fold changes normalized to the control values[18].

Table 1 | Oligonucleotide sequences for the quantitative real-time polymerase chain reaction analyses

Gene symbol	Forward primer	Reverse primer
β-actin	TGTTACCAACTGGGACGACA	GGGGTGTTGAAGGTCTCAAA
Aqp7	CTCGTGATAGGCATCCTTGT	AGCCAGCAATGAAAGTGAAC
Cpt1b	TATTACCGCATGGAGACATT	TTAGTTGCCCACCATGACTT
Lpl	TGCCTGAGTTGTAGAAAGAA	CTTTGTAGGGCATCTGAGAG
Cyp7a1	TTCAAGACCGCACATAAAGC	CGTTGAGAAACATGCGTAGA
PPARα	CTGTCCGCCACTTCGAGTC	GATACGCCCAAATGCACCAC
PPARΔ	TCACCAGCAGCCTAAAAGCA	CCGGCGAGAAGAAAGGAAGT
PPARγ	CGGGCTGAGAAGTCACGTT	TGCGAGTGGTCTTCCATCAC

Aqp7, aquaporin 7; Cpt1b, carnitine palmitoyltransferase 1b; Lpl, lipoprotein lipase; Cyp7a1, cytochrome P450, family 7, subfamily a, polypeptide 1; PPAR-α, peroxisome proliferator activator receptor alpha; PPAR-Δ, peroxisome proliferator activator receptor delta; PPAR-γ, peroxisome proliferator activator receptor gamma.

Statistical Analysis

The data were expressed as the mean ± standard deviation. Statistical analyses were carried out with Student's t-test between the two groups and two-way ANOVA was used to compare the glucose levels of IPGTT. A P-value <0.05 was considered statistically significant. All statistical analyses were calculated with SPSS 15.0 (SPSS, Inc., Chicago, IL, USA).

RESULTS

Maternal LPD Altered Birthweight, Bodyweight and Bodylength

The offspring of the dams fed a LPD had a lower birthweight compared with the dams fed a control diet ($P < 0.05$, Figure 1a). After 3 weeks, the bodyweights and bodylengths of the offspring were significantly lower compared with the
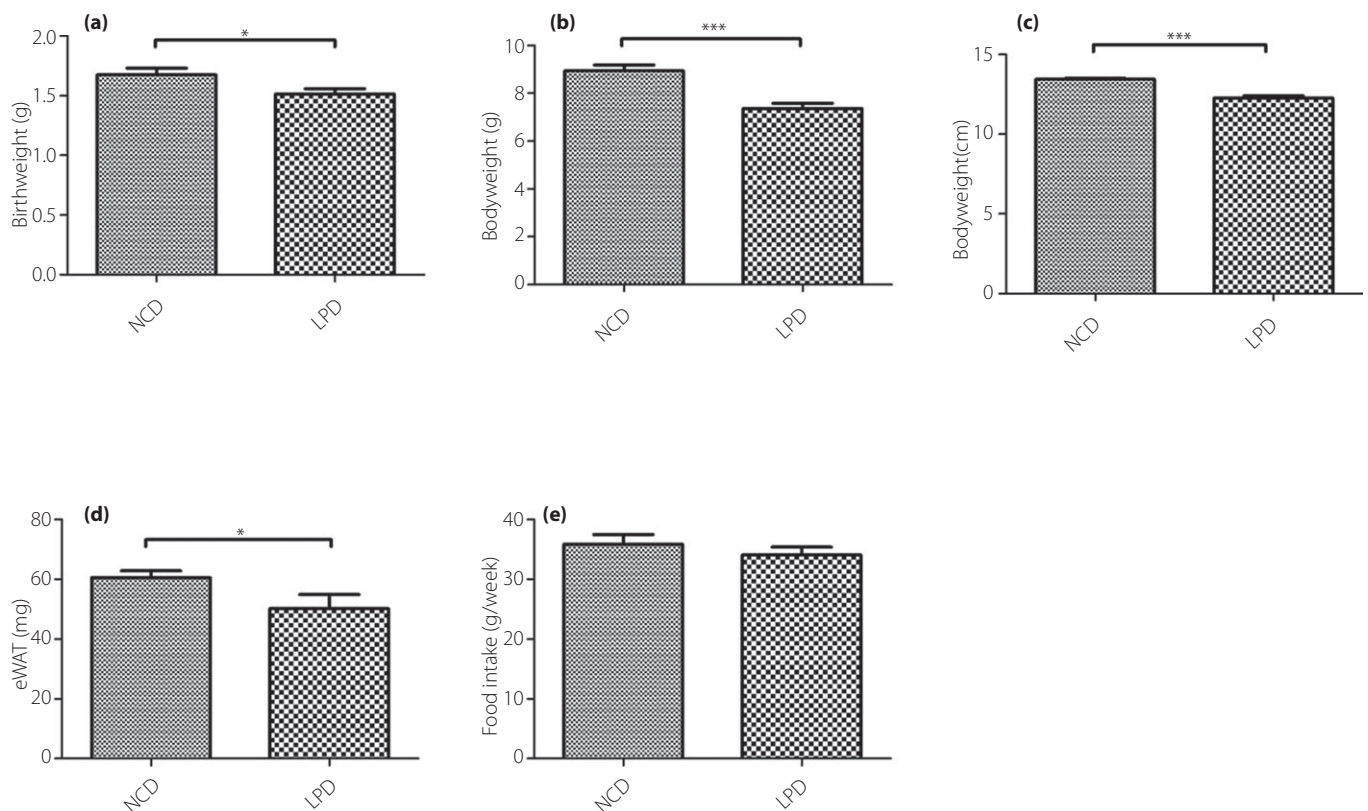

