6-7-2013 12:00 AM

The Role of the Liver X Receptor (LXR) in the Fetal Programming of Hepatic Gluconeogenesis

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Physiology

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(Thesis format: Integrated Article)

by

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Graduate Program in Physiology

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

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ABSTRACT

Chronic diseases such as type 2 diabetes and the Metabolic Syndrome create enormous burdens on society. Epidemiological studies now strongly implicate intrauterine growth restriction (IUGR) for increasing the risk of developing chronic diseases later on in life. However, the molecular mechanisms underlying how IUGR leads to the increased susceptibility to these metabolic diseases in adulthood is not well understood. The Liver-X-Receptor (LXR) is a nuclear receptor involved in cholesterol, glucose, and lipid metabolism. LXR acts to decrease gluconeogenesis through repression of glucose-6-phosphatase(G6Pase), phosphoenolpyruvate carboxykinase(PEPCK), and 11β-hydroxysteroid dehydrogenase type-1(11β-HSD1). Using a well-characterized model of maternal protein restriction in rats, this study attempts to elucidate the role of LXR in the long-term programming of impaired glucose homeostasis. It was discovered that altered expression of LXR during the gestational and neonatal period predisposes the fetus to impaired glucose tolerance in adult life through LXR-mediated activation of the gluconeogenic genes G6Pase, PEPCK, and 11β-HSD1.

KEYWORDS

Liver X Receptor, maternal protein restriction, intrauterine growth restriction, fetal programming, hepatic gluconeogenesis, 11β-hydroxysteroid dehydrogenase type-1, glucose-6-phosphatase, phosphoenolpyruvate carboxykinase, epigenetics, post-translational histone modifications, glucose intolerance, neonatal intervention

STATEMENT OF CO-AUTHORSHIP

The studies in Chapter 2 and 3 were primarily performed by (Peter) Thin Xuan Vo in the laboratory of Dr. Daniel Hardy, with the following additions:

- Figure 2.3: Gurjeev Sohi contributed a significant amount of work to the western immunoblotting studies examining markers of insulin resistance in Chapter 2 (done in the laboratory of Dr. Hardy)
- Figure 3.5 – Cynthia Sawyez was responsible for the measurement of insulin and triglycerides in Chapter 3 (done in the laboratories of the Robarts Research Institute)

Dr. Daniel Hardy contributed significantly to the design of all the studies, data analysis, and interpretation of results.

In Chapter 2, Andrew Revesz was primarily responsible for animal care and assisted with many of the intraperitoneal glucose tolerance tests. Noelle Ma also assisted with the intraperitoneal glucose tolerance tests.

In Chapter 3, (Peter) Thin Xuan Vo was primarily responsible for animal care with assistance from Andrew Revesz. Andrew Revesz, Noelle Ma, and Michael Wong assisted with the intraperitoneal glucose tolerance tests. Waseem Iqbal and Michael Wong assisted with liver and blood extractions.

ACKNOWLEDGEMENTS

First and foremost, this dissertation and the successful defence of my thesis would not have been possible without the mentorship and assistance of Dr. Daniel Hardy, my wonderful and exceptional supervisor. He has put as much work into this thesis as much as I have and has been extremely helpful, encouraging, understanding, and patient throughout the past few years. Not only has Dr. Daniel Hardy been a delightful supervisor, but a great mentor as well. Thanks to Dr. Hardy, I have learned what it means to be a good scientist, a good mentor, and a good person. His passion for science is contagious and his love for life is even more infectious. I can only hope that one day I will be able spread my passion for learning, teaching and life as much as Dr. Hardy does. Thank you Dr. Hardy. Like you said, the journey does not end here, it only continues. Although I know that our friendship will continue even after I leave the lab, I look forward to finally being Facebook friends with you.

My thesis also would not be possible without the advice and support of my advisory committee. Thank you Dr. Rommel Tirona and Dr. Peter Chidiac. You have both given me your insights, patience and encouragement, which helped me gain perspective on my research and fueled my progress along the way.

I would also like to thank various faculty members who have contributed to my research. Thank you Dr. Tim Regnault, you have been very supportive throughout my time in the lab. I can always count on you for when wit and dry humour are lacking in my day. Your dedication to teaching has also been inspiring. Working with you and being a teaching assistant for the steroid abuse lab has been a pleasure. Thank you Dr. Bryan

Richardson and Dr. Dean Betts for your input and insightful questions during the perinatal lab meetings.

Thank you to Dr. Lin Zhao, also known colloquially as “the guru”. Dr. Lin Zhao’s nickname is the guru for a reason. His knowledge seems to be endless. Lin’s invaluable advice, scientific expertise, and technical knowledge have been a blessing throughout my time in the lab. Thank you Lin, you are worth your weight in *Au*.

Thank you to all the members of the “DDT” lab that I have crossed paths with in the past four years. In particular, thank you Gurjeev Sohi and Andrew Revesz for teaching me almost everything I needed to know to be successful in the lab. I would not have been so disciplined with my lab technique and well-informed without you two. Gurjeev, you have so many ideas floating around in your head, I hope you will benefit humanity with at least one of them one day. Thank you Noelle Ma for being such a kind, supporting, and helpful colleague. I am glad we had the opportunity to experience this journey together. Best wishes as you embark on your new journey at U of T. Thank you Michael Wong for being so helpful, diligent, and tolerant while assisting me with my projects. Best of luck as you embark on your graduate studies and give new light to the Hardy Lab. Thank you Waseem Iqbal for just hanging out around the lab and office and keeping all of us company. I will really miss all of our barbeques (that masala marinade is unbelievable) and lunches. Thank you Jacky Chiu for the hilarious moments. Keep working hard. Thank you Katie Belgrave and Stephanie Hallows for the laughs and never letting the office have a dull moment. Thank you Heather Mulholland for the thought-provoking and philosophical conversations. Thank you Ian Tobias for the enlightening

conversations about basketball and other non-science subject matter. I couldn't have asked for a better desk neighbour. Best wishes to all of you in your future endeavours.

Thank you to all of the other graduate students and friends (the list is way too long) that I have crossed paths with during my studies here at Western. You have all helped make my time at Western a memorable and amazing experience. Western will always be my home away from home. Thanks for making this journey so special.

Thank you to Tom Stavraky and the Physiology and Pharmacology department for allowing me to be a teaching assistant for the Physiology 3130Y course. During my undergraduate studies, Physiology 3130Y was one of my favourite courses so it was truly a delight to be able to teach it back to younger, inquiring minds. To me, having the opportunity to teach was a huge part of my Master's and in turn has taught me a lot. As such, I must also thank all of the students that I had the opportunity to teach. It was a privilege.

Thank you to all of my friends back home in Toronto (and wherever else you are in the world right now). Particularly, I'd like to thank Taft Micks and Allen Ngo, two of my closest friends who have been tremendously loyal, understanding, and encouraging throughout the past 10 years. Maeng, Wang, Lisa, and Ali, thanks for all of the moments we've shared in London and across the world. To the guys who always visit London (you all know who you are), you guys have all been so supportive and have made my journey a rollercoaster of shenanigans and unforgettable moments. Thanks for visiting London and Western so much and keeping things interesting. Let's keep the good times rolling.

Thank you to Mrs. Saini, my wonderful high school biology teacher who really sparked my love for the biological sciences and physiology and kept that fire burning. Mrs. Saini, you are the definition of what a high school teacher should be like. Please never stop teaching.

A huge thank you to my girlfriend, Tiffany Chow, who has been there for me through thick and thin. Her tremendous support and encouragement throughout my studies has been invaluable. Thanks for being so tolerant and understanding and putting up with me during my worst moments. Thanks for endlessly encouraging me. You have made my journey so much easier and meaningful, T. Chow, thank you.

Finally, an enormous thank you to my wonderful family, for whom I will always be grateful. Without their unconditional love and unwavering support I would not be here today. Thank you to my mother, Kim Thi Nguyen, and father, Tan Quan Vo, for always encouraging me to do what I love. Thank you to my younger brother, David Vo. You have always been supportive of my ambitions and have always kept me grounded by challenging me in various areas of my life. Our arguments and talks about life will never get old, Dave. Keep them coming.

Thank you again to everyone. You have all contributed in some way, whether big or small, to who I am, and to my journey as a student and training scientist. My accomplishments are your accomplishments. I am in debt to all of you.

DEDICATION

This thesis is dedicated to my parents, Kim Thi Nguyen, and Tan Quan Vo for being the best parents they could be and working so hard to help me make my dreams a reality. I am proud of you both. Thanks mom and dad, I could not have done any of this without you.

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11β-HSD1	-11β-hydroxysteroid dehydrogenase type 1
ABCA1	-ATP-binding cassette transporter member 1
ABCG5	-ATP-binding cassette sub-family G member 5
ABCG8	-ATP-binding cassette sub-family G member 8
ACC	-acetyl-CoA carboxylase
Cyp7a1	-cholesterol 7α-hydroxylase
FAS	-fatty acid synthase
FBPase	-fructose biphosphatase
G6Pase	-glucose-6-phosphatase
GLP-1	-glucagon-like peptide 1
GLUT	-glucose transporter
GR	-glucocorticoid receptor
GSK3	-glycogen synthase kinase 3
HAT	-histone acetyltransferase
HDAC	-histone deacetylase
HMT	-histone methyltransferase
IgG	-immunoglobulin G
IRS-1	-insulin receptor substrate 1
LXR	-liver X receptor
LXRE	-liver X receptor element
PEPCK	-phosphoenolpyruvate carboxykinase
PI 3-Kinase	-phosphoinositide 3-kinase

PKB/Akt1	-protein kinase B
RXR	-retinoid X receptor
SCD-1	-stearoyl-CoA desaturase 1
SGA	-small for gestational age
SRE	-sterol regulatory element
SREBP-1	-sterol regulatory element binding protein type 1

Chapter One: Introduction - Literature Review

Excerpts of this chapter have been previously published: T. Vo & D.B. Hardy. Molecular mechanisms underlying the fetal programming of adult disease. Journal of Cell Communication and Signaling. (6)3: 139-53, 2012.

Introduction and Literature Review

1.1 Chronic Diseases and the Metabolic Syndrome

Chronic, non-communicable diseases create a vast burden on society, both socially and economically. Non-communicable diseases rather than infectious diseases are now the leading causes of death worldwide. For instance, in the United States, cardiovascular disease is the number one cause of death, responsible for almost 30% of all deaths in the country[1,2]. Other chronic illnesses include hypertension, hyperlipidemia, impaired glucose tolerance, and obesity, which, in combination, encompass the metabolic syndrome[3]. The metabolic syndrome is defined by the following criteria: abdominal obesity, dyslipidemia, hypertension, insulin resistance and/or glucose intolerance, a proinflammatory state, and a prothrombotic state[4]. More specifically, the metabolic syndrome is characterized by the following parameters: abdominal circumference ≥102 cm in men and ≥88cm in women; triglycerides ≥ 1.7mM in men and women; fasting glucose ≥ 5.6mM in men and women; HDL cholesterol ≤ 1.1mM in men and ≤ 1.3mM in women; and blood pressure ≥130/85mmHg in men and women[5]. Along with obesity, the metabolic syndrome greatly increases the risk of developing further diseases such as type 2 diabetes and cardiovascular disease[6]. To put a number on these figures, more than one in three Americans is obese[7], while in Canada, more than one in four Canadians is obese[8]. The extensive development of these chronic diseases is not only a problem in North America, but worldwide as well[2,9-12]. The increasing prevalence of the metabolic syndrome and obesity is becoming apparent even in the developing world, where under nutrition used to be of great concern[13-15]. Undoubtedly, the growing incidence of these

chronic diseases is a worldwide phenomenon that needs to be addressed. Yet, the burden of these diseases is extremely complex in nature and the solutions are no less complex.

