Maternal protein restriction leads to enhanced hepatic gluconeogenic gene expression in adult male rat offspring due to impaired expression of the liver X receptor

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Abstract

Epidemiological studies demonstrate that the link between impaired fetal development and glucose intolerance in later life is exacerbated by postnatal catch-up growth. Maternal protein restriction (MPR) during pregnancy and lactation in the rat has been previously demonstrated to lead to impaired glucose tolerance in adulthood, however the effects of protein restoration during weaning on glucose homeostasis are largely unknown. Recent in vitro studies have identified that the liver X receptor \( \alpha \) (LXR\( \alpha \)) maintains glucose homeostasis by inhibiting critical genes involved in gluconeogenesis including G6pase (G6pc), 11\( \beta \)-Hsd1 (Hsd11b1) and Pepck (Pck1). Therefore, we hypothesized that MPR with postnatal catch-up growth would impair LXR\( \alpha \) in vivo, which in turn would lead to augmented gluconeogenic LXR\( \alpha \)-target gene expression and glucose intolerance. To examine this hypothesis, pregnant Wistar rats were fed a control (20%) protein diet (C) or a low (8%) protein diet during pregnancy and switched to a control diet at birth (LP). At 4 months, the LP offspring had impaired glucose tolerance. In addition, LP offspring had decreased LXR\( \alpha \) expression, while hepatic expression of 11\( \beta \)-HSD1 and G6Pase was significantly higher. This was concomitant with decreased binding of LXR\( \alpha \) to the putative LXRE on 11\( \beta \)-Hsd1 and G6pase. Finally, we demonstrated that the acetylation of histone H3 (K9,14) surrounding the transcriptional start site of hepatic Lxr\( \alpha \) (Nr1h3) was decreased in LP offspring, suggesting MPR-induced epigenetic silencing of the Lxr\( \alpha \) promoter. In summary, our study demonstrates for the first time the important role of LXR\( \alpha \) in mediating enhanced hepatic gluconeogenic gene expression and consequent glucose intolerance in adult MPR offspring.

Key Words
- fetal programming
- liver X receptor
- gluconeogenesis
- nuclear receptors
- maternal protein restriction

Introduction

Epidemiological evidence suggests that adverse events in utero (e.g. placental insufficiency-induced intrauterine growth restriction (PI-IUGR)) can permanently alter physiological processes leading to hypertension and type 2 diabetes (Barker et al. 1993, Nilsson et al. 1997, Jaquet et al. 2000, Levitt et al. 2000, Huxley et al. 2007).
Previous animal models of maternal protein restriction (MPR) have consistently linked asymmetric IUGR (Desai & Hales 1997) with symptoms of type 2 diabetes long-term in the offspring. For example, Petrik et al. (1999) demonstrated a low protein diet during pregnancy and weaning induced a decrease in birth weight and disrupted pancreatic β-cell proliferation in the adult offspring. Other studies have found altered glucagon-stimulated and insulin-stimulated hepatic glucose output as well as reduced glucokinase expression and structural modifications in the livers of low protein offspring (Ozanne et al. 1996, Burns et al. 1997). In addition, Chamson-Reig et al. (2009) have demonstrated that low protein offspring have impaired glucose tolerance as early as 130 days of age in rat offspring. Thus, the evidence strongly suggests that maternal low protein-mediated IUGR in the rat predisposes the offspring to impaired glucose tolerance and a type 2 diabetes-like phenotype. However, the molecular mechanisms underlying these low protein-induced alterations in the output of hepatic glucose are not completely understood.

The liver X receptor (LXR) is a transcription factor belonging to the 1H subfamily of nuclear receptors. LXR exists as two isoforms: LXRα and LXRβ. LXRα is mainly expressed in the liver, adipose tissue, macrophages and intestines (Apfel et al. 1994, Willy et al. 1995), while LXRβ is ubiquitously expressed (Song et al. 1994). Endogenous ligands for LXR are mainly derivatives of cholesterol (i.e. oxysterols; Lehmann et al. 1997, Song & Liao 2000). Consequently, LXR has principally been implicated in regulating genes involved in the metabolism and transport of cholesterol (Lehmann et al. 1997, Venkateswaran et al. 2000) and in enhancing the expression of lipogenic enzymes (Repa et al. 2000). Recent studies have also demonstrated that LXR can silence genes involved in glucose production including phosphoenolpyruvate kinase (PEPCK) and glucose-6-phosphatase (G6Pase), both critical enzymes involved in the gluconeogenic pathway (Stulnig et al. 2002a,b, Cao et al. 2003). In addition, LXR has also been found to indirectly down-regulate hepatic glucose production through inhibition of the enzyme 11β-hydroxysteroid dehydrogenase-1 (11β-HSD1; Stulnig et al. 2002a). 11β-HSD1 reduces inactive corticosteroids to their active form (e.g. 11-dehydrocorticosterone to corticosterone in the rodent). Since active corticosteroids are responsible for increased glucose production, LXR-mediated inhibition of 11β-HSD1 would indirectly decrease glucose production.

