Maternal undernutrition leads to elevated hepatic triglycerides in male rat offspring due to increased expression of lipoprotein lipase

WEI-FEN ZHU¹, JIAN-FANG ZHU¹, LI LIANG¹, ZHENG SHEN² and YING-MIN WANG¹

¹Department of Pediatrics, The First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou, Zhejiang 310058; ²Department of Central Laboratory, Children’s Hospital of Zhejiang University School of Medicine, Hangzhou, Zhejiang 310003, P.R. China

Received February 12, 2015; Accepted December 8, 2016

DOI: 10.3892/mmr.2016.5040

Abstract. Small for gestational age (SGA) at birth increases the risk of developing metabolic syndrome, which encompasses various symptoms including hypertriglyceridemia. The aim of the present study was to determine whether maternal undernutrition during pregnancy may lead to alterations in hepatic triglyceride content and gene expression levels of hepatic lipoprotein lipase (LPL) in SGA male offspring. The present study focused on the male offspring in order to prevent confounding factors, such as estrus cycle and hormone profile. Female Sprague Dawley rats were arbitrarily assigned to receive an ad libitum chow diet or 50% food restricted diet from pregnancy day 1 until parturition. Reverse transcription quantitative polymerase chain reaction and western blot analysis were used to measure the gene expression levels of hepatic LPL at day 1 and upon completion of the third week of age. Chromatin immunoprecipitation quantified the binding activity of liver X receptor-α (LXR-α) gene to the LXR response elements (LXRE) on LPL promoter and LPL epigenetic characteristics. At 3 weeks of age, SGA male offspring exhibited significantly elevated levels of hepatic triglycerides, which was concomitant with increased expression levels of LPL. Since LPL is regulated by LXR-α, the expression levels of LXR-α were detected in appropriate for gestational age and SGA male offspring. Maternal undernutrition during pregnancy led to an increase in the hepatic expression levels of LXR-α, and enriched binding to the putative LXRE response elements in the LPL promoter regions in 3-week-old male offspring. In addition, enhanced acetylation of histone H3 [H3 lysine (K)9 and H3K14] was detected surrounding the LPL promoter. The results of the present study indicated that maternal undernutrition during pregnancy may lead to an increase in hepatic triglycerides, via alterations in the transcriptional and epigenetic regulation of the LPL gene.

Introduction

Fetal growth restriction is a common complication of pregnancy, and a significant cause of perinatal morbidity and mortality (1). Numerous studies have reported that adverse conditions during critical periods of development can alter physiological processes leading to metabolic diseases, including type 2 diabetes, hypertension, fatty liver disease and cardiovascular disease (2,3). Furthermore, the majority of small for gestational age (SGA) offspring exhibit compensatory growth during the first 2 years of life, which may contribute to a higher body fat mass from as young as 2-12 months of age (4), and increased body fatness and abdominal fat accumulation during childhood (5) and adulthood (6,7). Considering the social and economic burden of chronic metabolic disease, it is important to elucidate the underlying mechanisms and provide potential strategies for the prevention of long-term metabolic consequences in SGA offspring.

Lipoprotein lipase (LPL), which is a key lipid metabolism enzyme that hydrolyzes triglyceride (TG), provides free fatty acids (FFAs) for cells and affects the maturation of circulating lipoproteins (8,9). LPL has its own developmental genetic program, the activity and expression of which can vary greatly between tissues. In the liver tissue of fetal and neonatal rats high LPL activity has been detected (10); however, the expression of LPL progressively decreases and falls to nearly undetectable levels by the time of weaning (11), as determined by measuring age-related decreases in LPL activity, LPL synthesis and LPL mRNA expression (12). Numerous studies have suggested that, in the pathological state, LPL expression may undergo alterations resulting in various diseases, including atherosclerosis, obesity and diabetes (13-15). The present study aimed to determine whether hepatic LPL gene expression was altered in SGA male rat offspring, and to investigate the potential mechanisms underlying expression alterations.

Several nuclear receptors can activate the transcription of LPL, including peroxisome proliferator-activated receptors, sterol regulatory element-binding protein-1c and liver X receptors (LXRs) (13,16,17). LXRs are nuclear receptors
involved in the transcriptional regulation of de novo TG synthesis. Two isoforms of LXR: Liver X receptor-α (LXR-α) and LXR-β, have been identified in birds and mammals. As a more selective regulator of LPL than LXR-β, LXR-α binds to LXR response elements (LXRE), which contain a hexameric nucleotide direct repeat spaced by four bases (DR4), in the LPL promoter in order to govern regulation following activation by oxysterols (17). A previous study demonstrated that maternal protein restriction can alter rat LXR-α expression and lead to long-term epigenetic alterations in LXR target genes associated with lipid homeostasis (18). The present study hypothesized that maternal undernutrition during pregnancy-induced SGA male offspring would exhibit alterations in the expression of LPL, which may be mediated by increased or decreased binding of LXR-α to the LPL gene promoter.