Although the prevalence of these chronic and non-communicable diseases puts tremendous strain on the health care system and society, intervention with diet or drugs can play a significant role to reduce their incidence. For example, a meta-analysis prospective study, using data from 58 clinical trials as well as nine cohort studies, indicates that in patients with vascular disease, a 1.8 mM reduction in LDL cholesterol by statins resulted in a 17 % reduction in stroke and a 60% reduction in the risk of ischemic heart disease[16]. Current treatment of type 2 diabetes and the metabolic syndrome include improvements in lifestyle through healthy dieting and increasing exercise, along with the use of pharmaceuticals (*e.g.* metformin or glucagon-like peptide-1 (GLP-1) analogues). Unfortunately, these treatments are not efficacious for all individuals. For example, in some patients statin treatment can lead to rhabdomyolysis and hepatitis-associated liver failure[16]. As well, some patients of non-communicable diseases, such as type 2 diabetes, may become dependent on pharmaceuticals for their entire life and have to live with common side effects of the drugs (*e.g.* gastrointestinal discomfort, heartburn, and nausea), which can lead to a decreased quality of life[17]. Recent studies on the treatment of type 2 diabetes indicate that while there were improvements in risk factor control and lifestyle, nearly half of diabetic individuals did not reach their goals for control of their disease[18]. Thus, research is now focusing on strategies for disease prevention, in addition to the current interventions, to decrease the devastating burden of the non-communicable disease pandemic.

1.2 Low Birth Weight and Intrauterine Growth Restriction

The prevalence of low birth weight babies (defined as ≤2500 g or 5.5lbs) worldwide is estimated to be 15.5 %, and that number is greatly underestimated[19]. As a general indicator of public health, it is imperative that we study the etiology and outcomes of the individuals that develop as low birth weight babies. Low birth weight babies are often referred to as being "small for gestational age" (SGA) and are traditionally defined as being born with a birth weight ≤ 10th percentile[20]. Evidence strongly suggests that SGA infants are susceptible to higher rates of mortality and morbidity[21-23]. Several definitions have arisen to classify whether an infant should be constituted as SGA or not. The classic definition of an SGA infant was that its weight was in the lowest 10th percentile for gestational age[20]. However, this definition does not take into account constitutional factors such as ethnicity, infant sex, or parity. Thus, optimized and specific growth curves generated for infants and fetuses of different sex, ethnicity, and other factors have been adopted to better classify SGA infants[24-26].

SGA infants are often a result of intrauterine growth restriction (IUGR). IUGR infants are defined as infants who do not fully reach their growth potential due to genetic and/or environmental factors[27]. It is postulated that approximately one third of these IUGR infants arise due to genetic factors, while two thirds are a result of environmental influence[27]. IUGR can also be classified into two categories, symmetric and asymmetric. Symmetric IUGR occurs when the entire fetus' growth is stunted in a proportional manner. Asymmetric IUGR occurs when the fetus' growth is stunted in a disproportional manner, such that vital organs (*e.g.* the brain and heart) receive the most nutrients and energy at the expense of other organs (*e.g.* liver). The redistribution of blood flow from

the peripheral organs to the brain is also known as the "brain sparing effect"[28]. The asymmetric growth stunted fetus usually displays a normal head circumference with a reduced abdominal circumference. These fetuses usually arise from cases of placental insufficiency IUGR[29] and are at a higher risk of developing neonatal complications (*e.g.* respiratory distress, sepsis, and intraventricular hemorrhage) than their symmetric IUGR counterparts[30].

IUGR can arise from a variety of factors including, infection[31,32], chronic maternal hypoxia[33-35], maternal malnutrition[36,37], maternal body composition and gestational weight gain/loss[38,39], glucocorticoid exposure[40], and placental dysfunction[41] (Figure 1.1). Interestingly, the spacing of pregnancies may also influence the development and growth of fetuses[42,43], with decreased spacing between pregnancies correlating to subsequent lower birth weights. It should be noted that while any single one of these factors may influence fetal growth and development, these factors might also be compounded to impair fetal growth and development even further.

1.3 Developmental Origins of Health and Disease

The developmental origins of health and disease first stemmed from the "Barker Hypothesis" (or "Thrifty Phenotype Hypothesis"). The Barker Hypothesis suggests that impaired growth of the fetus during gestation strongly correlates to the development of chronic disease in later life[44,45]. One of the first pieces of evidence linking fetal life and chronic disease was a study done by Barker and Osmond (1986) where a strong positive correlation was found between the prevalence of ischemic heart disease and the

prevalence of neonatal and post neonatal mortality in populations throughout England and Wales[46]. Subsequent studies by Barker and colleagues found evidence that infants with the lowest birth weights possessed the highest blood pressures in adulthood and were the most likely to die from ischemic heart disease[47,48]. Further evidence also emerged that demonstrated links between low birth weight and impaired glucose tolerance at age 50[49] and an even stronger connection was found between low birth weight babies and the development of the metabolic syndrome[50]. Additional epidemiological studies have also demonstrated strong correlation between low birth weight infants and the development of impaired glucose tolerance and type 2 diabetes, cardiovascular disease, and hypertension[51-57]. Altogether, these studies provide considerable evidence that a relationship exists between prenatal growth and development and the development of chronic disease in later life.

It is postulated by the Barker Hypothesis that the fetus is physiologically "programmed" *in utero* to adapt to its environment[58-60]. In cases of maternal nutritional deficiency or placental insufficiency, the fetus must program itself for a poor nutritional postnatal environment. However, this adaptation becomes maladaptive when the infant is exposed to a dissimilar postnatal environment. An example is an environment of nutritional surplus. Evidence of this is supported by two studies of two different populations during World War II. First, a study examining the glucose tolerance of individuals born during the Dutch hunger winter (in World War II) found that these individuals had lower birth weights and impaired glucose tolerance compared to those born a year before or after the famine[52]. However, another study that examined the glucose tolerance of individuals from the Leningrad siege famine (also in World War II)

found no differences in glucose tolerance between infants born during the famine and the infants born outside of the siege (unexposed to the famine)[61]. A major difference between these two populations was that the Dutch hunger winter siege had lasted less than 6 months, while the Leningrad siege had lasted 28 months. Thus, infants from the Dutch hunger winter siege would have received a higher nutrient intake earlier than those infants from the Leningrad siege, who would have continued on a low nutrient diet for longer postnatally[62]. It is believed that the Dutch hunger winter infants experienced a mismatch in environment and "catch-up" growth, leading to the programmed glucose intolerance in adulthood, while the Leningrad infants did not experience the mismatch in environment until much later[62]. Thus, it is a mismatch in the prenatal and postnatal environment and the accompanying maladaptation during a critical time point that is strongly related to the development of chronic disease in later life.

Lastly, the concept of accelerated "catch-up" growth also appears to play a factor in the development of chronic disease and reduced lifespan[63]. Catch-up growth generally occurs when the development of a growth restricted organism is accelerated to compensate for its impaired growth in early life. While this compensation helps the organism grow in its early stages, this growth trajectory appears to exacerbate the programming of disease and decreased longevity in later life[63]. For instance an early study done by Crowther *et al.* (1998) found that low birth weight in addition to rapid childhood weight gain was closely associated with the development of impaired glucose tolerance[64]. Similarly, a study by Forsén and colleagues (1999) found that individuals at greatest risk for coronary heart disease were those who were born with low birth weights and experienced accelerated catch-up growth[65]. A subsequent study by Eriksson *et al.*

(2001) found similar results in males only[66], while Fewtrell *et al.* (2000) found that increased plasma insulin concentrations were associated with accelerated growth patterns during childhood[67]. Additionally, the development of childhood obesity is also strongly related to accelerated weight gain during the first 4 months of childhood, regardless of birth weight[68]. Finally, a study in which preterm infants (usually born low birth weight) were given a fortified formula diet after birth (accelerated growth) displayed higher markers of insulin resistance during adolescence than those given a lower nutrition donated breast milk diet[69]. Taken together, these human studies provide strong evidence for the role of accelerated postnatal growth in contributing to the development of adult chronic diseases, especially in cases of prenatal growth restriction.

Thus, it appears that there are two critical periods for the programming and development of chronic diseases in adulthood – the prenatal period and the neonatal period. The first few weeks of life appear to be especially sensitive to the effects of nutrition and catch-up growth[69]. This makes sense because the neonatal period is a period of tremendous growth and development[70,71]. Yet, the mechanisms behind how insults that occur during these critical time periods lead to the programming of adult disease are still under investigation. Consequently, many animal models of intrauterine growth restriction and fetal programming have been developed to study the physiology and pathophysiology of the developmental origins of health and disease.

1.4 Animal Models of IUGR

A variety of animal models have been developed to study the developmental origins of adult diseases and fetal programming. Experiments of IUGR in animal models provide further evidence to support the hypothesis that impaired growth *in utero* via various maternal deficiencies leads to impairment of glucose, cholesterol, and triglyceride metabolism in adulthood[72-75]. In addition, these animal models provide avenues to elucidate the mechanisms behind the fetal programming of adult diseases. *In utero* deficiencies that can lead to impaired growth in humans and animals include hypoxia[76], deficiencies in essential vitamins and minerals[77], diminished protein[75], total caloric restriction[78], excess glucocorticoids[79,80], and placental dysfunction[41] (Figure 1.1). Although the correlation between impaired fetal growth and the risk for developing chronic disease in adulthood is undoubtedly strong, the mechanisms behind these programming effects are only beginning to be elucidated. A few proposed mechanisms underlying the fetal programming of adult disease include altered epigenetic and transcriptional regulation, altered nuclear receptor activities, increased oxidative stress, and increased endoplasmic reticulum stress resulting in protein misfolding[81]. Studies have only begun to scratch the surface in understanding the molecular events responsible for the altered physiology and pathophysiology of these chronic diseases.

1.4.1 Maternal Protein Restriction

Maternal protein restriction (MPR) in animals, and especially rodents, is a well-established model of IUGR that is used to study the developmental origins of health and

disease. Due to the fact that placental insufficiency during pregnancy leads to protein and amino acid deficiencies in the developing fetus[82], the MPR model of IUGR shares many similarities with placental insufficiency-related IUGR[83]. In general, the model employs a protein-restricted diet (5-8% protein content) to mothers during the gestation and the weaning periods, which is up to three weeks after birth in rats. After the weaning period (or in some cases, after birth), the offspring are given a diet restored in protein (generally 15-20% protein content). Studies from our own laboratory have found that MPR offspring exhibit a 15% lower fetal to placenta weight ratio and a 40% decreased fetal liver to body weight ratio at embryonic day 19[75]. Notably, the MPR model does not alter the sex ratio of the offspring, litter size, or food intake in the offspring[75,84].

The first few studies of maternal protein restriction in rats found that the offspring were born low birth weight and displayed impaired pancreas development[85]. Further studies by the same group and others found impaired pancreas function and development and impaired glucose tolerance in later life[72,84]. Petrik *et al.* (1999) in particular found changes in β-cell replication, increased β-cell apoptosis, and decreased insulin growth factor-2 (IGF-2) expression in the pancreas. Hales and colleagues (1996) also found impaired glucose tolerance in MPR offspring (at a much later age) as well as predicting two different mechanisms for differences seen between the glucose intolerance in males versus females[86]. They postulate that males develop insulin resistance, while females develop glucose intolerance due to a lack of insulin. More recent studies by Chamson-Reig and colleagues (2009) have also found impaired glucose tolerance in MPR offspring occurring in a sexually dimorphic manner[87].

In addition to the pancreas, Burns and colleagues (1997) demonstrated impaired liver development and function in addition to hepatic structural changes in the offspring of MPR rats[88]. Another study found increased hepatic glycogen storage in young MPR rats[89]. Furthermore, studies from our lab recently demonstrated that MPR leads to epigenetic-mediated repression at the *Cyp7a1* promoter, an essential enzyme responsible for cholesterol conversion into bile acids, ultimately resulting in elevated cholesterol in adulthood[75]. Other organs that appear to be affected by MPR long-term include the heart[90,91] and kidneys[92,93].

The MPR model in rodents can also be used to examine the effect of IUGR and catch-up growth. It has been previously demonstrated in our laboratory that in a model of MPR where protein is restored at an earlier time point (*i.e.* immediately after birth rather than after the weaning period), the offspring exhibit rapid catch-up growth, such that by postnatal day 21 the body weight and liver to body weight ratios between the low protein offspring and control offspring are unchanged[75]. This was apparent in both males and females and persisted well into early adulthood at postnatal day 130, where the body weights and liver to body weight ratios of the catch-up growth animals did not differ from the control animals. In contrast, MPR offspring that continued to receive a low protein diet after birth until the end of the weaning period, exhibited decreased body weights at postnatal day 130, suggesting that they never catch up in body weight[75]. However, in these animals, the liver to body weight ratio at postnatal day 130 was unchanged, suggesting that the liver eventually did catch up in growth in the offspring restored on a control diet after weaning. This was true for both males and females[75].