Previous studies from our own laboratory have found that MPR leads to decreases in the expression of the LXR-target gene, Cyp7a1, the critical enzyme involved with cholesterol catabolism. The decrease in Cyp7a1 leads to hypercholesterolemia in male offspring by 4 months (Sohi et al. 2011). This was found to be due, in part, to repressive changes in histone modifications at the LXRE site of the Cyp7a1 promoter. Other studies in mice have demonstrated that MPR leads to hypermethylation of the Lxrα (Nr1h3) promoter in association with decreased Lxrα mRNA in the liver tissue of embryonic day 19.5 fetuses, however the effect on posttranslational histone modifications surrounding Lxrα remains elusive (van Straten et al. 2010). While others and we have demonstrated that MPR can lead to long-term epigenetic alterations of LXR-target genes involved in cholesterol and lipid homeostasis, it is not known if LXR-target genes impairing hepatic gluconeogenesis are altered.

The aims of the present study were to examine whether MPR alters LXRα-mediated gluconeogenesis in the liver. Given the role of LXRα in lipid, glucose and cholesterol homeostasis, it is an attractive candidate in elucidating the molecular mechanisms underlying IUGR-related fetal programming. We hypothesized that decreased maternal protein availability during gestation would impair hepatic gluconeogenesis in the adult offspring through decreases in LXRα and aberrant activity of its target genes (G6pase (G6pc), Pepck (Pck1) and 11β-Hsd1 (Hsd11b1)). Using a well-established model of MPR in rat pregnancy, we assessed the effects of a low protein diet in gestation on long-term glucose handling, LXRα activity, and the expression of hepatic LXR-target genes involved in gluconeogenesis. In the control group, dams were fed a 20% protein diet throughout life. Low protein dams received an 8% protein diet until birth of the offspring, followed by a 20% protein diet during the weaning period (until postnatal day 21). We decided to examine the effects of restoring protein immediately after birth as opposed to waiting until after the weaning period because we have already demonstrated that earlier restoration of protein promotes accelerated catch-up growth (Sohi et al. 2011). Moreover, postnatal accelerated growth of IUGR offspring has been demonstrated to exacerbate the effects of IUGR-related programming and reduce the lifespans of these offspring (Ozanne & Hales 2004, Chen et al. 2009, Tarry-Adkins et al. 2013).

**Materials and methods**

**Animal experiments and dietary regime**

All procedures were performed in accordance with the guidelines set by the Canadian Council of Animal Care and upon approval of the Animal Care Committee of the
University of Western Ontario. Male and female Wistar rats at breeding age (250 g) were purchased from Charles River (La Salle, St-Constant, QC, Canada) and were allowed to acclimatize to their new environment for 2 weeks. Rats were housed at room temperature on a 12 h light:12 h darkness cycle. Females were housed in separate cages and were cohabitated with a male for mating upon entering proestrus. Conception was confirmed by the presence of sperm in the vaginal smear the following day.

Dams and offspring received isocaloric diets (Bio-Serv, Frenchtown, NJ, USA) varying in protein composition, depending on their experimental group. Briefly, the control offspring and dams received 20% protein throughout life. Protein-restricted dams received low protein chow (8%) throughout gestation and then restored on a 20% protein chow immediately after birth (herein termed ‘LP’). All diets and water were administered ad libitum. Previous studies by our laboratory have demonstrated that the food intake between both offspring groups is practically identical (Sohi et al. 2011).

At embryonic day 19, a subset of dams (three control dams; four LP dams) was sacrificed and livers from the fetuses were extracted. The livers were flash frozen for further molecular analysis. The other subset of dams (four control dams; four LP dams) delivered spontaneously. All litters with less than ten pups were arbitrarily culled down to 9–10 pups to ensure a consistent litter size per dam.