Figure 1 | (a) Birthweight, (b) bodyweight, (c) body length, (d) epigonadal white adipose tissue (eWAT) and (e) food intake of the offspring at weaning. All values are the mean ± standard error of the mean (n = 12 mice/group). *$P < 0.05$ vs normal chow diet (NCD), ***$P < 0.001$ vs NCD assessed by Student's t-test. LPD, low-protein diet.

offspring of dams fed the NCD at weaning ($P < 0.001$; Figure 1b,c). The offspring of the LPD group also showed lower epigonadal white adipose tissue (eWAT) than the NCD group ($P < 0.05$; Figure 1d). During this period, the calorie intake and food consumption of the dams was not significantly different between the LPD and NCD groups ($P > 0.05$; Figure 1e).

Maternal LPD Impaired Glucose Tolerance and Decreased Insulin Sensitivity

The blood glucose levels of the offspring of the dams fed a high-fat diet at weaning were higher at 0 min ($P < 0.05$), 60 min ($P < 0.05$) and 120 min ($P < 0.05$) after intraperitoneal glucose administration compared with the NCD group (Figure 2a). The AUC was consistently larger for the LPD group compared with the NCD group ($P < 0.05$; Figure 2b). To determine whether the LPD suppressed insulin sensitivity in the offspring, the levels of fasting blood glucose (FBG) and
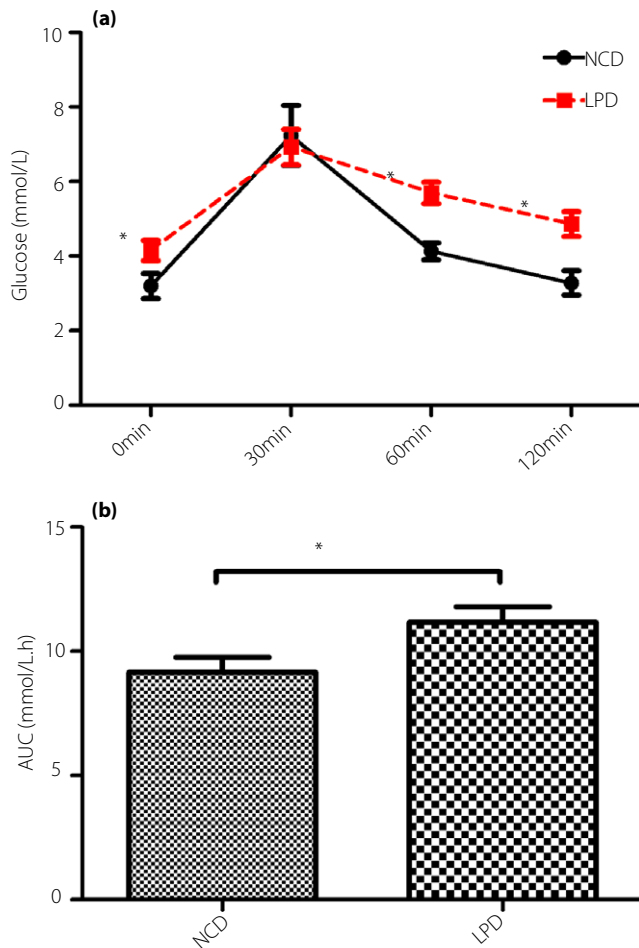