Offspring longevity also appears to be affected in catch-up growth models of MPR. In MPR offspring, when protein was restored earlier, the offspring displayed a significantly shortened lifespan, suggesting the possibility that accelerated catch-up growth may be quite detrimental to growth restricted offspring[86,94]. One proposed mechanism contributing to the decreased lifespan in these accelerated catch-up growth offspring is impaired mitochondrial function and increased oxidative stress in the kidneys[92,93]. Other possible mechanisms include altered insulin signaling and sensitivity and abrogated reactive oxygen species (ROS) handling in early life[95,96]. In the reverse situation, when the offspring were not protein restricted during gestation and were given a low protein diet after birth, they exhibited a longer lifespan[86]. Ozanne & Hales (2004) found similar results in terms of offspring longevity[97]. Interestingly, they also found that the "reverse protein" experimental group (normal protein during gestation and a low protein diet during weaning) was protected against the lifespan shortening effects of an obesogenic diet[97].

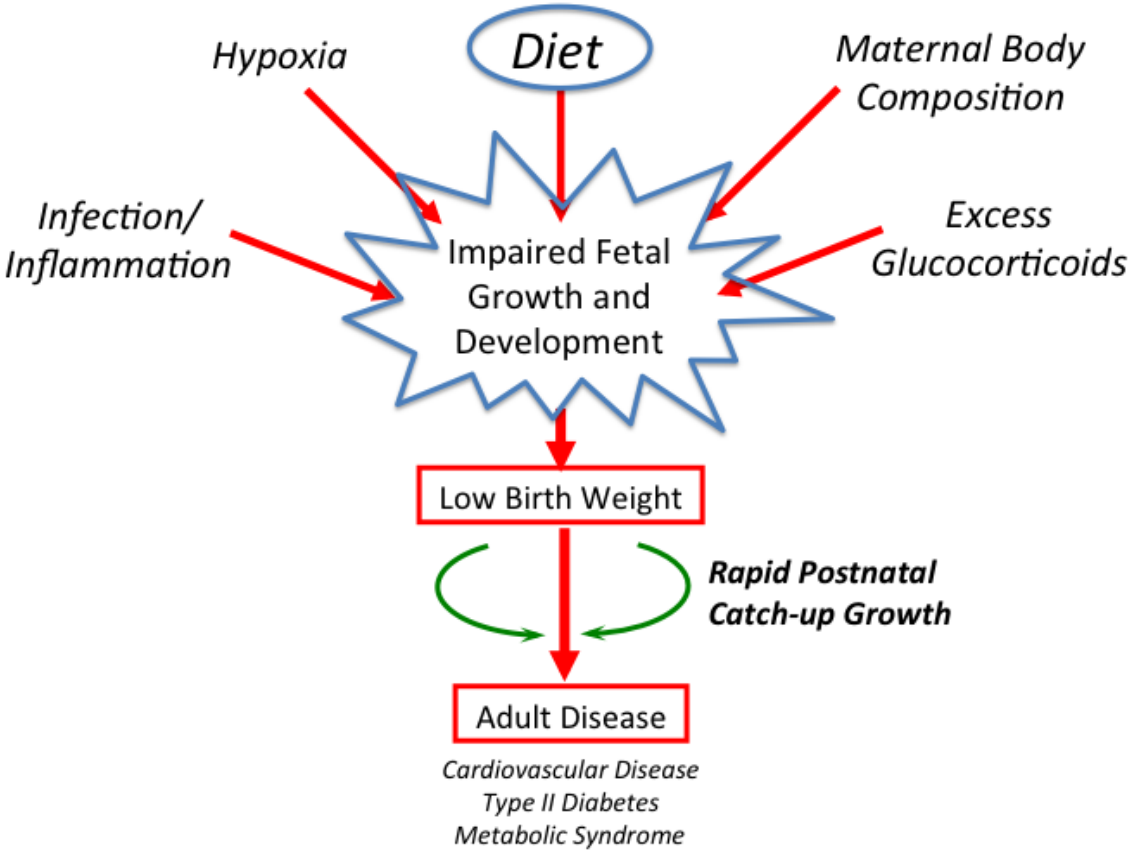

Figure 1.1: Factors That May Contribute to Low Birth Weight and The Developmental Origins of Health and Disease. A maternal insult (or a combination of several insults) generally leads to intrauterine growth restriction and low birth weight. This predisposes the infant to a higher risk of developing chronic disease in adulthood. The effect of rapid postnatal catch-up growth has been demonstrated to exacerbate the effects of programmed chronic disease.

1.5 The Liver

The liver is known for its plethora of functions in the body. It is involved in many complex processes including detoxification, red blood cell decomposition, glycogen storage, bile production, drug metabolism, and energy metabolism. With regards to energy metabolism, the liver is mainly responsible for carbohydrate and lipid metabolism[98]. Through coordinated regulation of carbohydrate and lipid metabolism, the liver contributes an essential role in the regulation of blood glucose levels. During the fasted state the liver maintains a steady supply of glucose to the body via hepatic gluconeogenesis. In the post-prandial state, the liver increases hepatic glucose uptake to stimulate glycogen production and increase lipogenesis. Perturbations in the regulation of hepatic carbohydrate and lipid metabolism leads to the development of many metabolic-related diseases such as type 2 diabetes[99].

1.5.1 Hepatic Gluconeogenesis

Two processes determine total hepatic glucose output: glycogenolysis, the breakdown of glycogen, and gluconeogenesis, the *de novo* production of glucose from non-carbohydrates (*e.g.* amino acids, pyruvate, lactate, and glycerol)[98]. Gluconeogenesis is influenced hormonally and by the body's nutritional state. The rate of gluconeogenesis is generally determined by the activities of phosphoenolpyruvate carboxykinase (PEPCK), G6Pase (glucose-6-phosphatase), and fructose-1,6-bisphosphatase (FBPase)[98]. For example, in the diabetic and fasted states, the activity of G6Pase is increased[100]. G6Pase is responsible for the enzymatic conversion of glucose-6-phosphate to glucose,

the last step of gluconeogenesis. PEPCK is the rate-limiting enzyme that converts oxaloacetate into phosphoenolpyruvate, committing oxaloacetate to gluconeogenesis.

Gluconeogenesis is mainly controlled by the actions of hormones such insulin, glucagon, and glucocorticoids. Insulin transcriptionally suppresses the expression of the gluconeogenic genes, PEPCK, G6Pase, and FBPase[101]. In contrast, glucocorticoids and glucagon stimulate gluconeogenesis. Insulin signaling appears to be essential in the control of hepatic glucose handling, as loss of insulin signaling in the liver leads to severe insulin resistance, hyperinsulinemia, impaired glucose intolerance, and an increase in the expression of G6Pase, and PEPCK in mice[102]. Regulation of hepatic gluconeogenesis is briefly summarized in Figure 1.2.

G6Pase is regulated through several pathways. Generally, the insulin-mediated suppression of G6Pase involves suppression of the forkhead transcription factor (FKHR/FOXO1) by protein kinase B-α (also known as Akt)[103]. FKHR transcriptionally activates G6Pase by binding to one of two insulin response units on the *G6Pase* promoter[104]. Insulin signaling causes phosphorylation of FKHR, which then leads to the expulsion of FKHR from the nucleus and eventual degradation in the cytosol[105]. Phosphoinositide 3-kinase (PI 3-kinase) also appears to be partly involved in insulin-mediated G6Pase suppression[106]. G6Pase expression can also be suppressed by the mitogen-activated protein kinase kinase (MEK) and extracellular signal-regulated kinase 1/2 (ERK 1/2) –mediated pathway, induced by the phorbol ester PMA[107]. Furthermore, G6Pase expression is downregulated by tumour necrosis factor-α (TNFα) through activation of necrosis factor κB (NFκB), although not through direct binding of the

G6Pase promoter[108]. Lastly, G6Pase expression has also been demonstrated to be repressed by the liver X receptor (LXR)[109,110].

In contrast, G6Pase expression is induced by glucocorticoids. Administration of dexamethasone has been shown to increase G6Pase expression and putative glucocorticoid response elements (GRE) have been identified on the *G6Pase* promoter[111]. Furthermore, the accessory protein hepatic nuclear factor (HNF) appears to be required for glucocorticoid-mediated stimulation of G6Pase (and PEPCK)[112]. Moreover, there are cAMP response elements on the *G6Pase* promoter that are responsive to cAMP Responsive Element Binding Protein (CREBP) binding[113].

PEPCK is transcriptionally regulated in a similar fashion to G6Pase. The main suppressor of PEPCK transcription and activation is insulin[114]. Insulin mediates its gluconeogenic suppressive effects through several downstream pathways. One pathway, similar to G6Pase regulation, is through the activation of PI 3-kinase[113]. Furthermore, inhibition of FKHR/FOXO1 appears to play a role in the insulin-mediated repression of PEPCK, though through a different mechanism than G6Pase[115]. The transcription factor sterol regulatory element binding protein-1 (SREBP-1) also plays a role in the suppression of PEPCK expression[116]. However, SREBP-1-mediated suppression is likely to be another intermediate in the insulin-mediated suppression of gluconeogenic genes[117]. Further studies have found that insulin activity stimulates hepatic SREBP-1 expression, which then binds to sterol regulatory elements (SRE) on the *PEPCK* promoter. This mechanism represses PEPCK expression by blocking the binding of the stimulatory transcription factor SP-1[118]. Another proposed mechanism for SREBP-1-mediated repression of PEPCK is through interference with the peroxisome proliferator-activated

receptor coactivator-1 (PGC-1) and hepatic nuclear factor-4 (HNF-4) activation pathway of PEPCK[119]. Like G6Pase, PEPCK is also under transcriptional repression by LXR[109,110].

PEPCK is stimulated by glucagon, cyclic-AMP (cAMP) and transcription factors such as the glucocorticoid receptor (GR)[113]. Evidence also suggests that FKHR is involved in the transcriptional activation of PEPCK, although through an indirect and different pathway than G6Pase[115,120]. PGC-1 has also been found to be a key co-activator in the induction of PEPCK and G6Pase by binding to and co-activating FKHR[121]. PGC-1 co-activation of HNF-4 and GR is also required for cAMP- and glucocorticoid- mediated activation of PEPCK and G6Pase[122]. Furthermore, PGC-1 interacts with CREBP to activate gluconeogenesis through PEPCK and G6Pase[123].

Aberrant overexpression of the gluconeogenic genes, G6Pase and PEPCK, has been found to produce glucose intolerance[124,125]. Rodent models of diabetes include the overexpression of G6Pase[126,127]. In fact, it is believed that while PEPCK is the rate-limiting step of gluconeogenesis in the normal state, G6Pase may be the rate-limiting step of gluconeogenesis in the diabetic state[128]. Constant overexpression of G6Pase would then lead to chronic increased hepatic glucose output and decreased hepatic glycogen storage. It is interesting to note that overexpression of G6Pase does not necessarily lead to increases in resting glucose levels but it does lead to elevated glucose levels during oral glucose tolerance tests[125]. Yet, in a mouse model of PEPCK overexpression, basal hepatic glucose production was increased but glucose tolerance was not affected during a hyperinsulinemic-euglycemic clamp experiment[129]. Furthermore, these PEPCK-overexpressing mice demonstrated increased expression of both G6Pase and PEPCK

along with insulin resistance specific only to insulin-mediated G6Pase and PEPCK signaling (insulin-mediated signaling of GLUT2 and glucokinase were not affected)[129]. These findings highlight the fact that while expression of G6Pase and PEPCK are coordinated and tightly regulated through similar pathways, they also demonstrate the ability to exert vastly different effects due to the many pathways involved in their individual expression.