After their intraperitoneal glucose tolerance tests (IPGTT) at postnatal days 120–125, all offspring were sacrificed using a lethal dose (50 mg/kg) of Euthanyl forte pentobarbital sodium (Bimeda-MTC, Cambridge, ON, Canada) at postnatal day 130. This age was chosen because previous studies have demonstrated that the food intake between both offspring groups is practically identical (Sohi et al. 2011). Total RNA was subsequently treated with deoxyribonuclease to eliminate contaminating DNA. Four micrograms of total RNA were then reverse transcribed to cDNA using random primers and Superscript II RNase H-reverse transcriptase (Invitrogen). TaqMan probes and sequences for the genes of interest (11β-Hsd1, G6pase, Lxrα, Pepck and β-actin (Actb)) and TaqMan Universal Master Mix were obtained from Invitrogen. Quantitative analysis of mRNA expression was measured using the Bio-Rad CFX384 Real Time System. The cycling conditions were as follows: polymerase activation (95 °C for 10 min) followed by 40 cycles of denaturing (95 °C for 15 s) and annealing (60 °C for 1 min).

Glucose tolerance tests

At postnatal days 120–125, male offspring were subject to an IPGTT. Prior to the IPGTT, the animals were fasted overnight for 14–16 h. Animals were awake throughout the experiment. Blood glucose measurements were obtained using a Bayer Breeze 2 Blood Glucose Meter (Bayer). Fasted blood glucose levels were obtained prior to the glucose injection. Animals then received 2 g/kg of glucose via injection into the i.p. cavity. Blood glucose was sampled at the tail vein at t = 0, 5, 10, 15, 30, 60 and 120 min. Area under the curve of each animal was calculated using (GraphPad Prism Software, San Diego, California, USA, www.grapahpad.com). IPGTTs were performed on six control males and ten LP males.

Quantitative real-time PCR for gene expression analysis

Total RNA was extracted from the medial lobe of offspring livers at embryonic day 19 and postnatal day 130 as previously described, using the one-step TRizol (Invitrogen) method (Sohi et al. 2011). Total RNA was subsequently treated with deoxyribonuclease to eliminate contaminating DNA. Four micrograms of total RNA were then reverse transcribed to cDNA using random primers and Superscript II RNase H-reverse transcriptase (Invitrogen). TaqMan probes and sequences for the genes of interest (11β-Hsd1, G6pase, Lxrα, Pepck and β-actin (Actb)) and TaqMan Universal Master Mix were obtained from Invitrogen. Quantitative analysis of mRNA expression was measured using the Bio-Rad CFX384 Real Time System. The cycling conditions were as follows: polymerase activation (95 °C for 10 min) followed by 40 cycles of denaturing (95 °C for 15 s) and annealing (60 °C for 1 min). The cycle threshold was set where the exponential increase in amplification was equivalent between all samples. Relative fold changes were calculated using the comparative cycle times (Ct) method with β-actin as the reference gene. ΔCt values for each probe set were standardized to the experimental samples with the lowest transcript abundance (highest Ct value). The relative abundance of each primer set compared with calibrator was determined by the formula, 2^ΔΔCt, where ΔΔCt was the standardized Ct value.

Tissue protein extraction and western immunoblotting

Tissue protein was extracted from the medial lobe of snap-frozen offspring livers using a lysis buffer solution (pH 7.4, Tris–HCl 50 mM, NP-40 1%, sodium deoxycholate 0.25%,...
NaCl 150 mM, EDTA 1 mM, NaF 50 mM, Na₂VO₄ 1 mM and β-glycerophosphate 25 mM). Prior to tissue homogenization, a mini protease inhibitor tablet was added to the lysis buffer.

First, a small chunk of snap-frozen liver was added to 600 µl of RIPA buffer. The tissue was then homogenized with the IKA T10 Basic S1 Dispersing Tool (IKA Works, Inc., Wilmington, NC, USA) for 10–15 s at speed 6. After letting the homogenized tissue sit on ice for 5 min, the tissue was then sonicated. Following sonication, the tissue was rotated at 4 °C for 5 min and then centrifuged for 15 min at 300 g and 4 °C. The supernatant was retained for further centrifugation at 20 000 g for 20 min at 4 °C. The final supernatant was retained for protein quantification and western immunoblotting.

Equal concentrations of total protein were normalized using a colorimetric BCA Protein Assay (Pierce Corp., Madison, WI, USA). Proteins were then fractionated in 17-well gradient polyacrylamide gels (Invitrogen) and transferred onto PVDF membrane (Millipore, Etobicoke, ON, Canada). Amido black and Coomassie brilliant blue staining confirmed sufficient transfer of proteins onto the membrane.