Figure 2 | (a) Intraperitoneal glucose tolerance test (IPGTT) and (b) area under the curve (AUC) of the offspring at weaning. All values are the mean ± standard error of the mean ($n = 12$ mice/group). *$P < 0.01$ vs normal chow diet (NCD) assessed by Student's t-test and two-way ANOVA. LPD, low-protein diet.
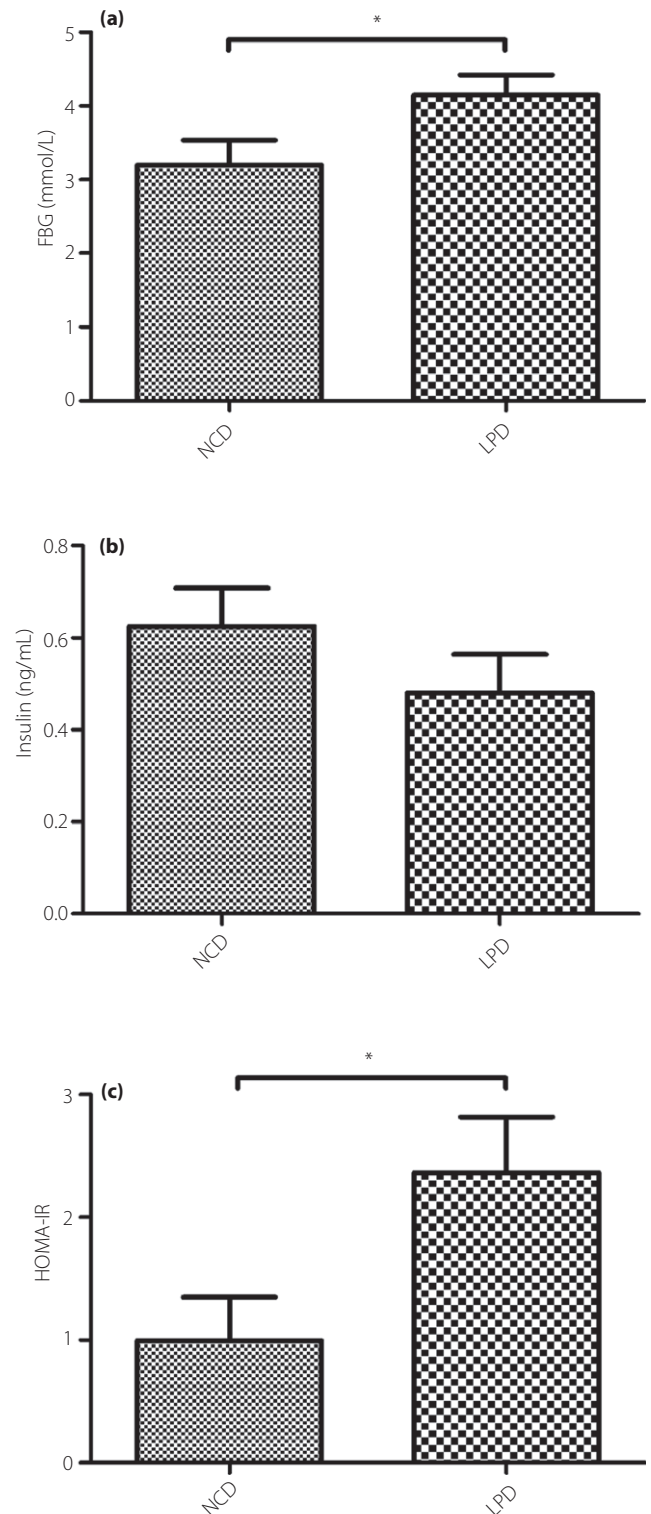

Figure 3 | (a) Fasting blood glucose, (b) serum insulin levels (c) and homeostasis model assessment of insulin resistance (HOMA-IR) of the offspring at weaning. All values are the mean ± standard error of the mean ($n = 12$ mice/group). *$P < 0.05$ vs normal chow diet (NCD) assessed by Student's t-test. FBG, fasting blood glucose; LPD, low-protein diet.

serum insulin were determined. The FBG was higher for the LPD group ($P < 0.05$; Figure 3a), and there were no significant differences between the two groups (Figure 3b). The HOMA-IR was significantly higher in the offspring of the dams fed the LPD compared with the NCD group at weaning ($P < 0.05$; Figure 3c).

Maternal LPD Decreased Total Serum Cholesterol Levels

We determined that the total cholesterol was decreased in the offspring of the dams fed the LPD compared with the NCD during pregnancy and lactation ($P < 0.05$; Figure 4a). However, no significant differences in serum triacylglycerol were observed between the two groups (Figure 4b). The hepatic cholesterol and triacylglycerol levels showed no significant differences between the two groups (data not shown).