Epigenetics serves a critical function in affecting gene transcription. Previous studies have focused on the identification of epigenetic dysregulation at the promoters of certain genes in SGA offspring. Park et al (19) demonstrated that histone modifications are involved in the effects of uteroplacental insufficiency on islet pancreatic and duodenal homeobox gene 1 transcription in rats. Sohi et al (18) indicated that maternal protein restriction leads to long-term decreases in histone H3 lysine (K)9 (H3K9) and H3K14 surrounding the promoter of the LXR target gene cholesterol 7α-hydroxylase, resulting in hypercholesterolemia in SGA offspring. The present study aimed to determine whether post-translational histone modifications may also influence the expression of LPL in SGA male rat offspring.

**Materials and methods**

**Animal model.** Animal experiments were performed at the Laboratory Animal Center of Zhejiang University (Hangzhou, China). All animal experimental procedures were approved by the Animal Ethics Committee of Zhejiang University, School of Medicine.

Briefly, 24 Sprague Dawley rats (16 male and 8 female; SLRC Laboratory Animal Co., Ltd., Shanghai, China) were housed under standard conditions (room temperature, 20-22°C; humidity, 40-60%). After 1 week of acclimation, male and female rats were mated overnight, and the presence of sperm in a vaginal smear was designated as gestational day 1. Pregnant rats were arbitrarily divided into two groups: The control group continued to receive an ad libitum diet throughout pregnancy; the maternal protein restriction group received 50% of their usual daily intake until parturition. The pregnant rats delivered spontaneously, and the litter size was randomly culled to eight per mother at birth, in accordance with the criteria for AGA and SGA, were culled. The criteria for AGA were as follows: Offspring of normal intake and birth weight -2 SD of the AGA group. Following parturition, mothers from the food restricted mothers were sacrificed by the administration of 20 ml chloral hydrate (J&I Biological, Shanghai, China). The pups were cross fostered from food-restricted mothers to ad libitum-fed mothers. Both groups were given ad libitum access to food. At 1 day and 3 weeks of age, the rats were sacrificed by anesthesia (chloral hydrate; dose, 2 ml 1-day-old rats and 10 ml for 3-week-old rats). The ad libitum mothers were sacrificed by the administration of 20 ml chloral hydrate. Liver tissue samples were harvested and snap-frozen in liquid nitrogen for subsequent processing.

**Hepatic TG content.** Total hepatic TG content was determined using a GPO-PAP enzymatic assay (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Briefly, liver tissue (30-50 mg) was homogenized in ethanol (9 ml ethanol, 1 g liver sample), the mixture was microfuged at 664 x g for 10 min at 4°C, and the supernatant was transferred to new tubes. The reaction system was prepared according to the manufacturer’s instructions. The GPO-PAP enzymatic assay protocol includes the following steps: First, the lipids are broken down via the hydrolysis of triglycerides into glycerol and FFAs. Then the glycerol is converted to glycerol 3-phosphate via adenosine triphosphate and glycerol kinase, and is further converted to dihydroxycetone phosphate and hydrogen peroxide via glycerophosphate oxidase. Under the effect of peroxidase, red quinones are produced when hydrogen peroxide meets 4-amino-antipyrine and 4-chlorophenol. The color degree of quinones is proportional to triglyceride concentration. Following a 5 min incubation at 37°C, the final mixtures in 96-cell plates were rapidly quantified at 500 nm using a microplate reader (Varioskan Flash 3001, Thermo Fisher Scientific, Inc., Waltham, MA, USA). Quantification of TG content was based on the following calculation: ([Sample optical density (OD) value - blank OD value] × calibration OD value - blank OD value) x calibration concentration. Finally, data were expressed as mg/g of liver.

**RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis.** Briefly, total RNA was isolated from the liver tissue samples using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). High-Capacity cDNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used to reverse transcribe 2 μg total RNA using the following program: 25°C for 10 min, 37°C for 120 min, 85°C for 5 min, and a 4°C hold. To measure the relative mRNA expression levels, RT-qPCR was performed using an Applied Biosystems StepOnePlus™ Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The PCR cycling conditions were as follows: 94°C for 10 min, followed by 40 cycles at 94°C for 20 sec and 60°C for 1 min. RT-qPCR was performed using SYBR® Select Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) All reactions were performed in triplicate. The relative mRNA expression levels were calculated using the 2^ΔΔCt method (20). The primer sequences were as follows: LXR-α, forward 5’-GAGAGCATCCACCTTTCAAG-3’; reverse 5’-CTACGGATCTGGAGACCTCAAG-3’; LPL, forward 5’-ACAGGGCAATTCAAGAG-3’; reverse 5’-CCTTCA GCCACGTGCCCATA-3’; and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), forward 5’-GACACATTGGGC ATCGTGGA-3’ and reverse: 5’-ATGCAGGGATGATGT
Western blotting. The liver tissue samples were homogenized in lysis buffer (Beyotime Institute of Biotechnology, Hangzhou, China) and centrifuged at 12,000 x g for 10 min at 4°C. The protein concentration was determined using a Bicinchoninic Acid Protein Assay kit (Beyotime Institute of Biotechnology). Equal amounts of protein (80 µg) were separated by 10% sodium dodecyl sulfate (SDS)-polyacylamide gel electrophoresis and electroblotted onto a polyvinylidene difluoride membrane (0.2 µm pore size; EMD Millipore, Billerica, MA, USA). The membrane was blocked in 5% non-fat-milk for 2 h at room temperature. Following blocking, the membrane was incubated at 4°C with anti-LXR-α (dilution 1:3,000; cat. no. ab41902; Abcam (Hong Kong) Ltd., Hong Kong, China), anti-LPL (dilution 1:200; cat. no. sc-32885; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and anti-GAPDH antibodies (dilution 1:5,000; cat. no. 5174; Cell Signaling Technology, Inc., Danvers, MA USA) for 12 h. The blots were analyzed by ImageJ version 1.39 software (National Institutes of Health, Bethesda, MD, USA). Subsequently, the membrane was incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (IgG) (HRP-labeled (cat. no., A0208; dilution 1:2,000; Beyotime Institute of Biotechnology) and HRP-labeled goat anti-mouse IgG secondary antibodies (cat. no., A0216; dilution 1:2,000; Beyotime Institute of Biotechnology) for 2 h at room temperature. Signals were detected using enhanced chemiluminescence, according to the manufacturer's protocol (SuperSignal chemiluminescent substrates; Pierce; Thermo Fisher Scientific, Inc.).

Chromatin immunoprecipitation (ChIP). The ChIP assay was performed according to the manufacturer's protocol (EZ-ChIP kit; EMD Millipore). Liver tissue samples (40 mg) were fixed in 1% formaldehyde for 10 min at room temperature. Cross-linking was terminated by the addition of glycine (1 M). Following two washes with cold phosphate-buffered saline, the liver tissue was resuspended in 1 ml SDS lysis buffer supplemented with 5 µl 1X protease inhibitor cocktail II. The lysates were aliquoted to 300–400 µl per microfuge tube and were sonicated on ice, in order to shear the DNA to a length between 200 and 1,000 bp. The sheared crosslinked chromatin was diluted 10-fold in dilution buffer containing protease inhibitor cocktail II. The chromatin solution was pre-cleared with 60 µl protein G agarose at 4°C for 1 h with rotation. The chromatin solutions were then microfuged at 4,000 x g for 1 min at 4°C to pellet agarose, and the supernatant was placed in new tubes, with 10 µl removed as input. The supernatant fractions were incubated overnight on a rocking platform with antibodies against acetylated histone H3K9 [4 µg; cat. no. ab10812; Abcam (Hong Kong) Ltd.], acetylated histone H3K14 [4 µg, cat. no. ab52946; Abcam (Hong Kong) Ltd.] and ChIP-grade LXR-α [5 µg; cat. no. ab41902; Abcam (Hong Kong) Ltd.] at 4°C. Subsequently, 60 µl protein G agarose was added to the tubes, which were incubated on a rocking platform for 1 h at 4°C. Following centrifugation at 4,000 x g at 4°C for 1 min, the agarose beads containing the immunoprecipitated complexes were washed sequentially in Low Salt Immune Complex Wash Buffer, High Salt Immune Complex Wash Buffer, LiCl Immune Complex Wash Buffer, and 2X TE buffer. The immune complexes were eluted twice with 200 µl elution buffer (10 µl 20% SDS, 20 µl 1 M NaHCO₃, 170 µl sterile distilled water) at room temperature. Elution buffer (200 µl) was also added to the input tubes. Subsequently, 8 µl 5 M NaCl was added to the elute and the cross-linking of the immunoprecipitated chromatin complexes and input controls were reversed by heating at 65°C for 5 h. Following treatment with Proteinase K, Tris-HCl and EDTA for 2 h at 45°C, the DNA was purified, according to the protocol of the manufacturer of the EZ-CHIP kit (EMD Millipore, Billerica, MA, USA).