Immunoblots were probed using LXRα (LXR; 1:1000; cat no. sc-13068), PEPCK (1:200; cat no. sc-32879), G6Pase-α (1:1000; cat no. sc-25840), PI3-kinase p85α (Z-8; 1:1000; cat no. sc-423) and 11β-HSD1 (1:800; cat no. sc-20175) all purchased from Santa Cruz Biotechnology. In addition, p-Akt1 (Serine 473; 1:1000; cat no. ab66138), p-Akt (Threonine 308; 1:500; cat no. 4796) and Akt (1:125; cat no. ab6076) antibodies used to assess hepatic insulin sensitivity were purchased from Abcam, Inc. (Cambridge, MA, USA). In addition, we also assessed insulin sensitivity by antibodies against p-IRS-1 (Serine 302; 1:500; cat no. 2384), p-IRS-1 (Serine 1101; 1:500; cat no. 2385) and IRS-1 (1:500; cat no. 2382) all purchased from Cell Signaling (Danvers, MA, USA). Monoclonal HRP conjugated β-actin (1:50 000; cat no. A3854; Sigma–Aldrich) diluted in 5% milk–TBS–TWEEN-20 (0.1%) buffer and HRP conjugated donkey anti-rabbit IgG (1:10 000, cat no. 711-035-152; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) diluted in 5% milk–TBS–TWEEN-20 (0.1%) buffer were used as the secondary antibodies. Finally, immunostained bands were then visualized using an ECL detection system (Thermo Scientific, Waltham, MA, USA).

Chromatin immunoprecipitation

Chromatin was extracted from the medial lobe of offspring livers as previously described (Sohi et al. 2011). Briefly, a small piece of snap-frozen liver was homogenized and incubated with 1% formaldehyde for 10 min at room temperature to cross-link proteins and DNA. Cross-linking was terminated by the addition of glycine (0.125 M, final concentration). The liver tissue was washed once with cold PBS and placed in 500 µl of SDS lysis buffer (Millipore) with a protease inhibitor cocktail (Roche). The lysates were sonicated on ice to produce sheared, soluble chromatin. The lysates were diluted 10 times with the addition of chromatin immunoprecipitation (ChIP) dilution buffer (Millipore) and aliquoted to 400 µl amounts. Each of the aliquots was precleared with protein A/G Plus agarose beads (40 µl; Millipore) at 4 °C for 30 min. The samples were centrifuged at 20 000 g to pellet the beads, and the supernatant containing the sheared chromatin was placed in new tubes. The aliquots were incubated with 4 µg of antibodies against RNA polymerase II (cat no. 05-623B; Millipore), trimethylated histone H3 lysine 4 (K4; cat no. ab1012; Abcam, Inc.), acetylated histone H3 lysine 9,14 (K9,14; cat no. 05-399; Millipore), trimethylated histone H3 lysine 9 (K9; cat no. 07-442; Millipore) and ChIP-grade LXRα (cat no. sc-13068x; Santa Cruz Biotechnology) at 4 °C overnight. Two aliquots were reserved as ‘controls’ – one incubated without antibody and the other with nonimmune IgG (Millipore). Protein A/G Plus agarose beads (60 µl) were added to each tube, the mixtures incubated for 1 h at 4 °C and the immune complexes collected by centrifugation. The beads containing the immunoprecipitated complexes were washed sequentially for 5 min in wash buffer I (20 mM Tris–HCl, pH 8.1, 2 mM EDTA, 0.1% SDS, 1% Triton X-100 and 150 mM NaCl), wash buffer II (same as I, except containing 500 mM NaCl), wash buffer III (10 mM Tris–HCl, pH 8.1, 1 mM EDTA, 1% NP-40, 1% deoxycholate and 0.25 M LiCl), and in 2× TE buffer. The beads were eluted with 250 µl elution buffer (1% SDS, 0.1 mM NaHCO₃+20 g salmon sperm DNA (Sigma–Aldrich)) at room temperature. This was repeated once and eluates were then combined. Cross-linking of the immunoprecipitated chromatin complexes and ‘input controls’ (10% of the total soluble chromatin) was reversed by heating the samples at 65 °C for 4 h. Proteinase K (15 µg; Invitrogen) was added to each sample in buffer (50 mM Tris–HCl, pH 8.5, 1% SDS and 10 mM EDTA) and incubated for 1 h at 45 °C. The DNA was purified by phenol–chloroform extraction and precipitated in EtOH overnight at 20 °C. Samples and ‘input controls’ were diluted in 10–100 µl TE buffer just prior to quantitative real-time PCR (qRT-PCR).