Maternal LPD Showed no Influence on Liver Histology

Histological examination of the liver tissues showed that the offspring of the dams fed the LPD and the NCD both had normal liver structures at weaning (Figure 5).
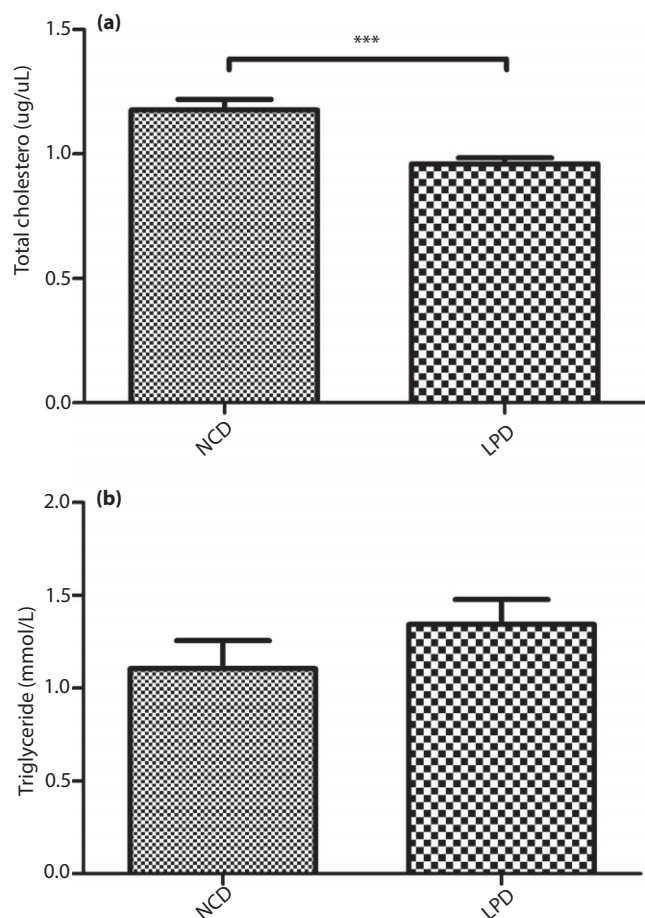

Figure 4 | (a) Serum triacylglycerol and (b) total cholesterol levels of the offspring at weaning. All values are the mean ± standard error of the mean ($n = 12$ mice/group). ***$P < 0.001$ vs normal chow diet (NCD) assessed by Student's t-test. LPD, low-protein diet.

Genes Differentially Regulated by Maternal LPD

A total of 253 genes were differentially expressed between the offspring of the dams fed the LPD and the NCD at weaning. A total of 146 genes (57.7%) were upregulated, and 107 genes (42.8%) were downregulated in the LPD group. Hierarchical clustering based on similarity in gene expression using all differentially expressed genes highlighted the difference in the transcriptional profiles between the LPD and NCD groups (Figure 5). WebGestalt analyses of all differentially expressed genes in the LPD group yielded 40 Gene Ontology categories of biological processes (Table 2). The WebGestalt annotation tool was used for the identification of the putative Kyoto Encyclopedia of Genes and Genomes pathways. The genes were mapped to 11 pathways ($P < 0.05$; Table 3). The most common type of enriched pathway was related to 'metabolic pathways' ($P = 1.16\text{E-}10$). The second and third most abundant pathways were related to the 'Jak-STAT signaling pathway' ($P = 9.50\text{E-}5$) and the 'PPAR signaling pathway' ($P = 0.0036$), respectively. Other pathways are shown in Table 3. We focused on the 'PPAR signaling pathway,' because PPARs have emerged as central regulators of glucose and lipid homeostasis. Four genes in the PPAR signaling pathway, including *Aqp7* (aquaporin 7), *Cpt1b* (carnitine palmitoyltransferase 1b), *Lpl* (lipoprotein
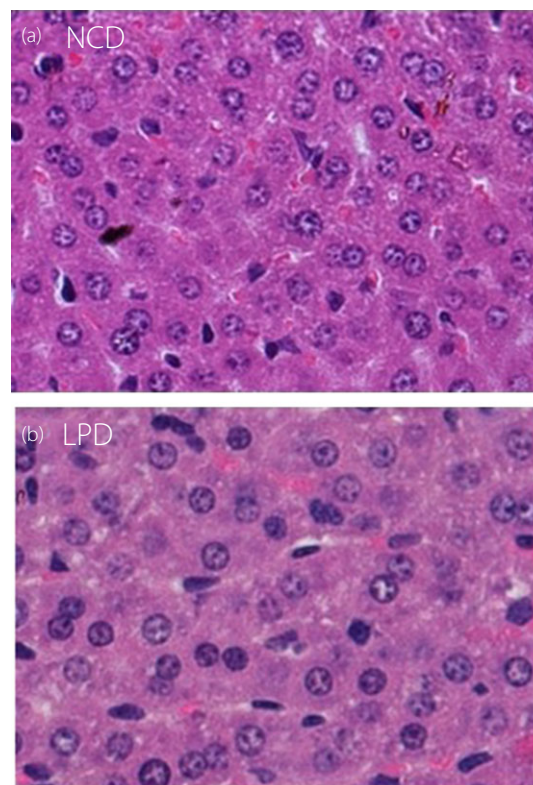

Figure 5 | Hematoxylin–eosin staining of liver tissues of the offspring at weaning. Liver histology in offspring at weaning from dams fed (a) normal chow diet (NCD) and (b) low-protein diet (LPD) both had normal liver structure. Original magnification, ×40.