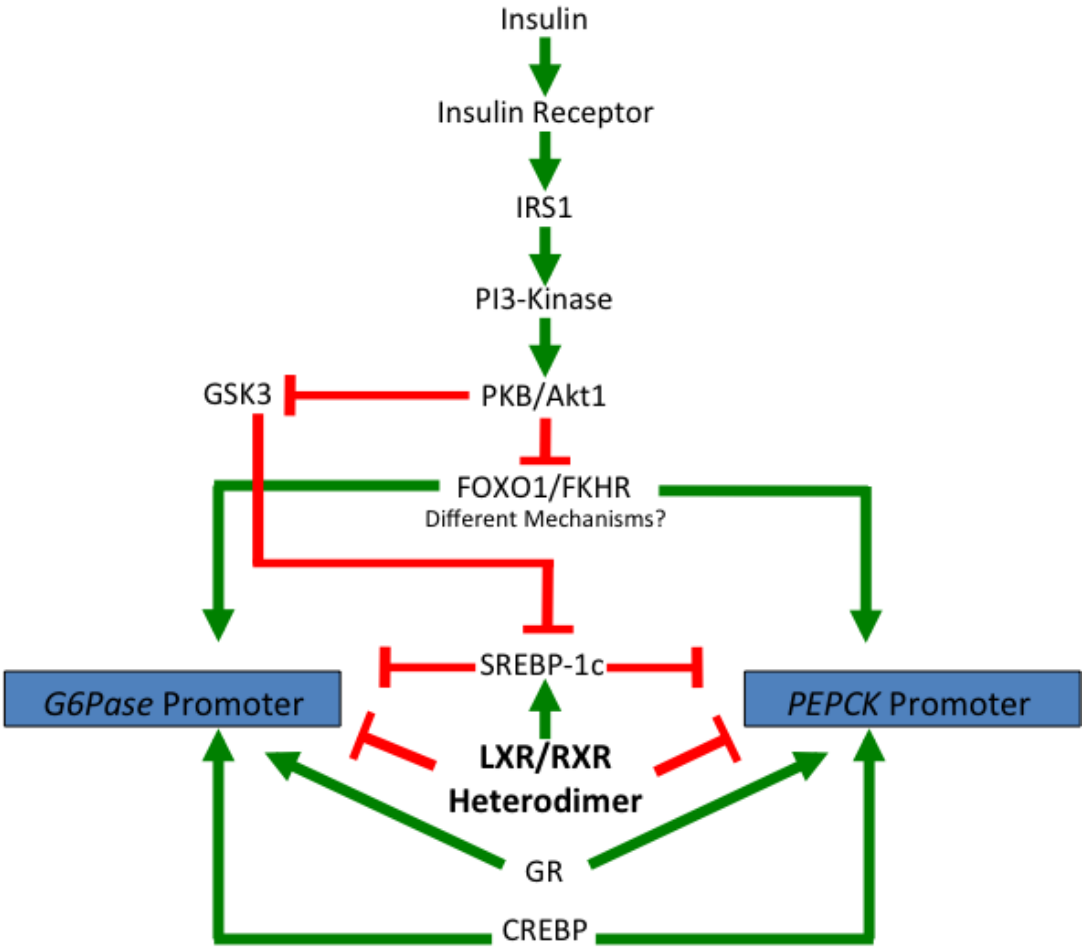

Figure 1.2: A Brief Overview of the Regulation of Hepatic Gluconeogenesis. The promoters of *G6Pase* and *PEPCK* are regulated through many pathways including the insulin-mediated pathway, LXR-mediated pathway, glucocorticoid-mediated pathway, and cAMP-mediated pathway. This list is not exhaustive and other mechanisms and pathways are also involved.

1.5.2 Hepatic Lipogenesis

The liver plays a critical role in the maintenance of triglyceride levels in the body. The overall level of fatty acids and triglycerides in the body is dependent on the balance between lipogenesis and lipolysis. The two main sites of lipogenesis are the liver and adipose tissue[130]. Together, these two tissues are responsible for the coordinated regulation of fatty acids and triglycerides in the body. In addition, hepatic lipogenesis is also tightly associated with the regulation of hepatic carbohydrate metabolism. For instance, one of the major functions of hepatic glycolysis is to provide carbon atoms (in the form of acetyl-CoA) for *de novo* lipogenesis[98].

Lipogenesis is highly dependent on nutritional status. For instance, carbohydrate intake is a major stimulator of hepatic and adipocyte lipogenesis. An increase in carbohydrate intake leads to an insulin spike and insulin is one of the most potent stimulators of lipogenesis[130]. Hyperinsulinemia in rats has been found to increase the long-term expression and activity of hepatic lipogenic genes such as fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC)[131]. Furthermore, it appears that insulin-mediated lipogenesis requires the induction of the transcription factor sterol regulatory element binding protein-1, specifically the 1c isoform (SREBP-1c)[132]. To further link carbohydrate and lipid metabolism, Foretz and colleagues (1999) also found that SREBP-1c was required for the insulin-mediated activation of glucokinase and lipogenic genes[133]. It is believed that the insulin-mediated induction of SREBP-1 is facilitated through the PI 3-kinase pathway[134].

Transcriptional regulation of hepatic lipogenesis is largely mediated by SREBP-1c, the "master lipid regulator" and an isoform of the SREBP family of proteins[135]. Many of the genes involved in fatty acid synthesis possess SRE or EBOX-motifs on their promoters, essential sites for SREBP-1 binding[98,134,136]. For instance, the promoters of *FAS* and *ACC* possess binding sites for SREBP-1[137,138]. Furthermore, mice lacking SREBP-1 expression display a severe impairment of lipogenic gene expression[135]. The generation of fatty acids is an essential prerequisite for the generation of triglycerides. The rate-limiting step of long-chain fatty acid synthesis is mediated by ACC through catalyzing the conversion of acetyl-CoA to malonyl-CoA[139]. The enzyme FAS is then responsible for the repeated addition of malonyl-CoA subunits to acetyl-CoA through condensation reactions. After seven cycles, FAS forms its primary product, palmitate (or palmitic acid), a saturated 16-carbon fatty acid[140]. Stearoyl-CoA desaturase-1 (SCD-1) is a rate-limiting enzyme for the formation of monounsaturated and polyunsaturated fatty acids. It is responsible for adding a *cis*-orientation double bond to carbons 9 and 10 on a variety of acyl-CoAs but prefers palmitoyl- and stearoyl-CoA, which form palmitoleoyl- and oleoyl-CoA, respectively[141]. The resulting monounsaturated fatty acids formed by SCD-1 go on to form essential substrates for the production of other unsaturated fatty acids, triglycerides, phospholipids, and cholesterol esters[141]. Rodent studies have strongly suggested the overexpression and hyperactivity of these lipogenic genes in the development of hypertriglyceridemia and obesity due to their essential role in the formation of triglycerides through increased fatty acid production[142-145]. Hepatic lipogenesis is briefly summarized in Figure 1.3.

While carbohydrate intake stimulates the induction of lipogenesis, the presence of polyunsaturated fatty acids leads to suppression of lipogenesis. This process appears to be mediated through both transcriptional and post-transcriptional mechanisms[146-148]. Lipogenic genes are also suppressed by the presence of polyunsaturated fatty acids through decreases in SREBP-1 expression[149]. Interestingly, the presence of saturated or monounsaturated fatty acids do not appear to affect hepatic lipogenesis[149].

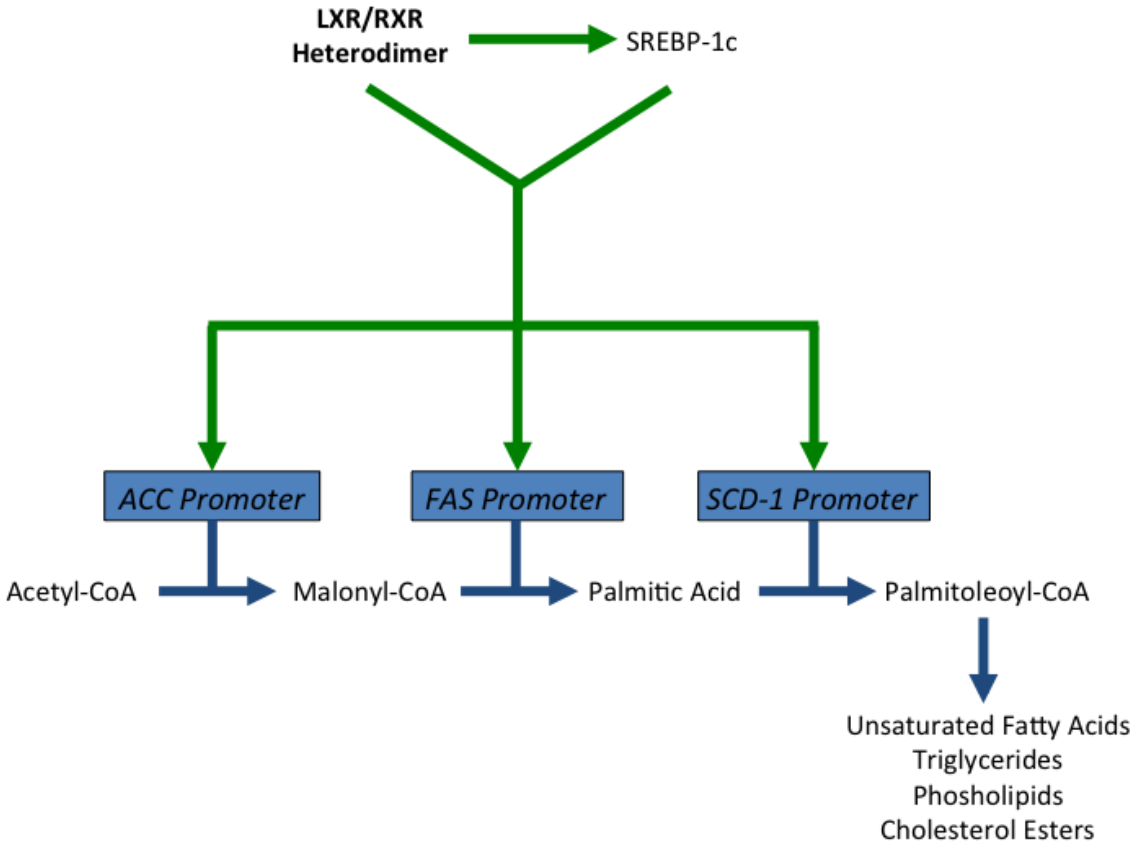

Figure 1.3: A Brief Overview of Hepatic Lipogenesis. Both LXR and SREBP-1c are able to bind the promoters of *ACC, FAS,* and *SCD-1*, while LXR has been found to induce the expression of SREBP-1c.

1.5.3 Hepatic Cholesterol Regulation

In addition to its role in glucose and lipid homeostasis, the liver plays a vital role in the regulation of cholesterol metabolism and transport. Maintenance of proper cholesterol levels is vital to the functioning of an organism. Cholesterol is an essential component of the cell membrane and is the precursor to bile acids, steroids, and vitamins[150]. Three processes, *de novo* cholesterol synthesis, cholesterol catabolism, and cholesterol absorption, mediate cholesterol regulation. The two main sources of cholesterol in the body come from dietary sources and *de novo* cholesterol synthesis. Although virtually every cell in the body can synthesize cholesterol, the principle site of *de novo* cholesterol synthesis is the liver[150]. Since cholesterol can be synthesized *de novo* in the body it is not considered an essential nutrient.

The transcriptional regulation of cholesterol synthesis is principally mediated through the actions of the transcription factor SREBP-1[151]. When the cell detects low level of sterols, SREBP-1 is cleaved from the endoplasmic reticulum and translocates to the nucleus where it activates transcription of essentially all genes involved in the synthesis of cholesterol from acetyl-CoA. These genes include, 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) synthase, HMG-CoA reductase, farnesyl pyrophosphate (FPP) synthase, 7-dehydrocholesterol reductase, squalene synthase, and lanosterol 14 α-demethylase[152,153].