Putative LXR-binding sites (threshold of 0.7) on the promoters of G6pase and 11β-Hsd1 were determined using...
the MatInspector Software (Genomatix, Munich, Germany). The MatInspector Software was used to match the LXR consensus binding site (AGGTCA_DR-4_AGGTCA; Willy et al. 1995) with putative transcription factor binding sequences based on algorithms as described by Cartharius et al. (2005). qRT-PCR was employed using forward (5'-GGTCACCTGATGACACAGG-3') and reverse (5'-CCTTGGAATCCAGAATGCTC-3') primers that amplify a −35 to +92 bp region encompassing the rat G6pase LXRE site (+22 to +46 bp), and forward (5'-TTCGCCAAACTCTGACCTCT-3') and reverse (5'-ACAGTTTTGGCCTGGAT-3') primers that amplify a −115 to −7 bp region encompassing the rat 11ß-Hsd1 LXRE site (−114 to −90 bp) (PE Applied Biosystems). The LXRs (gene: Nr1h3) transcriptional start site (TSS) was found using the Ensembl Genome Browser (http://www.ensembl.org). Forward (5'-GGTCACCTGATGACACAGG-3') and reverse (5'-AGGGGGTTGATTCTTGAGGT-3') primers were designed to amplify the −135 to +144 bp region surrounding the +1 bp TSS of Lxrα. Recent evidence indicates that there is epigenetic regulation in the CG-rich regions of the Lxrα promoter around the TSS in another rodent model of MPR (van Straten et al. 2010). Thus, primers around the promoter were used to examine the binding of RNA polymerase II, acetylation of histone H3 (K9,14), methylation of histone H3 (K4) and trimethylation of histone H3 (K9) at the TSS of Lxrα.

The aforementioned constructed ChIP primers were then used in conjunction with Sso-Fast EvaGreen Supermix (Bio-Rad) to perform qRT-PCR. Similar to the gene expression assays, the relative abundance of the immunoprecipitated chromatin compared with input chromatin was determined using the 2^ΔΔCT method.

**Statistical analysis**

All data are represented as a mean of an arbitrary value ± S.E.M. IPGTT, areas under the curve, qRT-PCR (including ChIP) and quantified western immunoblot bands were analyzed using the unpaired Student’s t-test. All data with a P value <0.05 were considered statistically significant.

**Results**

**MPR leads to impaired glucose tolerance at 4 months of age in male offspring**

At 4 months of age, all of the male offspring underwent an IPGTT to assess fasted glucose tolerance after an administered glucose load. Resting levels of glucose were not significantly different between control and LP animals. After administration of the glucose (2 g/kg), measured blood glucose levels were significantly elevated (P<0.05) in LP animals at the 10-, 15-, 30- and 60-min time points (Fig. 1A). Blood glucose levels in the LP animals returned to the same levels as the control animals by the 120-min time point. At the end of the experiment, both control and LP animals had similar blood glucose levels. The area under the curve for the LP animals was increased by 32.8% (P<0.05) compared with the control animals (Fig. 1B), further indicating impaired glucose tolerance at 4 months of age. Although we did not perform insulin tolerance tests, hepatic insulin sensitivity was assessed through western immunoblot detection of phosphorylated-Akt1 (S473 and T308), the p85 subunit of phosphoinositide 3-kinase (p85), and the phosphorylated IRS-1 (S302 and S1101), all markers of insulin sensitivity (Guo et al. 2009,
The steady-state levels of hepatic Lxrα mRNA are decreased, concomitant with an increase in G6pase and 11β-Hsd1 mRNA in LP animals by 4 months of age

Given the LP animals exhibited glucose intolerance at 4 months of age, we subsequently investigated the expression of hepatic LXRα and its target genes involved in gluconeogenesis. To determine differences in in vivo hepatic mRNA levels of Lxrα, G6pase, 11β-Hsd1 and Pepck at 4 months of age in the male offspring, qRT-PCR was employed with TaqMan probes for each gene. Lxrα mRNA was significantly decreased by 45% (P<0.05) in the LP offspring, while Pepck mRNA was unchanged between groups (Fig. 2). Hepatic G6pase and 11β-Hsd1 mRNA were significantly increased (P<0.05, 1.6-fold) in the LP offspring (Fig. 2).