Table 2 | Gene ontology (biological process) of differentially expressed genes between the two groups

GO classification	GO term	GO ID	C	O	E	R	rawP	adjP
Biological process	Sterol metabolic process	GO:0016125	107	12	1.07	11.2	8.29E-10	1.06E-06
	Cholesterol metabolic process	GO:0008203	100	11	1	10.98	5.24E-09	3.35E-06
	Steroid metabolic process	GO:0008202	238	14	2.38	5.87	1.40E-07	5.96E-05
	Regulation of immune system process	GO:0002682	695	23	6.96	3.3	5.97E-07	0.0002
	Lipid metabolic process	GO:0006629	944	27	9.46	2.86	1.03E-06	0.0003
	Alcohol metabolic process	GO:0006066	254	13	2.54	5.11	1.89E-06	0.0004
	Monocarboxylic acid metabolic process	GO:0032787	367	15	3.68	4.08	4.94E-06	0.0008
	Sterol biosynthetic process	GO:0016126	43	6	0.43	13.93	4.24E-06	0.0008
	Oxidation-reduction process	GO:0055114	919	25	9.21	2.72	6.38E-06	0.0009
	Single-organism biosynthetic process	GO:0044711	385	15	3.86	3.89	8.76E-06	0.0011
	Mitotic cell cycle	GO:0000278	542	18	5.43	3.32	1.00E-05	0.0012
	Oxoacid metabolic process	GO:0043436	718	21	7.19	2.92	1.25E-05	0.0012
	Cytolysis	GO:0019835	30	5	0.3	16.64	1.12E-05	0.0012
	Organic acid metabolic process	GO:0006082	732	21	7.33	2.86	1.67E-05	0.0015
	Carboxylic acid metabolic process	GO:0019752	676	20	6.77	2.95	1.72E-05	0.0015
	Cell cycle	GO:0007049	1136	27	11.38	2.37	3.00E-05	0.0021
	Small molecule biosynthetic process	GO:0044283	373	14	3.74	3.75	2.64E-05	0.0021
	Lipid biosynthetic process	GO:0008610	428	15	4.29	3.5	3.02E-05	0.0021
	Cholesterol biosynthetic process	GO:0006695	37	5	0.37	13.49	3.25E-05	0.0022
	Steroid biosynthetic process	GO:0006694	125	8	1.25	6.39	3.87E-05	0.0025
	Regulation of lipid biosynthetic process	GO:0046890	96	7	0.96	7.28	5.18E-05	0.0031
	Triglyceride metabolic process	GO:0006641	69	6	0.69	8.68	6.71E-05	0.0039
	Small molecule metabolic process	GO:0044281	1824	36	18.27	1.97	7.08E-05	0.0039
	Triglyceride biosynthetic process	GO:0019432	23	4	0.23	17.36	7.49E-05	0.004
	Immune system process	GO:0002376	1353	29	13.55	2.14	9.52E-05	0.0041
	Neutral lipid biosynthetic process	GO:0046460	26	4	0.26	15.36	0.0001	0.0041
	Acylglycerol metabolic process	GO:0006639	79	6	0.79	7.58	0.0001	0.0041
	Complement activation, alternative pathway	GO:0006957	10	3	0.1	29.95	0.0001	0.0041
	Acylglycerol biosynthetic process	GO:0046463	26	4	0.26	15.36	0.0001	0.0041
	Alcohol biosynthetic process	GO:0046165	109	7	1.09	6.41	0.0001	0.0041
	Cellular lipid metabolic process	GO:0044255	667	18	6.68	2.69	0.0001	0.0041
	Neutral lipid metabolic process	GO:0006638	81	6	0.81	7.39	0.0002	0.0075
	Cell cycle phase	GO:0022403	617	17	6.18	2.75	0.0002	0.0075
	Positive regulation of immune system process	GO:0002684	440	14	4.41	3.18	0.0002	0.0075
	Retinoic acid catabolic process	GO:0034653	3	2	0.03	66.55	0.0003	0.0101
	Saturated monocarboxylic acid metabolic process	GO:0032788	3	2	0.03	66.55	0.0003	0.0101
	Unsaturated monocarboxylic acid metabolic process	GO:0032789	3	2	0.03	66.55	0.0003	0.0101
	Diterpenoid catabolic process	GO:0016103	3	2	0.03	66.55	0.0003	0.0101
	Death	GO:0016265	1475	29	14.78	1.96	0.0004	0.0122
	Myeloid leukocyte differentiation	GO:0002573	131	7	1.31	5.33	0.0004	0.0122