The regulation of sterol (including cholesterol) absorption in the body is another essential point of regulation in cholesterol homeostasis, although much of the process is still not very well understood. A majority of the cholesterol ingested into the body is not

readily absorbed since it is a relatively inefficient process[150]. Evidence for the importance of cholesterol and sterol absorption regulation comes from studies of sitosterolemia, a rare autosomal recessive genetic disorder in which there is a mutation in ATP-binding cassette sub-family G member 5 (ABCG5) and/or ABCG8 genes. They encode the proteins sterolin-1 and sterolin-2, respectively, and are both expressed exclusively in the liver and intestines where they increase the intake and excretion of sterols[154]. Patients with sitosterolemia exhibit elevated circulating cholesterol, and premature atherosclerosis. Further evidence for the role of these transporters in sterol regulation comes from a study where human ABCG5 and ABCG8 were overexpressed in mice, leading to decreased intestinal cholesterol absorption and increased secretion of biliary sterols[155]. Moreover, evidence also suggests that the ATP-binding cassette transporter ABCA1 may also play a role in the excretion of dietary cholesterol, in addition to its role in "reverse cholesterol transport" (the process of transporting cholesterol from the periphery to the liver via the formation of high density lipoproteins)[156]. These transporters, ABCA1, ABCG5, and ABCG8 are principally regulated by a group of nuclear receptors known as the liver X receptors[157-159].

Lastly, cholesterol can be eliminated in the body through bile acid synthesis, a process that occurs solely in the liver[150]. Bile acid synthesis occurs through two pathways, the classic pathway and the alternate pathway[160]. Although several enzymes exist in the bile acid synthesis pathways (*e.g.* cholesterol 7α-hydroxylase (Cyp7a1), 25-hydroxycholesterol 7α-hydroxylase (Cyp7b1), sterol 27-hydroxylase (Cyp27), and sterol 12α-hydroxylase (Cyp8b)), Cyp7a1 is the most studied and is the rate-limiting enzyme in the production of bile acids from cholesterol through the classic pathway[161]. Cyp7a1 is

responsible for the enzymatic conversion of cholesterol to form 7α-hydroxycholesterol. Activity of Cyp7a1 is controlled by the ratio of cholesterol to bile acids in the liver and is sensitive to the changing concentrations of oxysterols (derivatives of cholesterol) and cholesterol[162]. Increasing oxysterol concentrations mediate increased Cyp7a1 transcription through LXR, while transcriptional repression is indirectly mediated through the bile acid receptor known as the farnesoid X receptor (FXR)[162]. Other nuclear receptors involved in Cyp7a1 transcriptional regulation include the promiscuous nuclear receptor known as the retinoid X receptor (RXR), involved in heterodimer formation with LXR, the liver receptor homologue-1 (LRH-1), responsible for basal Cyp7a1 induction, and the small heterodimer partner (SHP), responsible for antagonizing the actions of LRH-1, and ultimately decreasing Cyp7a1 expression[162].

1.5.4 Transcriptional Regulation of Hepatic Gene Expression: The Liver X Receptor

The LXRs (LXRα and LXRβ), part of the 1H subfamily of nuclear receptors, are ligand-activated transcription factors. They have long been implicated in the homeostasis of cholesterol and fatty acids[163,164]. Although both LXRs share similar homology (~78%), they are expressed in different tissues and are differentially regulated in terms of nuclear and cytosolic trafficking[150,165]. Furthermore, studies have also found that both isoforms may be involved in different pathways in the regulation of cholesterol and triglycerides[166]. LXR transcriptionally regulates its downstream target genes by heterodimerizing with the retinoid X receptor (RXR) and binding to the LXR Element (LXRE) on the promoters of these genes. The LXRE consists of a direct repeat gene sequence containing the Direct Repeat-4 (DR-4) motif AGGTCA_4n_AGGTCA, where

'4n' represents a random nucleotide sequence[167]. When LXR and RXR are bound to each other, they can be activated by ligands for either partner[168]. LXRα is mainly expressed in the liver, adipose tissue, spleen, and lungs[168,169], while LXRβ is expressed ubiquitously[170].

Known endogenous ligands for LXR include the oxysterols, which are essentially derivatives of cholesterol. These oxysterols include 24(S),25-epoxycholesterol and 24(S)-hydroxycholesterol[164]. In general, most oxysterols have similar affinities for both LXR isoforms with the exception of 6α-hydroxy bile acids, which have a higher affinity for LXRα[171]. In addition to the endogenous oxysterol ligands for LXR, the non-steroidal agonists GW3965 and T0901317 are potent activators of LXR[172,173]. Natural antagonists for LXR include constituents of mevalonate metabolism (*e.g.* geranylgeraniol and geranylgeranyl pyrophosphate), 5α,6α-epoxycholesterol-3-sulfate (ECHS), and 7-ketocholesterol-3-sulfate[174-176]. Studies have shown that LXR also possesses the ability to autoregulate itself[177-179]. These studies have demonstrated that there are LXREs present on the *LXR* promoter itself and that both endogenous and synthetic ligands for LXR can induce transcription of LXR. However, it appears that this mechanism of autoregulation is found largely in the human LXR gene, and more specifically in macrophages. Interestingly, peroxisome proliferator-activated receptor (PPAR) response elements (*PPRE*) have also been found on the *LXR* promoter indicating that ligands for PPAR (*e.g.* PPARγ) can also induce the transcription of LXR[177,180].

Owing to its activation by oxysterols and its presence in the liver and macrophages, LXR has principally been implicated in regulating genes involved in the metabolism and transport of cholesterol[157,164,181] (Figure 1.2). LXR was first found to

enhance expression of cholesterol 7α-hydroxylase, also known as Cyp7a1[164]. Cyp7a1 is responsible for the enzymatic conversion of cholesterol to 7α-hydroxycholesterol, the rate-limiting step in the classic conversion of cholesterol to bile acids. In addition to the role of LXR in upregulating the conversion of cholesterol to bile acids, it is also involved in the transport and excretion of cholesterol[158,159]. LXR has also been demonstrated to increase the transcription of the "half ATP-binding cassette transporters" G5 and G8 (ABCG5 and ABCG8)[158,159], responsible for the excretion of cholesterol and other sterols from the liver and intestines. Furthermore, LXR also increases the transcription of ATP-binding cassette transporter A1 (ABCA1), which is also responsible for cellular cholesterol efflux to the protein, apolipoprotein A-1 (Apo-A1), an important step in reverse cholesterol transport[157,180,182]. Overall, the role of LXR in the transcriptional regulation of cholesterol metabolism and transport is one of great importance and is essential to maintaining adequate levels of cholesterol. Studies have demonstrated that LXRα$^{-/-}$ deficient mice display the complete inability to accommodate increased cholesterol loads[183].

In addition to its involvement in cholesterol metabolism and transport, LXR has also been implicated in the regulation of enzymes involved in fatty acid synthesis (Figure 1.4). The main target of LXR in the transcriptional regulation of fatty acid metabolism is sterol regulatory element binding protein-1c, SREBP-1c[184-186]. SREBP-1c, also known as the "master lipid regulator", is responsible for transcriptionally inducing many of the essential hepatic lipogenic genes (Figure 1.4). These genes include fatty acid synthase (FAS), stearoyl-CoA desaturase-1 (SCD-1), and acetyl-CoA carboxylase (ACC) among others[137,187,188]. Out of these genes, SCD-1 appears to be one of the main mediators in

LXR-mediated hepatic triglyceride accumulation[145]. Chu *et al*. (2006) demonstrated that SCD-1 deficient mice were protected against LXR-mediated lipogenic effects and even exhibited increased plasma HDL[145]. However, in addition to being regulated by SREBP-1c, these genes (FAS, ACC, SCD-1) are also directly regulated by LXR, as they all possess functional LXREs in addition to functional SREs[145,189,190]. This is exemplified in mice lacking LXR, which exhibit decreased production of hepatic fatty acids[184]. Thus, the control of hepatic lipogenesis is under coordinated and complementary transcriptional regulation between both SREBP-1 and LXR. For instance, LXR has also been demonstrated to act as an intermediary for insulin-mediated SREBP-1c activation[191]. Tobin *et al*. (2002) found that the insulin-mediated regulatory effect on SREBP-1c was completely eliminated in LXR deficient mice[191]. This study was further supported by Chen *et al*. (2004), which demonstrated impaired activation of SREBP-1c when the LXREs on the promoter of *SREBP-1c* were disrupted[192]. Interestingly, lipogenic effects mediated by LXR appear to be primarily mediated by the LXRα isoform[166,193]. Studies done by Lund *et al* (2006) and Quinet *et al* (2006), both demonstrated that selective pharmacological activation of LXRβ could induce the cholesterol-related effects of LXR but not the lipogenic effects.

Recently, it has been found that LXR may also act as a glucose sensor by binding directly to glucose and influencing the expression of genes involved in glucose homeostasis[194] (Figure 1.4). Mitro *et al*. (2007) found that in addition to the known oxysterols, glucose (D-glucose and D-glucose-6-phosphate) is also very likely to be an endogenous ligand for LXR at physiological concentrations comparable to those of the oxysterols[194]. One of the earliest pieces of evidence demonstrating LXR involvement in

glucose homeostasis first stemmed from Stulnig *et al*. (2002), in which a genome-wide gene expression analysis was performed in wild-type and LXR knockout mice given an LXR agonist[109]. The study found decreases in the expression of several genes involved in hepatic gluconeogenesis including glucose-6-phosphatase (G6Pase), phosphoenolpyruvate carboxykinase (PEPCK), and fructose-1,6-biphosphatase (FBPase-1) in wild-type mice but not LXR deficient mice. Following the Stulnig *et al*. (2002) study, multiple studies were done where pharmacological administration of LXR agonists in diabetic phenotype mice led to the stabilization of blood glucose levels and improved insulin sensitivity[110,195,196]. Administration of LXR agonists in non-diabetic mice did not appear to affect the blood glucose levels[110]. However, administration of LXR agonists in obese phenotype mice led to stabilization of blood glucose levels and increased insulin sensitivity[197]. These studies proposed LXR-mediated suppression of hepatic gluconeogenesis (reduced PEPCK and G6Pase activity) as a possible mechanism for the normalization of blood glucose levels in the diabetic mice. It was also suggested that 11β-hydroxysteroid dehydrogenase 1 (11β-HSD1) and the glucocorticoid receptor (GR) might be essential in facilitating the decreased hepatic gluconeogenesis associated with LXR activation[196]. Since LXR has been found to repress 11β-HSD1 expression, a key enzyme in the conversion of inactive corticosteroids to active corticosteroids[198], the effects of decreased glucocorticoid production may also contribute to the observed decrease in hepatic gluconeogenesis. As seen with the LXR-mediated lipogenic effects, it appears that LXR-mediated effects on glucose metabolism are primarily mediated by the LXRα isoform[199]. On the molecular level, it is still unclear how LXR suppresses hepatic

gluconeogenesis, but there is likely interplay between the transcription factors LXR, SREBP-1, and GR.

Finally, LXR has been demonstrated to influence the peripheral uptake of glucose, chiefly in peripheral adipose tissue, through the GLUT4 receptor[195,197,199]. Additionally, GLUT1 expression also appears to be induced by increased LXR activation[200]. Studies examining the promoter of *GLUT4* and its expression in response to LXR agonists have found functional LXREs and direct interactions between LXR and the *GLUT4* promoter[195,201]. Interestingly, the role of LXR in adipose tissue seems to contrast that of its role in the liver, suggesting that LXR metabolic effects are tissue specific. Ross *et al.* (2002) suggest that in adipose tissue, LXR mediates the uptake of glucose and increases lipolysis and glycogen synthesis[200]. This contrasts the role of LXR in the liver, which is to increase hepatic lipogenesis. Furthermore, while GLUT4 is also expressed in muscles, it appears that activation of LXR does not influence the regulation of GLUT4 in muscle cells[195,199]. However, there is some disagreement here as Dalen *et al.* (2003) have found that pharmacological activation of LXR does indeed lead to an increase in GLUT4 mRNA in muscle cells. Conflicting evidence also comes from Kase *et al*. in 2005, where administration of an LXR agonist to human myotubes increased GLUT4 and GLUT1 mRNA[202].