The levels of hepatic LXRα protein are decreased, concomitant with an increase in G6PASE and 11β-HSD1 protein levels in LP animals by 4 months of age

To assess the effect of a maternal low protein diet on the protein levels of LXRα and LXR-target genes in 4-month-old offspring, we performed western immunoblotting to determine if there would be similar trends to what was observed in the steady-state mRNA levels. At 4 months of age, LXRα protein expression was decreased by 40% (P<0.05), while both G6PASE and 11β-HSD1 protein levels were increased (P<0.05, 1.5- and 1.6-fold respectively) in the LP animals compared with the control animals (Fig. 3). PEPCK protein expression was not different between the two groups.

LXRα binding to the LXRE on the promoters of G6pase and 11β-Hsd1 is decreased by 4 months of age in the LP offspring

To investigate whether the changes in the expression of G6Pase and 11β-HSD1 between the control and LP offspring were due to the decreased binding of LXRα to the promoters of G6pase and 11β-Hsd1, we employed ChIP

Figure 2
The effect of LP on in vivo hepatic levels of (A) Lxrα mRNA, (B) Pepck mRNA, (C) G6pase mRNA and (D) 11β-Hsd1 mRNA in control and LP offspring at 4 months of age. Data were quantified from qRT-PCR (TaqMan) and the ΔΔCt method. Data are represented as arbitrary values and were analyzed using the Student’s unpaired t-test. Results are expressed as the mean±s.e.m. *Statistically significant. n=4–6 per experimental group.
to immunoprecipitate LXRα. After using MatInspector (Genomatix) to find putative LXREs on the promoters of G6pase and 11β-Hsd1, qRT-PCR was employed to examine LXRα binding at these putative LXRE sites. By 4 months of age, the LP animals exhibited a marked decrease in the binding of LXRα to the promoter of 11β-Hsd1 (45% decrease) and G6pase (50% decrease) compared with the control animals (P<0.05) (Fig. 4). The nonspecific binding of IgG was tested and found to be minimal (Ct value >34, data not shown).

Acetylation of lysine residues 9 and 14 on histone H3 is decreased surrounding the TSS of Lxrα in LP offspring by 4 months of age

We further employed ChIP to examine the epigenetic regulation of Lxrα at its TSS. By immunoprecipitating chromatin with antibodies specific to RNA polymerase II, trimethylated histone H3 (K9), acetylated histone H3 (K9,14) and trimethylated histone H3 (K4) we were able to examine the transcriptional and epigenetic regulation of Lxrα in our model of MPR. Using primers specific to the −144 to +134 region of the Lxrα gene promoter and qRT-PCR, we found a significant 45% reduction (P<0.05) in the acetylation of histone H3 (K9,14), a hallmark of chromatin silencing, near the TSS of Lxrα (Fig. 5C). While not significant, we also found a decreasing trend in the recruitment of RNA polymerase II binding and histone H3 trimethylation (K4) at the same site (Fig. 5A and B). Again, the nonspecific binding of IgG was tested and found to be minimal (Ct value >34, data not shown). These results, in combination, support the notion that Lxrα is transcriptionally and epigenetically silenced long-term in our MPR model of IUGR.

The steady-state levels of hepatic Lxrα mRNA are unchanged between control and LP offspring concomitant with a decrease in G6Pase and 11β-Hsd1 mRNA in LP animals at embryonic day 19

To assess the direct effects of the LP diet on Lxrα and LXR-target gene expression during gestation and prior to birth, we analyzed the livers of fetuses sacrificed at embryonic day 19. qRT-PCR was employed with TaqMan probes for (Lxrα, G6pase and 11β-HSD1 (Pepck could not be detected in our embryonic liver tissue). At embryonic day 19, there were no differences in Lxrα mRNA expression between the control and LP animals. However,
both G6pase and 11β-Hsd1 mRNA expression were decreased in LP offspring compared with control offspring (P<0.05; Fig. 6).

Discussion

Our present study demonstrates that male offspring of LP dams exhibit increased expression of hepatic gluconeogenic genes due to aberrant expression of hepatic LXRα. This is of great interest considering that previous studies have indicated that MPR leads to glucose dysregulation (Ozanne et al. 1996, Burns et al. 1997, Chamson-Reig et al. 2009). We present evidence for the first time that suppressed expression of LXRα may mediate the enhanced transcription of the gluconeogenic genes G6pase and 11β-Hsd1 due to its decreased binding on these promoters, ultimately removing its ability to suppress hepatic gluconeogenesis (Stulnig et al. 2002a, Cao et al. 2003, Laffitte et al. 2003, Mitro et al. 2007).