Fold change >1.5, $P < 0.05$. adjP, P-value adjusted by the multiple test adjustment; C, the number of reference genes in the category; GO, Gene Ontology, O, the number of genes in the gene set and category; E, the expected number in the category; R, ratio of enrichment; rawP, P-value from hypergeometric test.

lipase) and *Cyp7a1* (cytochrome P450, family 7, subfamily a, polypeptide 1), were significantly differentially expressed on the gene array. The expression ratios are presented as the $\log_2$ (fold change; Figure 6).

Hepatic Gene Expression for Validation of Array Analyses
We used qRT–PCR assays to verify a subset of genes included in the array results. Four genes (*Aqp7*, *Cpt1b*, *Lpl* and *Cyp7a1*) were selected for verification by expanding the quantity samples (*n* = 12 per group) because of their true positive expression

differences in the 'PPAR signaling pathway,' which is essential for glucose and lipid metabolism. *Aqp7*, *Cpt1b* and *Lpl* were significantly upregulated, and *Cyp7a1* was downregulated in the LPD group. Strong consistency between the array and qRT–PCR results was observed for all four genes, which shows the reliability of our array assays. It was shown that PPAR-γ increased significantly in the LPD group, but PPAR-α was similar between the two groups. The expression of PPAR-δ was very low, which could not even be detected in some samples (Table 4).

Table 3 | Kyoto Encyclopedia of Genes and Genomes pathways with differentially expressed genes between the two groups

KEGG ID	Pathway name	Gene symbols	C	O	E	R	rawP	adjP
01100	Metabolic pathways	Idi1, Ido2, Chkb, Cyp51, Nsdhl, Cyp2c54, Sds, Nans, Ces3b, Cyp7a1, Asns, Pnliprp1, Sc4 mol, Nnmt, Fdps, Cryl1, Sqle, Ggt6, Cyp2c38, Ces3a, Hsd3b5, Ccbl1, Agpat9, Bdh2, Ldhb, Acss3, Acmsd, Cyp2u1	1184	28	5.4	5.18	1.78E-12	1.16E-10
04630	Jak-STAT signaling pathway	Lepr, Socs2, Irf9, Myc, Pim1, Ccnd1, Cish	153	7	0.7	10	7.31E-06	9.50E-05
03320	PPAR signaling pathway	Cyp7a1, Aqp7, Lpl, Cpt1b	80	4	0.4	11	0.0005	0.0036
00590	Arachidonic acid metabolism	Cyp2c54, Cyp2c38, Ggt6, Cyp2u1	90	4	0.4	9.74	0.0008	0.0047
00561	Glycerolipid metabolism	Agpat9, Lpl, Pnliprp1	51	3	0.2	12.9	0.0017	0.0069
00564	Glycerophospholipid metabolism	Agpat9, Chkb, Gpd2	80	3	0.4	8.22	0.006	0.015
01040	Biosynthesis of unsaturated fatty acids	Acot4, Acot3	25	2	0.1	17.5	0.0058	0.015
04950	Maturity onset diabetes of the young	Hhex, Hes1	26	2	0.1	16.9	0.0063	0.0152
04975	Fat digestion and absorption	Pnliprp1, Apoa4	45	2	0.2	9.74	0.018	0.0355
00591	Linoleic acid metabolism	Cyp2c54, Cyp2c38	46	2	0.2	9.53	0.0188	0.0359
00140	Steroid hormone biosynthesis	Hsd3b5, Cyp7a1	55	2	0.3	7.97	0.0263	0.0442

Fold change >1.5, $P < 0.05$. adjP, P-value adjusted by the multiple test adjustment; C, the number of reference genes in the category; KEGG, Kyoto Encyclopedia of Genes and Genomes; O, the number of genes in the gene set and category; E, the expected number in the category; R, ratio of enrichment; rawP, P-value from hypergeometric test.

DISCUSSION

Maternal undernutrition during pregnancy and/or lactation can alter the offspring's responses to environmental challenges, and thus increases the risk of the development of metabolic diseases at a later age[19]. A systematic review of substantial epidemiological studies and large-scale clinical cohorts showed that birthweight was inversely related to type 2 diabetes risk in most populations studied[20], which shows that maternal undernutrition might be more inclined to increase the predisposition to metabolic diseases in adulthood compared with maternal overnutrition. Therefore, studies that examine the effect of maternal undernutrition on offspring are particularly important in light of the increasing awareness of maternal malnutrition, and the concern about the critical periods of gestation and the suckling period.