The putative LXR-binding site in the promoter region of LPL was determined using MatInspector Software (http://www.genomatix.de/online_help/help_matinspector/matinspector_help.html). Primers were purchased from Invitrogen (Thermo Fisher Scientific, Inc.) and were as follows: Forward (5’-ATT CTCACACTTGTTCCTTTG-3’) and reverse (5’-GCT TGA GGT TGC TGC TGC GAA CT-3’) primers that amplify -3438 to -3423 promoter regions encompassing the rat LPL LXRE site (GAG GCC_DR4_GAGG), and primers (promoter A1, forward 5’-TCTGCTTTTGTCTGGAACT-3’, reverse 5’-AGACGA AACGACACCTGA-3’, promoter A2, forward 5’-CACTGT AACGGGCTCAACG-3’, reverse 5’-GTGACATTTGCT CGAGTTCG-3’, and promoter A3, forward 5’-GAGCGAAG ATCATGTTGAAATA-3’ and reverse 5’-CTCCGTCTTCTCA GTACGATTAT-3’) surrounding the promoter were used to examine the acetylation status of acetylation of histone H3 (K9, K14) at the promoter of LPL. For negative controls, ChIP assays were performed using an immunoglobulin G antibody (1 µg; part of the EZ-ChIP kit) to determine the immunospecificity of the antibodies for the LPL promoter. The DNA samples from the input, unbound and bound fractions were determined by qPCR, according to the previously described protocol. The relative abundance of the immunoprecipitated chromatin, as compared with the input chromatin was determined using the 2^ΔΔCt method (20).
Statistical analysis. SPSS 18.0 (SPSS, Inc., Chicago, IL, USA) was used for data analysis. All data are expressed as the mean ± standard error of the mean. Statistical significance was calculated using Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

Hepatic TG content. At 1 day of age, no differences were evident in hepatic TG levels between the AGA and SGA male rats (AGA, 17.39±3.25 mg/g; SGA, 18.68±2.05 mg/g; P>0.05) (Fig. 1). At 3 weeks of age, the hepatic TG levels were increased in the SGA male rats (AGA, 5.18±0.63 mg/g; SGA, 7.73±0.91 mg/g; P<0.05), as compared with the AGA rats.

Hepatic LXR-α mRNA expression levels are increased, concomitant with an increase in LPL mRNA in 3-week-old SGA rats. To obtain further information regarding the differences in protein expression, western blotting was performed (Fig. 3). The protein expression levels of LXR-α and LPL were increased (P<0.05) in the SGA male rats, as compared with the AGA rats at 3 weeks of age (Fig. 3B and D); however, no differences were detected between the SGA and AGA male rats at 1 day of age (Fig. 3A and C).

LXR-α binding to the LXRE in the promoter regions of LPL is increased in 3-week-old SGA rats. To determine whether there were alterations in the recruitment of LXR-α to the promoter regions of LPL containing a well-characterized LXRE site, ChIP analyses were performed with antibodies specific for LXR-α. Negative controls demonstrated that the immunoprecipitations were specific for the indicated antibodies. At 3 weeks of age, the SGA male rats exhibited a marked increase in the binding of LXR-α to the promoter regions of LPL, as compared with in the AGA rats (P<0.05) (Fig. 4).
Acetylation of lysine residues 9 and 14 on histone H3 surrounding the promoter regions of LPL is increased in 3-week-old SGA rats. ChIP was further used to investigate whether chromatin remodeling could be a factor influencing the observed increase in LPL mRNA and protein expression levels in 3-week-old male SGA rats. Three sites (A1: -87 to +177; A2: -136 to -533 and A3: -644 to -896) along the LPL promoter were analyzed. The hepatic levels of acetylated histone H3K9 in the LPL promoter A1, A2 and A3 regions of SGA rats were increased 1.71-fold, 2.73-fold and 2.50-fold, respectively (P<0.05), as compared with the AGA rats (Fig. 5A). The hepatic levels of acetylated histone H3K14 in the LPL promoter A1, A2 and A3 regions of SGA rats were increased 1.69-fold, 1.86-fold and 2.67-fold, respectively (P<0.05), as compared with the AGA rats (Fig. 5B).