Given placental insufficiency in humans can produce protein deficiency in the fetus (Crosby 1991), this LP model shares features in common with PI-IUGR (Ross & Beall 2008). Previous studies done in our laboratory with the same cohort of animals have already demonstrated that LP offspring exhibit a 15% lower fetal to placental weight ratio and a 40% decreased fetal liver to body weight ratio at embryonic day 19 (Sohi et al. 2011). While switching the low protein offspring to a control diet at weaning led to glucose intolerance (Chamson-Reig et al. 2009), little is known about how catch-up growth due to early restoration of protein (Sohi et al. 2011) influences their hepatic glucose handling by adulthood. In our LP model, after switching to a control (20% protein) diet at birth, the animals exhibited full catch-up growth by 3 weeks of age (Sohi et al. 2011). Moreover, by 4 months, these offspring exhibited impaired glucose tolerance with no evidence of hepatic insulin insensitivity. Given that glucose intolerance precedes insulin resistance, it is likely that these MPR offspring will develop insulin resistance at a later time point. Interestingly, the impaired glucose tolerance was similar, not worse, to low protein offspring at 4 months whereby their diet was switched to 20% at weaning (Chamson-Reig et al. 2009). Collectively, both studies further support of the main tenets of the Thrifty Phenotype hypothesis (Hales & Barker 1992).

We previously found that LXRα expression and binding could be influenced by maternal diet by 3 weeks of age in the offspring (Sohi et al. 2011), but the expression of LXRα at 4 months was unknown. In this study we demonstrated that in LP offspring, hepatic Lxrα mRNA and
protein were decreased at 4 months of age compared with control offspring. Given aberrant LXRα expression and activity can alter the expression of genes involved in hepatic gluconeogenesis (e.g. Pepck, G6pase and 11β-Hsd1; Stulnig et al., 2002a, b, Cao et al., 2003, Laffitte et al., 2003), we next examined whether the protein expression of these LXR-target genes was altered in LP offspring. At 4 months, we found increases in the steady-state mRNA and protein levels of G6PASE in LP male rats. This is of great interest considering that this LXR-target gene is responsible for the final catalytic step of gluconeogenesis, the conversion of glucose-6-phosphate to glucose. Moreover, overproduction of G6Pase does not necessarily lead to increases in fasting glucose levels, which we also observed, but it does lead to an enhanced glucose response (e.g. a greater area under the curve during GTT; Trinh et al. 1998). To directly implicate whether alterations in LXRα expression influenced the binding of LXRα to the promoter of G6pase, we then employed ChIP to examine the in vivo binding of LXRα to its putative LXRE on the promoter of G6pase. At 4 months of age, we observed a decrease in the binding of LXRα to the LXRE site (C22 to C46) of the G6pase promoter. These data suggests that the increase of G6pase expression seen in protein restricted offspring is at least partly due to the decreased binding of LXRα to the putative G6pase promoter. Overexpression of PEPCK has also been demonstrated to impair glucose tolerance and leads to noninsulin-dependent diabetes (Valera et al., 1994), however we did not find any significant alterations in Pepck mRNA or protein. This is in contrast to other studies whereby hepatic PEPCK activity increased in 3-week-old and 11-month-old offspring fed a low protein
increase in 11β-Hsd1 mRNA along with elevated 11β-HSD1 protein levels in the LP offspring. Interestingly, while previous nutrient restriction models have demonstrated no change in 11β-HSD1 expression in the adipose tissue of adult rat offspring (Dutriez-Casteloot et al. 2008), its expression in the liver was not examined. Similar to G6pase, we proposed that a decrease in LXRα expression and binding would lead to the loss of inhibitory action on the 11β-Hsd1 promoter and a subsequent increase in 11β-Hsd1 gene expression. Our ChIP experiments confirmed our speculation by demonstrating a decrease in LXRα binding to the putative LXRE (−114 to −90) on 11β-Hsd1. With increased expression of 11β-HSD1, it is conceivable that there would be enhanced conversion of inactive glucocorticoids to active glucocorticoids. Since glucocorticoids have stimulatory effects on the expression of gluconeogenic genes such as G6pase and Pepck (Sasaki et al. 1984, Lange et al. 1994, Lin et al. 1998), the sustained overproduction of glucocorticoids would lead to an augmented glucose response, as observed in the IPGTTs. Collectively, it is likely that the overproduction of G6Pase may not only occur due to the direct actions of LXRα, but also indirectly through enhanced 11β-HSD1 expression.