Due to the role of LXR in increasing peripheral glucose uptake through the GLUT receptors and limiting hepatic glucose production through inhibition of the hepatic gluconeogenic genes (G6Pase and PEPCK), LXR agonists have been considered as a therapeutic agent in the treatment of diabetes[110,195]. However, due to the lipogenic

properties of LXR agonists[203], more investigation is required before the therapeutic benefits of LXR can be truly considered.

The role of LXR in regulating glucose, lipid, and cholesterol metabolism is summarized in Figure 1.4.

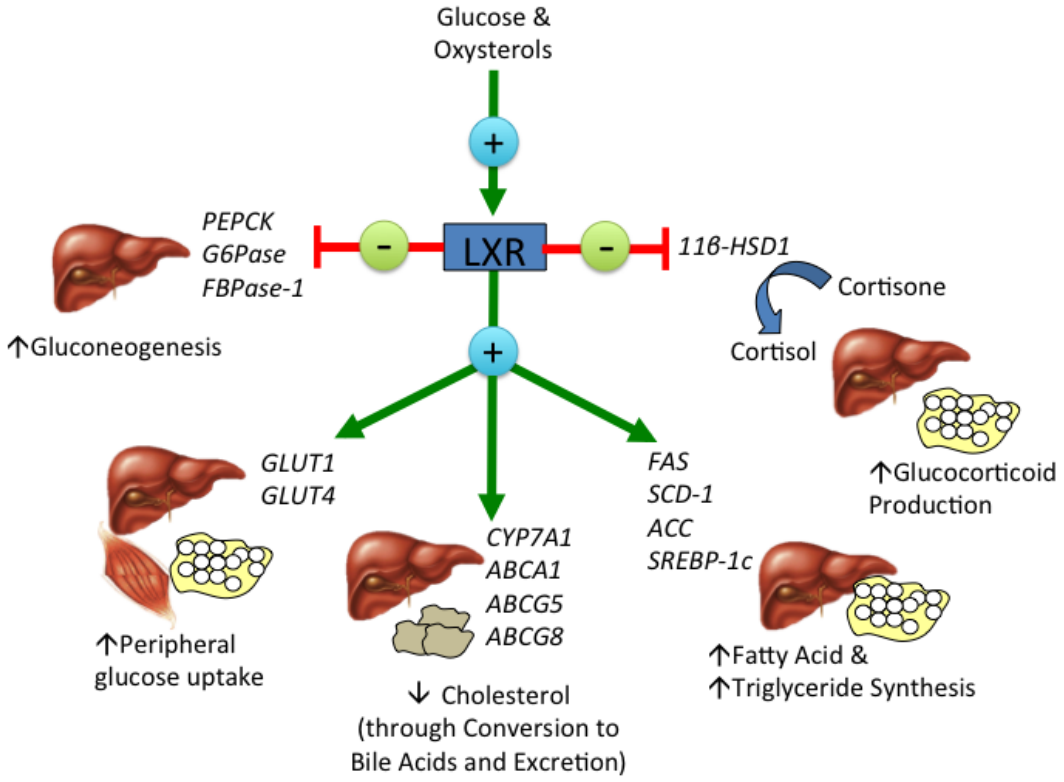

Figure 1.4: The Role of the Liver X Receptor in Regulating Glucose, Cholesterol and Lipid Homeostasis. In general, LXR acts to decrease the level of glucose in the blood through two mechanisms. The first is through increasing peripheral glucose uptake in the body (through induction of the GLUT receptors). The second mechanism is through the suppression of glucose production (via repression of the gluconeogenic genes G6Pase and PEPCK) and suppression of glucocorticoid production (via repression of 11β-HSD1). LXR also decreases cholesterol in the body through the induction of various cholesterol metabolism genes: Cyp7a1 (responsible for conversion of cholesterol into bile acids) and ABCA1, ABCG5, and ABCG8 (responsible for cholesterol efflux). Finally, LXR is involved in the induction of genes involved in the production of fatty acids and ultimately triglycerides. These genes include FAS, ACC, SCD-1, and the "master lipid regulator" SREBP-1.

1.6 Transcriptional Regulation of Hepatic Gene Expression: The Role of Epigenetics

The development of many complex and chronic diseases cannot be simply explained with genomic heritability alone[204]. Epigenetics has emerged as an important mechanism in adjusting the expression patterns of genes in a site and tissue specific manner as an adaptive response to insults during the developmental period. Epigenetic mechanisms essentially influence the long-term expression of a gene by altering the ability of the transcriptional machinery to interact with the chromatin environment. Moreover, they influence heritable changes in phenotype without altering the genetic sequence of an organism. Epigenetic changes can be both transient and persist for long periods of time[205,206]. Mechanisms of epigenetic action include direct DNA methylation, post-translational histone modifications, and more recently discovered microRNA-mediated repression and activation.

1.6.1 Post-Translational Histone Modifications

In the eukaryote nucleus, genomic DNA is combined with numerous different proteins, including histones, to form chromatin. One purpose of chromatin, among many, is to regulate gene expression. The most basic unit of chromatin is the "nucleosome", a length of DNA that is 146 base pairs long and surrounds eight core histones (a pair of each of the histones H2A, H2B, H3, and H4)[207]. Each core nucleosome contains two functional domains: a "histone-fold" motif for histone-histone and histone-DNA interaction within the nucleosome and a histone tail composed of a terminal $–NH_2$ group and –COOH group[208]. Nucleosomes are linked together by "linker DNA", which also

interacts with histone H1. At the lowest level of organization, genomic DNA surrounds the nucleosome to form a structure resembling “beads on a string”. As chromatin condenses into higher order structures, it becomes more complex in nature due to the countless interactions between the genomic DNA, histones, and a vast array of proteins associated with the histones. Furthermore, condensed chromatin is less accessible, more stable, and is generally considered transcriptionally inactive. With that being said, even today, the precise structure of higher order condensed chromatin is still in question.

In general, there are two main forms of chromatin. Euchromatin, the least dense form of chromatin, is said to be more transcriptionally active as its open structure allows easier accessibility for transcriptional machinery and protein interaction. Euchromatin is generally associated with increased histone acetylation profiles, a hallmark of chromatin opening. The more condensed form of chromatin is known as heterochromatin[209,210]. Unlike euchromatin, heterochromatin is less accessible by transcriptional machinery and generally considered transcriptionally inactive. Heterochromatin is generally associated with a decreased histone acetylation profile and an increased histone methylation profile, which is representative of more stable and inaccessible chromatin.

A major epigenetic mechanism involves influencing the chromatin environment through a number of post-translational modifications on the histone tails (-NH_2 domain on the histone), including methylation, acetylation, phosphorylation, ubiquitination and ADP-ribosylation of histones[211,212]. Histone tail modifications such as phosphorylation and acetylation are more transient, while methylation is considered more stable in nature[213,214]. The combinatorial and unique nature of these covalent modifications reveal a “histone code”, which may serve critical as an adaptive regulatory mechanism that can

also influence gene expression in a tissue- and gene-specific manner at times of insult during development. Furthermore, these histone modifications occur and are maintained by a diverse range of histone modifying enzymes including families of histone acetylases and methyltransferases[215], whose levels may also be altered as a result of a developmental insult. It is important to realize that the different prenatal insults that lead to IUGR offspring seem to have both common and distinct adaptive responses initiated via epigenetic mechanisms. Therefore IUGR offspring derived from different insults may differ or be similar due to global, tissue, or site-directed epigenetic modifications.

1.6.2 Histone Acetylation and Increased Gene Activity

The first evidence of histone acetylation as a mechanism for transcriptional activation came from a studies done by Allfrey and colleagues[216,217]. Later studies went on to support the notion that histone acetylation was strongly correlated with active genes and increased transcriptional activity[218,219]. Additional studies then found increased interaction between transcription factors and chromatin sites where the histones were highly acetylated, further indicating that sites of increased acetylation facilitated transcription, likely through increased accessibility for transcriptional machinery (*e.g.* co-activators, signaling proteins, and RNA polymerase II)[220,221].

There are several hypotheses that may explain why acetylation of histone tails on the nucleosome would lead to increased transcription[222]. The first hypothesis, as mentioned earlier, is based on the belief that acetylation of lysine residues on the histone tail leads to the neutralization of the positive charges at these tails and subsequently less

interaction between the histone tails and DNA. This decreased interaction would then lead to increased accessibility for the transcriptional machinery to bind DNA and facilitate increased transcriptional activity. The second hypothesis is that the acetylation of lysine residues on the histones (and occasionally non-histones) surrounding the gene in a site-specific pattern acts as a signal for corresponding transcriptional machinery. For instance, co-activators or co-repressors of a transcription factor may recognize specific acetylation patterns for different histones and facilitate or repress transcription. The third hypothesis does not involve acetylation of the histone tail itself but acetylation of non-histone proteins that may associate with the core histones and/or transcriptional machinery and facilitate transcription. It should be noted that these hypotheses are not mutually exclusive and are very likely acting in concert[222].

The two main families of enzymes responsible for the acetylation and deacetylation of histone tails are the histone acetyltransferases (HAT) and histone deacetylases (HDAC), respectively (Figure 1.5). The steady-state acetylation profile of histones depends on the balance between the activities of HATs and HDACs. Interestingly, prior to the discovery of these HATs and HDACs, many of these proteins were already known to be functionally involved in transcriptional regulation[223]. Studies found that HATs were generally associated with co-activators, while HDACs were associated with generally co-repressors, lending further evidence to the permissive actions of histone acetylation and the repressive actions of histone deacetylation[223,224]. HATs, and more specifically, A-type HATs (or HAT-A) are responsible for transferring acetyl groups from acetyl-CoA to specific lysine groups on histone tails[224]. Common sites for histone acetylation are lysine residues 9, 14, 18, and 23 on histone H3[225,226]. HDACs

are part of a superfamily and are composed of several different classes, which are involved in different cellular processes. HDACs are responsible for the removal of acetyl groups from lysine residues on histones (and non-histones), leading to hypoacetylated chromatin[227]. Hypoacetylated chromatin is generally more condensed due to increased interaction between the positively charged lysine residues and the negatively charged genomic DNA. This decreases the accessibility of transcriptional machinery. To add to the already complex nature of histone modifications and transcriptional activation, studies have also demonstrated that HDAC activity may also be required for transcriptional *activation* of certain genes[228]. The opposite has also been found, where histone acetylation was required for transcriptional silencing[229]. Thus, transcriptional regulation on the chromatin and histone level requires a delicate balance between different acetylation patterns. It should also be noted that some transcription factors themselves appear to possess histone acetyltransferase activity[222]. Finally, though histone acetylation is characterized as a highly dynamic and transient histone modification, evidence suggests there are also cases where acetylation persists for longer periods of time (*e.g.* mitosis)[230].

1.6.3 Histone Methylation and Altered Gene Activity

While histone hyperacetylation is strongly linked to increased chromatin accessibility and increased transcriptional activity, histone hypermethylation is generally associated with decreased transcriptional activity. Unlike the well-studied effects of histone acetylation, histone methylation is a relatively new field of study. Chen *et al.*

(1999) did one of the first studies linking histone methylation with transcription, whereby they found a strong connection between transcriptional co-activators, methyltransferase activity on histone H3, and levels of transcription[231]. Intriguingly, this first piece of evidence linked methylation with increased transcription rather than decreased transcription. A further study done by Rea *et al.* (2000) suggested that methylation of histone H3 on lysine 9 was associated with the formation of heterochromatin, discouraging the recruitment of transcriptional activators[232]. Unlike histone acetylation, lysine residues can be mono-, di-, and tri- methylated. Furthermore, while enzymes have been found to be able to reverse mono- and di- methylation, trimethylation appears to be generally irreversible[233]. This suggests histone trimethylation as a very stable histone modification.