Discussion

The present study demonstrated that 3-week-old SGA male offspring exhibited increased hepatic TG levels. In liver tissue, the fatty acid biosynthesis pathway facilitates excess energy storage, either as cytosolic lipid droplets or circulating TG-rich lipoproteins (21). These TG may provide energy during times of deficiency following oxidation; however, the excess accumulation of hepatic TG is a risk factor for cardiovascular disease. Therefore, a better understanding regarding the molecular determinants that control fatty acid metabolism and hepatic TG levels may facilitate the development of effective interventions that reduce the metabolic risk factors for SGA male offspring.

The results of the present study demonstrated that alterations in hepatic TG content were closely paralleled with changes in hepatic mRNA and protein expression levels of LPL, which implicated LPL in the development of hepatic lipid dysregulation. A previous study reported that in fetal plasma from pregnancies characterized by SGA, altered LPL plasma exchange, which may contribute to an abnormal lipid profile (22). Kim et al (23) demonstrated that mice with...
liver-specific LPL overexpression manifested hepatic steatosis and insulin resistance. In addition, at the time of weaning, LPL mRNA expression is nearly undetectable in normal rat livers; therefore, the hepatic expression of LPL in SGA male rats may not only enable the liver to hydrolyze TG from chylomicrons and very-low-density lipoprotein, but may also lead to an increase in the hepatic uptake of FFA, which may induce hepatic steatosis (24).

To the best of our knowledge, LPL mRNA expression levels have been widely evaluated in the SGA placenta. Gauster et al. (25) compared the LPL expression between normal pregnancies and those complicated with SGA; the results demonstrated that LPL was markedly increased (2.4-fold; P<0.015) in SGA placentas. In addition, Tabano et al. (26) detected an increase in LPL mRNA expression in severe SGA cases with abnormal umbilical blood flow, as compared with AGA placentas. The results of the present study combined with findings from previous studies led us to hypothesize that increased expression levels of LPL may persist into postnatal life, and may be involved in the development of metabolic disease in SGA male offspring.

It is well known that the regulation of LPL gene expression is complex, occurring at transcriptional, translational and post-translational levels. The present study hypothesized that alterations in LPL expression in SGA rats may occur via sensitive upstream transcriptional regulators, such as the LXR-α gene. To further characterize the mechanism involved, ChIP coupled with qPCR methods were used to study the exact mechanism. The results suggested that LPL expression is mediated by LXR-α, which interacts with LXRE sequences spanning the -3438 to -3423 promoter regions in hepatic LPL. The nuclear receptor LXR-α is emerging as a key regulator of lipid homeostasis, which is primarily expressed in the liver, intestine, adipose tissue and macrophages. In addition to LPL, LXR-α also regulates numerous genes involved in fatty acid synthesis, including fatty acid synthase, acetyl CoA carboxylase and the sterol-regulatory element binding protein 1 (27-29). Previous animal studies have demonstrated that administration of the LXR ligand, TO-901317, may cause severe fatty liver and obesity (30,31). Since overexpression of the LXR-α gene may increase fatty acid synthase via its target genes, it may be considered a suitable target for therapeutic intervention, in order to prevent hepatic fatty infiltration in SGA male offspring.

The present study demonstrated that gene expression is not the only molecular phenotype affected by maternal nutritional manipulation. Epigenetic states can also be modified by environmental factors, resulting in transcriptional expression or silencing. Acetylation of H3K9 and H3K14 is generally believed to be associated with actively transcribed genes (32,33), which are congruent with the increased mRNA and protein expression levels of LPL observed in the present study. Since hepatic development occurs throughout neonatal and early postnatal life, it is plausible that targeting this short period of development may help reverse or prevent adverse hepatic epigenetic phenotypes in SGA offspring.

In conclusion, the present study demonstrated that maternal undernutrition during pregnancy and subsequent SGA may result in postnatal alterations in the epigenetic characteristics
of the LPL gene, and increased binding of LXR-α to the LXRE in LPL promoter regions. These alterations were associated with predictable changes in LPL mRNA and protein expression levels, and may result in elevated hepatic TG content.

Acknowledgements

The present study was supported by the National Science Foundation of China (grant nos. 81170733 and 81000267).

References