Previous studies strongly suggest the role of epigenetics in mediating the effects of fetal programming long-term into adulthood (Rees et al. 2000, Lillycrop et al. 2005, Burdge et al. 2007, Park et al. 2008, van Straten et al. 2010). By 4 months of age, we demonstrated that LP males exhibited significantly decreased acetylation of histone H3 (K9,14) associated with trends of decreased RNA polymerase II recruitment and decreased methylation of histone H3 (K4) surrounding the promoter of Lxrα. Considering acetylation of histone H3 (K9,14) and methylation of histone H3 (K4) are both known to be hallmarks of chromatin opening (Jenuwein & Allis 2001, Santos-Rosa et al. 2002, Yan & Boyd 2006), our findings suggest that the Lxrα TSS is silenced through epigenetic mechanisms by adulthood. These findings are congruent with the decreased levels of Lxrα mRNA and protein expression observed. We previously demonstrated that MPR leads to long-term decreases in histone H3 acetylation (K9,14) surrounding the promoter of the LXR-target gene Cyp7a1 resulting in hypercholesterolemia in these offspring (Sohi et al. 2011). To address whether the low protein diet itself directly alters hepatic LXRα and LXR-target genes in vivo, we measured their fetal expression (embryonic day 19) during the low protein insult. Interestingly, the low protein diet impaired these hepatic genes involved in gluconeogenesis without changes to LXRα expression. This suggests that the augmented expression of LXRα, G6Pase

diet during gestation (Desai et al. 1995), and low protein offspring fed a high sucrose diet (500 g/kg) postpartum (Burdge et al. 2007). The difference in the former study may be due to the fact that the offspring were cross-fostered to dams not subjected to a low protein diet, potentially leading to even greater catch-up growth.

11β-HSD1 may also play a role in impairing glucose homeostasis in our LP model. Our study demonstrated an
and 11β-HSD1 observed in adulthood is more likely due to the indirect actions of the low protein diet, namely, a protein mismatch in postnatal life associated with rapid catch-up growth.

In view of the fact that LXRz suppresses glucose production, it may be a suitable target as a therapeutic intervention to prevent glucose intolerance. Animal studies have widely demonstrated that the administration of LXR agonists (e.g. GW3965 and T0901317) leads to improved glucose tolerance (Cao et al. 2003, Laffitte et al. 2003). Given that during the newborn period in the rat there is a high rate of replication, neogenesis and apoptosis leading to extensive liver remodeling (Cascio & Zaret 1991), this period represents a critical, but opportune window for therapy designed to improve hepatic growth and function long-term. For example, it has been demonstrated in IUGR rats derived from uterine artery-ligated dams that neonatal administration of Exendin-4 (a GLP-1 analog) prevented the development of hepatic oxidative stress and insulin resistance (Raab et al. 2009). Therefore it is plausible that LXR agonists, administered in neonatal life, a period of liver plasticity, may prevent the development of glucose intolerance long-term through activation of hepatic LRXz. While LXR agonists appear promising, the negative effects of LRXz activation must also be considered, given that it can activate lipogenesis through increased expression of fatty acid synthase, acetyl-CoA carboxylase and the master lipid regulator, sterol regulatory element binding protein-1c (Steffensen & Gustafsson 2004).

In summary, our study demonstrates for the first time the role of LRXz in mediating the transcriptional regulation of hepatic gluconeogenic genes in our rat LP model. In these offspring, decreased expression of hepatic LRXz reduced the transcriptional inhibition of hepatic G6Pase and 11β-HSD1. This increased expression of G6Pase and 11β-HSD1 in LP offspring would contribute, in part, to the aberrant glucose handling observed in these animals. Given the role of hepatic LRXz in reducing glucose production, it serves as a possible therapeutic target of intervention due to its anti-diabetic properties. Further studies will be necessary to find a suitable balance between anti-diabetic and lipogenic actions of LRXz before it could be considered as an ideal candidate for preventing glucose intolerance in IUGR offspring.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
T X V designed and performed all the experiments in this study and was the primary author in writing this manuscript. A R and N M assisted in the glucose tolerance tests and tissue collection, while G S assisted in the molecular analyses experiments (qRT-PCR and western immunoblotting). D B H assisted in the designing of the experiments and in the preparation of this manuscript.

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