The main family of enzymes involved in the methylation of histones is the histone methyltransferase (HMT) group (Figure 1.5). HMTs catalyze the transfer of methyl groups from the methyl donor S-Adenosyl methionine (SAM) to lysine residues or arginine residues on histones[233]. HMTs are commonly known to add methyl groups to lysine residues 4, 9, 27, and 36 on histone H3, and lysine residue 20 on histone H4[233]. The specific methylation of lysine residue 9 on histone H3 has been established as a recognition site for heterochromatin protein 1 (HP1), a protein involved in heterochromatin formation and stabilization as well as gene silencing[234,235]. HP1 recognition and recruitment has not been observed for other lysine residues, such as lysine 4 on histone H3[236]. In addition to methylation of lysine 9 on histone H3, methylation of histone H3 lysine residue 27 has also been found to be involved in gene silencing[237], although through a different mechanism. Methylation of lysine 27 on histone

H3 was found to facilitate transcriptional repression through recruitment of Polycomb group protein complexes[237].

In contrast to methylation of lysine residues 9 and 27, methylation of residue 4 on histone H3 has been found to be involved with increased gene transcription[238,239]. Bernstein and colleagues (2002) postulate that methylation of histone H3 lysine 4 facilitates transcription by protecting the lysine group from deacetylation[238]. Interestingly, Santos-Rosa *et al*. (2002) found that trimethylation of histone H3 lysine 4 was purely associated with increased gene transcription, while dimethylation of histone H3 lysine 4 was associated with both gene activation and repression[239]. Furthermore, studies have demonstrated that hypermethylation of histone H3 lysine 4 may act as a marker or placeholder for genes that were recently transcribed[240,241]. Given the stable nature of histone methylation compared to the other post-translational modifications (*e.g.* acetylation, phosphorylation, ubiquitination), it is the most likely candidate to act as a memory marker for transcriptional activity and other vital processes related to the genome.

Recently, the discovery of histone demethylases has provided much insight into the regulation of histone methylation profiles[242,243] (Figure 1.5). Prior to the discovery of these histone demethylases, it was postulated that methyl groups were removed through complete histone replacement and methylation profiles were modified through histone turnover[233,244]. The first lysine specific demethylase to be discovered was lysine-specific demethylase 1 (LSD1), which was found to specifically demethylate mono- or di- methyl groups from only histone H3 lysine 4[242]. Further studies identified another group of histone demethylases. Histone demethylase JmjC domain-containing histone demethylase

1 (JHDM1) was found to be responsible for the demethylation of mono- and di- methyl groups on histone H3 lysine 36[245], while JHDM2 was found to demethylate mono- and di- methyl groups on histone H3 lysine 9[246]. Further studies uncovered the JMJD2 subfamily consisting of JMJD2A, JMJD2B, JMJD2C, and JMJD2D[247]. All members of the JMJD2 subfamily have been found to demethylate trimethyl groups on histone H3 lysine 9[248-251]. JMJD2A and JMJD2C activity appear to favour the formation of a dimethyl group, while JMJD2D activity favours the formation of a single methyl group. Taken together, it is highly likely that the delicate balance of gene transcription and repression relies on the complex interplay between HATs, HDACs, HMTs, and histone deacetylases (Figure 1.5).

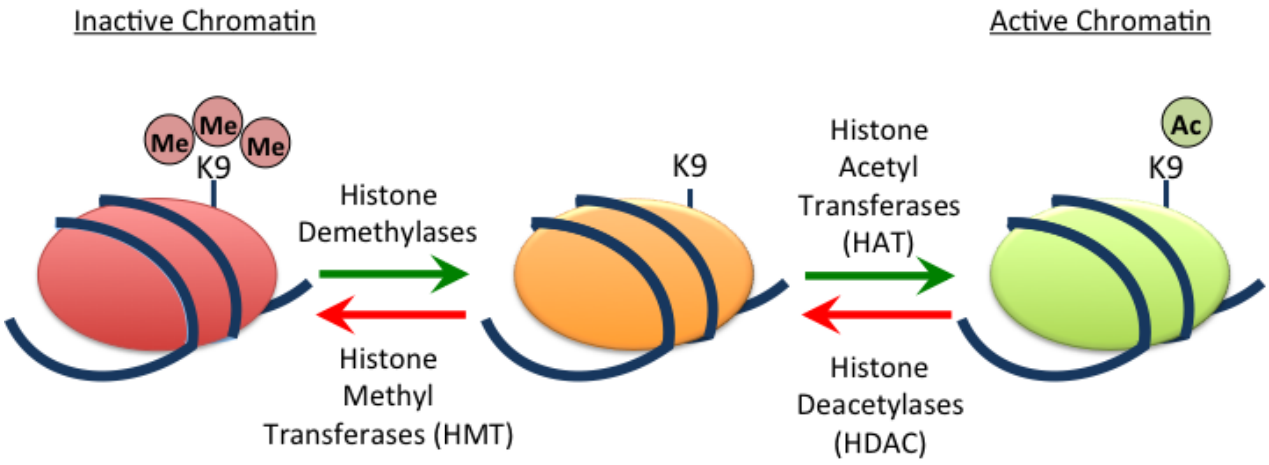

Figure 1.5: Post-Translational Histone Modifications Involved in Chromatin Remodeling. Histone demethylases and histone acetyl transferases (HAT) modify chromatin to "open" it up and provide access for transcriptional machinery. In contrast, histone methyl transferases (HMT) and histone deacetylases (HDAC) modify chromatin such that it is in a "closed" state, decreasing the likelihood of transcriptional machinery interaction.

1.7 Tissue Plasticity: Reversing the *in utero* Origins of Adult Disease

The development of many organs occurs both prenatally and postnatally. For example, in the liver, development consists of embryonic cell specification, budding, and differentiation[252]. Until birth, the liver has major hematopoietic function[253], but by mid-gestation in rodents, the liver bud is formed containing bipotential progenitor cells that differentiate into either hepatocytes or ductal cells[254]. In the last three days of gestation in the rat, liver mass triples due to a high rate of fetal hepatocyte proliferation[255], followed by a transition of fetal to adult rat hepatocytes in the first week of postnatal life[256]. Given that during this neonatal period there is a high rate of replication, neogenesis and apoptosis[255] leading to extensive liver remodeling, this period represents a critical window for therapy designed to improve hepatic growth and function long-term. For example, it has been demonstrated in IUGR rat offspring derived from uterine artery ligated dams, that neonatal administration of Exendin-4™, a glucagon-like peptide 1 (GLP-1) analogue, prevents the development of diabetes due to the restoration of the transcription factor pancreatic and duodenal homeobox 1 (Pdx-1), and ultimately cell function[257]. Moreover, Exendin-4™ treatment during this neonatal period also prevented the development of hepatic oxidative stress and insulin resistance[258]. This indicates quite remarkably that neonatal intervention in rats can influence both pancreatic and liver development long-term, and possibly reverse adverse events encountered during gestation. Therefore the goal of future studies is to understand how we can exploit this plasticity in organ development to correct the short- and long-term abnormalities resulting from an adverse *in utero* environment. Given that the rat liver develops at a very similar timeframe compared to the human liver[252], further insights into the reversibility of

fetal programming effects on liver development offers promise in human IUGR pregnancies.

Our recent studies indicate that restoration of maternal protein intake during lactation can rescue liver growth and prevent the development of hypercholesterolemia long-term in the offspring of protein-restricted dams[75]. However the underlying epigenetic and transcriptional mechanisms are unknown. While LXR agonists have been demonstrated to activate acetylation of LXR-target promoters and lower LDL cholesterol in atherosclerosis-prone adult mice[190], their use in neonatal life is limited[259]. Given Cyp7a1 expression is enhanced by histone hyperacetylation[260], it is conceivable that LXR agonist administration *in vivo* could boost the expression of LXR target genes, via increases in both LXR binding and histone acetylation surrounding the LXRE sites. Preliminary evidence from our laboratory suggests that 3-week-old MPR offspring treated with an LXR agonist (GW3965) from postnatal day 5 to 15 display decreased circulating cholesterol to HDL ratios compared to vehicle treated MPR offspring[261]. These offspring also displayed increased hepatic expression of Cyp7a1, concomitant with increased recruitment of RNA polymerase II and acetylation of histone H3 (lysine 9,14) surrounding the *Cyp7a1* promoter by 3 weeks of age[261]. Additionally, studies of MPR in embryonic day 19.5 mice have demonstrated that LXR expression is decreased in MPR offspring during the neonatal period[262]. Given the fact that LXR agonists exert antidiabetic effects when administered in rodents[110,195], it is possible that administration of an LXR agonist during the neonatal period may rescue the long-term repression of LXR and prevent the development of programmed impaired glucose tolerance in later adulthood[86,87].

While the effects of neonatal LXR agonist administration on glucose and cholesterol homeostasis still need to be assessed long-term, preliminary data suggest that LXR and other nuclear receptor agonists may play a promising role in reversing the long-term adverse effects of impaired fetal development. However, caution must still be taken since LXR agonist administration does lead to the elevated expression of hepatic lipogenic genes[203].

1.8 Rationale, Hypothesis, and Objectives

1.8.1 Rationale and Hypothesis: IUGR is now closely linked to the increased risk of developing chronic disease in later life[44,45]. A mismatch in environment, as proposed by the Barker Hypothesis, is responsible for the programming of these diseases[58-60]. Moreover, the concept of catch-up growth appears to exacerbate the risks of developing chronic diseases[62,69]. Yet, the molecular mechanisms underlying the programming of impaired glucose homeostasis, an essential symptom of the metabolic syndrome, remain elusive. Given the role of LXR in modulating the metabolism of glucose, lipids, and cholesterol, it is an attractive candidate to study in order to elucidate the molecular mechanisms behind the programming of diseases such as the metabolic syndrome. Evidence from our laboratory and others suggests that LXR expression and activity may be repressed in rodent models of IUGR, especially maternal protein restriction[75,261,262]. Further considering the role of LXR agonists in exerting antidiabetic effects in rodents[110,195], there is strong evidence that LXR may be involved in the programming of impaired glucose metabolism. ***Using MPR in the rat as a model of growth restriction, I***

hypothesize that MPR does indeed lead to impaired glucose homeostasis in the offspring and that impairment of glucose homeostasis is at least partly mediated through altered actions of LXR.

1.8.2 Objectives:

The first objective of the study is to determine the effects of MPR on impairing glucose homeostasis in offspring by examining glucose tolerance of offspring in adulthood.

The second objective is to determine the role of LXR in the programming of impaired glucose homeostasis by examining: A) expression profiles of LXR and LXR-target genes involved gluconeogenesis (*e.g.* G6Pase, PEPCK, and 11β-HSD1); B) the active and repressive roles of LXR on a transcriptional level and; C) the long-term effect of administration of an LXR agonist (GW3965) during neonatal life, a period of developmental plasticity.

1.9 References

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Chapter Two:

Maternal Protein Restriction Leads to Enhanced Hepatic Gluconeogenic Gene Expression in Adult Male Rat Offspring Due to Impaired Expression of the Liver X Receptor

A version of this chapter has been previously published: T.X. Vo, A. Revesz, G. Sohi, N. Ma, D. Hardy. Maternal protein restriction leads to enhanced hepatic gluconeogenic gene expression in adult male rat offspring due to impaired expression of the liver X receptor. Journal of Endocrinology. 218(1):85-97, 2013.

2.1 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 II diabetes[1-5]. Previous animal models of maternal protein restriction have consistently linked asymmetric IUGR[6] with symptoms of type II 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[7]. 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[8,9]. 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[10]. 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[11,12], while LXRβ is ubiquitously expressed[13]. Endogenous ligands for LXR are mainly derivatives of cholesterol (i.e., oxysterols)[14,15]. Consequently, LXR has principally been implicated in regulating genes involved in the metabolism and transport of cholesterol[14,16] and in

enhancing the expression of lipogenic enzymes[17]. 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[18-20]. In addition, LXR has also been found to indirectly suppress hepatic glucose production through inhibition of the enzyme 11β-hydroxysteroid dehydrogenase-1 (11β-HSD1)[18]. 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. Moreover, LXR has been implicated in the regulation of the glucocorticoid receptor (GR), further encompassing its activity in the regulation of glucocorticoids and glucose homeostasis[21].