In the present study, we examined the consequences to the offspring of a maternal LPD regimen during pregnancy and lactation periods in a mouse model. The findings showed that a short-term maternal LPD can contribute to a lower birthweight, lower bodyweight at weaning, impaired glucose tolerance and decreased insulin sensitivity compared with the NCD-fed group. This finding is in contrast with earlier animal experiments, which focused on the consequences for the offspring of long-term maternal LP feeding. However, these studies of the long-term effects of maternal undernutrition were limited to adult rodents[8, 21]. More importantly, studies focused on adults might lose the critical period for early prevention and intervention.

In addition to the body mass and specific markers of glucose metabolism, we also evaluated the lipid metabolism in mice born to mothers given a LPD during pregnancy and lactation. We determined that the total cholesterol was decreased in the offspring of the dams fed LPDs compared with the control group, and there were no significant differences in the serum triacylglycerol between the two groups. The maternal LPD consistently showed no influence on liver histology, and both groups had normal liver structures at weaning. These findings were consistent with a previous study that showed that elevated plasma triglycerides, cholesterol and hepatic steatosis emerged only with aging, but not in the early life of the offspring of the mothers fed a LPD[8]. Therefore, a maternal LPD regimen throughout pregnancy and lactation can promote early-onset impaired glucose homeostasis, but does not induce aberrant lipid metabolism in the offspring mice. Most studies have shown that a maternal LPD led to both aberrant glucose and lipid metabolism, such as
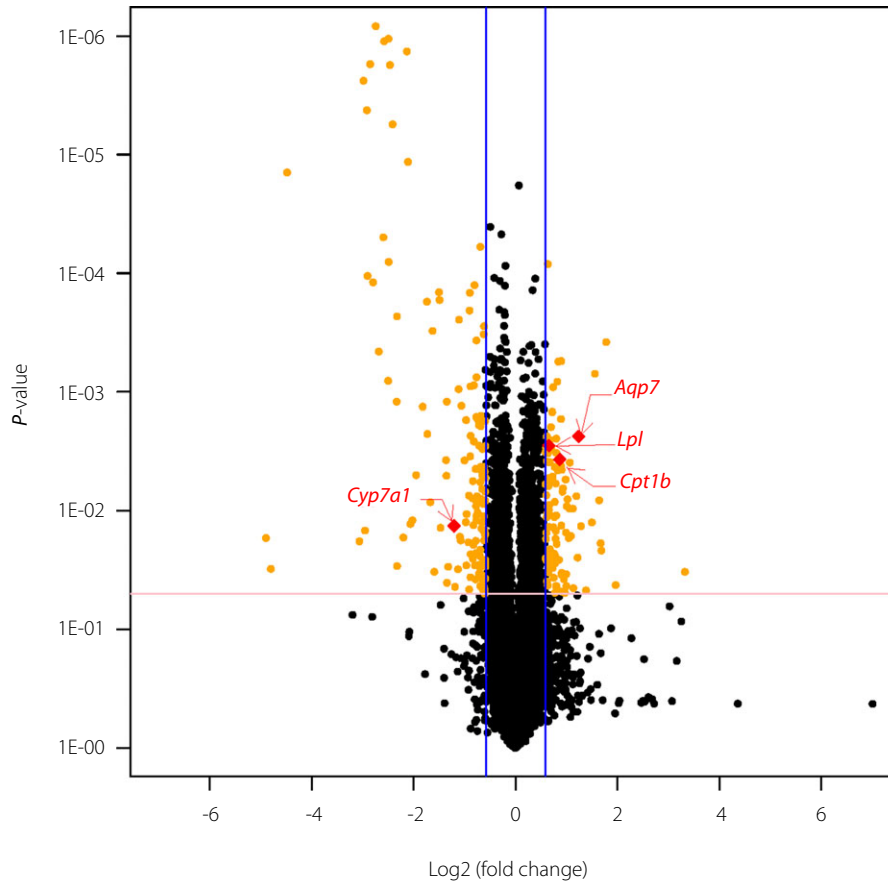
Figure 6 | The volcano plot graph of the differentially expressed genes of the offspring from the maternal low-protein diet (LPD) vs normal chow diet (NCD) groups at weaning. The volcano plot graph shows all differentially expressed genes based on the fold change and P-value. The blue lines show that the fold change in the gene expression threshold is 1.5 (log2 fold change = 0.59). The pink line shows that the P-value of the t-test threshold is 0.05. There were four genes located in the peroxisome proliferator-activated receptor signaling pathway. Aqp7, aquaporin 7; Cpt1b, carnitine palmitoyltransferase 1b; Lpl, lipoprotein lipase; Cyp7a1, cytochrome P450, family 7, subfamily a, polypeptide 1.

Table 4 | Fold change of gene expression measured through gene array and validated by quantitative real-time polymerase chain reaction

Gene symbol	Fold change (gene array)	P-value (gene array)	Fold change (qRT–PCR)	P-value (qRT–PCR)
Aqp7	2.358	0.002353	2.6 ± 0.52	0.0064*
Cpt1b	1.824	0.003674	1.7 ± 0.34	0.013*
Lpl	1.571	0.002817	1.6 ± 0.28	0.0284*
Cyp7a1	−2.308	0.01338	−2.5 ± 0.16	0.026*
PPAR-α	1.12	0.2977	1.07 ± 0.18	0.38
PPAR-γ	1.403	0.062	1.48 ± 0.03	0.046*

*$P < 0.05$ vs normal chow diet assessed by Student's t-test. All values are the mean ± standard error of the mean ($n = 12$ mice/group). Aqp7, aquaporin 7; Cpt1b, carnitine palmitoyltransferase 1b; Lpl, lipoprotein lipase; Cyp7a1, cytochrome P450, family 7, subfamily a, polypeptide 1; PPAR-α, peroxisome proliferator activator receptor alpha; PPAR-γ, peroxisome proliferator activator receptor gamma; qRT–PCR, quantitative real-time polymerase chain reaction.

obesity, hypertriglyceridemia, hepatic steatosis, insulin resistance, impaired glucose tolerance and diabetes, in the aging offspring[8]. Our present study provided the possibility that the occurrence of abnormal glucose metabolism occurs before aberrant lipid metabolism.

In our gene array experiments, the WebGestalt and Kyoto Encyclopedia of Genes and Genomes pathway analyses showed that all differentially expressed genes between the two groups were mapped to 11 pathways. The third most significant pathway was the 'PPAR signaling pathway,' and the four

differentially expressed genes in this pathway were further validated by qRT–PCR. Furthermore, PPAR isoforms α, δ and γ were also examined, because they are the critical genes in the PPAR signaling pathway. The present study shows for the first time that a maternal LPD restricted to the pregnancy and lactation periods can promote early-onset glucose intolerance in the offspring through the PPAR signaling pathway. In our study, it was shown that PPAR-γ increased significantly in the LPD group. Similarly, one study showed that prenatal exposure to a LPD programs disordered regulation of lipid metabolism and elevated PPAR-γ in the aging rat[8]. A previous study in rats also showed that a maternal LPD leads to alterations in PPAR gene expression (PPAR-α and PPAR-γ) in the offspring at 18 months-of-age[8]. Another study showed that hepatic PPAR-α gene expression was significantly higher later in the life of the offspring[22]. However, these studies only reflected a long-term effect during the life. Therefore, our study shows that PPAR-α and PPAR-γ might play a significant role in the glucose and lipid metabolism in the early life stage of the offspring.

The PPARs are members of the nuclear receptor superfamily of the ligand-inducible transcription factors, which exist in three isoforms, including PPAR-α, PPAR-β/δ and PPAR-γ[23]. PPARs have emerged as major regulators of carbohydrate and lipid metabolism[24]. Recently, they have been considered central regulators of glucose and lipid homeostasis, and molecular targets for drugs to treat hypertriglyceridemia and type 2 diabetes mellitus[25]. Thiazolidinediones (PPAR-γ agonists) are potent insulin sensitizers that are highly effective oral medications for type 2 diabetes[26]. A recent study showed that maternal diet-induced obesity contributes to metabolism disorders, and bezafibrate (a pan-PPAR agonist) can ameliorate these hepatic metabolic abnormalities[27]. These findings show that the PPAR signaling pathway could be of vital importance in regulating glucose homeostasis. Furthermore, the PPARs and their ligands have been proposed for the treatment of dyslipidemia, impaired glucose tolerance and type 2 diabetes mellitus[25].

In conclusion, our data suggest that a maternal LPD during pregnancy and lactation predisposes the offspring to the development of glucose intolerance and decreased insulin sensitivity in early life. Furthermore, the altered hepatic genes expression in the PPAR signaling pathway could play role in regulating this phenomenon. More importantly, these findings might be utilized as convincing evidence of the early intervention and prevention of some metabolic diseases during the early stage of life. Ultimately, selective and potent PPAR agonists might be useful agents to treat metabolic disorders based on a better understanding of the physiology of the PPAR signaling pathway and the molecular networks.

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