Previous studies from our own laboratory have found that maternal protein restriction (MPR) leads to decreases in the expression of the LXR-target gene, *Cyp7a1*, the critical enzyme involved with cholesterol catabolism. The decrease in Cyp7a1 led to hypercholesterolemia in male offspring by 4 months[22]. 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* promoter in association with decreased LXRα mRNA in the liver tissue of embryonic day 19.5 fetuses, however the effect on post-translational histone modifications surrounding *LXRα* remain elusive[23]. While we and others have demonstrated that MPR can lead to long-term epigenetic alterations of LXR-target genes

involved with cholesterol and lipid homeostasis, it is not known if LXR-target genes impairing hepatic gluconeogenesis are altered.

The aims of the current study were to examine whether maternal protein restriction 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, PEPCK, 11β-HSD1). Using a well-established model of maternal protein restriction 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 earlier restoration of protein promotes accelerated catch-up growth[22]. 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[24-26].

2.2 Materials and Methods

2.2.1 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, Quebec, Canada) and were allowed to acclimatize to their new environment for two weeks. Rats were housed at room temperature on a 12-12 hour light-dark cycle. Females were housed in separate cages and were cohabitated with a male for mating upon entering pro-estrous. Conception was confirmed by 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[22]. The experimental model is exemplified in Figure 2.1.

At embryonic day 19, a subset of dams (3 control dams; 4 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 (4 control dams; 4 LP dams)

delivered spontaneously. All litters with greater than 10 pups were arbitrarily culled down to 9-10 pups to ensure a consistent litter size per dam.

After the intraperitoneal glucose tolerance tests at postnatal day 120-125, all offspring were sacrificed using a lethal dose (50mg/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 in other models of protein restriction, impaired glucose tolerance was not observed earlier than 4 months[10]. Following sacrifice, liver and blood were immediately extracted and flash frozen at -80^{o}C for molecular analysis. We did not examine the female offspring in this study to prevent confounding factors related to their estrous cycle and hormone profile. More importantly, the maternal low protein model has been demonstrated to exhibit early life programming effects in a sexually dimorphic manner, which was not the focus of this investigation[10,22,27]. For molecular analysis, one to two male pups from each of four separate dams were arbitrarily chosen. All available male pups were used for the intraperitoneal glucose tolerance tests.

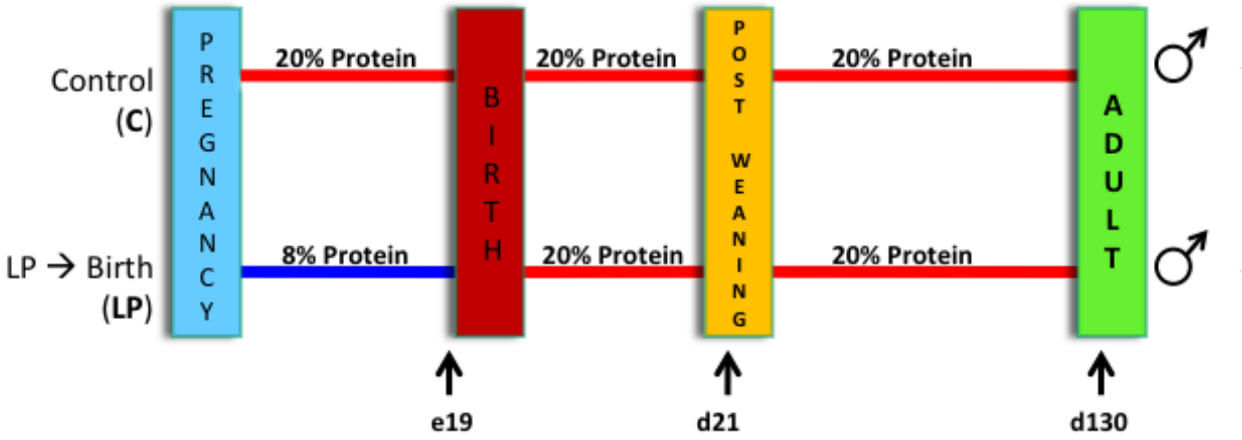

Figure 2.1: Experimental Paradigm of the Maternal Protein Restricted Model. Briefly, control (C) dams and offspring received a control (20%) diet throughout life, while low protein (LP) dams received a low protein (8%) diet throughout gestation. At birth, the LP dams were immediately placed on a control diet to restore protein and promote accelerated growth in the offspring. Offspring of LP dams received a control diet at the end of the weaning period (postnatal day 21).

2.2.2 Glucose Tolerance Tests

At postnatal day 120-125, male offspring were subject to an intraperitoneal glucose tolerance test (IPGTT). Prior to the IPGTT, the animals were fasted overnight for 14-16 hours. Animals were awake throughout the experiment. Blood glucose measurements were obtained using a Bayer Breeze® 2 Blood Glucose Meter (Bayer, New York, USA). Fasted blood glucose levels were obtained prior to the glucose injection. Animals then received 2g/kg of glucose via injection into the intraperitoneal cavity. Blood glucose was sampled at the tail vein at t= 0, 5, 10, 15, 30, 60, and 120 minutes. Area under the curve of each animal was calculated using *GraphPad Prism™* software. IPGTT were performed on 6 control males and 10 LP males.

2.2.3 Quantitative Real Time PCR (qRT-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, Carlsbad, CA, USA) method[22]. Total RNA was subsequently treated with deoxyribonuclease to eliminate contaminating DNA. 4µg of total RNA was then reverse transcribed to cDNA using random primers and Superscript II RNase H-reverse transcriptase (Invitrogen, Carlsbad, CA, USA). *Taqman®* probes and sequences for the genes of interest (11β-HSD1, G6Pase, LXRα, PEPCK, β-Actin) 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^{o}C for 10 minutes) followed by 40 cycles of denaturing (95^{o}C for 15 seconds) and annealing (60^{o}C for one minute). 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, $2^{\Delta\Delta Ct}$, where ΔΔCt was the standardized Ct value.

2.2.4 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 50mM, NP-40 1%, Sodium-deoxycholate 0.25%, NaCl 150mM, EDTA 1mM, NaF 50mM, Na3VO4 1mM 1mM, β-Glycerophosphate 25mM). Prior to tissue homogenization, a mini protease inhibitor tablet was added to the lysis buffer.

Firstly, a small chunk of snap frozen liver was added to 600µl of RIPA buffer. The tissue was then homogenized with the IKA T10 Basic S1 Dispersing Tool (IKA Works Inc, Wilmington, NC) for 10-15 seconds at speed 6. After letting the homogenized tissue sit on ice for 5 minutes, the tissue was then sonicated. Following sonication, the tissue was rotated at 4^{o}C for 5 minutes and then centrifuged for 15 minutes at 300g and 4^{o}C. The supernatant was retained for further centrifugation at 20,000g for 20 minutes at 4^{o}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, Carlsbad, CA, USA) and transferred onto PVDF membrane (Millipore, Etobicoke, Ontario, Canada). Amido black staining and Coomassie brilliant blue staining confirmed sufficient transfer of proteins onto the membrane.

Immunoblots were probed using LXRα (Liver X Receptor (1:1000; cat# sc-13068)), PEPCK (1:2000; cat# sc-32879), G6Pase-α (1:1000; cat# sc-25840), PI3-kinase p85α (Z-8) (1:1000; cat# sc-423) and 11β-HSD1 (1:800; cat# sc-20175) all from Santa Cruz Biotechnology (Santa Cruz, California). In addition, p-Akt1 (Serine 473) (1:1000; cat# ab66138), p-Akt1 (Threonine 308) (1:500; cat #4796) and Akt1 (1:125; cat# ab6076) antibodies used to assess hepatic insulin sensitivity, were purchased from Abcam Inc, Cambridge, Massachusetts. In addition, we also assessed insulin sensitivity by using antibodies against p-IRS-1 (Serine 302) (1:500; cat# 2384), p-IRS-1 (Serine 1101) (1:500; cat# 2385), and IRS-1 (1:500; cat# 2382) all purchased from Cell Signaling, Danvers, Massachusetts Monoclonal HRP conjugated β-Actin (1:50,000; cat#A3854, Sigma-Aldrich, Oakville, Ontario, Canada) diluted in 5% milk-TBS-Tween-20(0.1%) buffer and HRP conjugated donkey anti-rabbit IgG (1:10,000, cat# 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 enhanced chemiluminescence detection system (Thermo Scientific, Waltham, MA, USA).

2.2.5 Chromatin Immunoprecipitation (ChIP)

Chromatin was extracted from the medial lobe of offspring livers as previously described[22]. Briefly, a small piece of snap frozen liver was homogenized and incubated with 1% formaldehyde for 10 minutes at room temperature to cross-link proteins and DNA. Crosslinking was terminated by the addition of glycine (0.125M, final concentration). The liver tissue was washed once with cold PBS and placed in 500 µl of SDS lysis buffer (Millipore, Etobicoke, Ontario, Canada) with a protease inhibitor cocktail (Roche, Mississauga, Ontario, Canada). The lysates were sonicated on ice to produce sheared, soluble chromatin. The lysates were diluted ten times with the addition of ChIP dilution buffer (Millipore, Etobicoke, Ontario, Canada) and aliquoted to 400 µl amounts. Each of the aliquots was precleared with protein A/G Plus agarose beads (40 µl, Millipore, Etobicoke, Ontario, Canada) at 4°C for 30 minutes. The samples were centrifuged at 20,000g 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 Polyermase II (cat #05-623B, Millipore, Canada), trimethylated histone H3 lysine 4 [K4] (cat #ab1012, Abcam, Canada), acetylated histone H3 lysine 9,14 [K9,14] (cat #05-399, Millipore, Canada), trimethylated histone H3 lysine 9 [K9] (cat# 07-442, Millipore, Canada), and ChIP-grade LXRα (cat# sc-13068x, Santa Cruz Biotechnology, Santa Cruz, California) at 4°C overnight. Two aliquots were reserved as 'controls' – one incubated without antibody and the other with non-immune IgG (Millipore, Etobicoke, Ontario, Canada). 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

minutes in wash buffer I (20 mM Tris-HCl, pH 8.1, 2 mM EDTA, 0.1% SDS, 1% Triton X-100, 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, 0.25 M LiCl), and in 2 × TE buffer. The beads were eluted with 250 µl elution buffer (1% SDS, 0.1mM $NaHCO_3$ + 20 µg salmon sperm DNA (Sigma-Aldrich, Oakville, Ontario, Canada)) at room temperature. This was repeated once and eluates were then combined. Crosslinking 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, Carlsbad, CA, USA) was added to each sample in buffer (50 mM Tris-HCl, pH 8.5, 1% SDS, 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 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)[12] with putative transcription factor binding sequences based on algorithms as described by Cartharius *et al.*[28]. Quantitative real-time PCR was employed using forward (5'-GGTCACTGCATGATCACAGG-3') and reverse (5'-CCTTGGAATCCAGAATGCTC-3') primers that amplify a *-35* bp to *+92* bp region encompassing the rat *G6Pase* LXRE site (+22 bp to +46 bp), and forward (5'-TTCGCCAAACTCTGACCTCT-3') and reverse (5'-ACAGGTTTGGCCTGGAT-GT-3') primers that amplify a *-115* bp to *-7* bp region encompassing the rat *11β-HSD1* LXRE

site (-114 bp to -90 bp) (PE Applied Biosystems, Boston, MA, USA). The LXRα (Gene: NR1H3) transcriptional start site (TSS) was found using the Ensembl Genome Browser (http://www.ensembl.org). Forward (5'- GGCTTCACTGGTTGATCCAT-3') and reverse (5'-AGGGGGTTGATTCTTGAGGT-3') primers were designed to amplify the -135 bp 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 maternal protein restriction[23]. 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, Mississauga, Ontario, Canada) to perform qRT-PCR. Similar to the gene expression assays, the relative abundance of the immunoprecipitated chromatin compared to input chromatin was determined using the $2^{\Delta\Delta Ct}$ method.

2.2.6 Statistical Analysis

All data is represented as a mean of an arbitrary value ± Standard Error of the Mean (SEM). Glucose tolerance tests, areas under the curve, quantitative real-time PCR (including ChIP), and quantified western immunoblot bands were analyzed using the unpaired *Student's t-test*. All data with a *p*-value less than 0.05 were considered statistically significant.

2.3 Results

2.3.1 Maternal protein restriction with earlier protein restoration after birth leads to liver and body weight catch up growth by 3 weeks of age

As previously reported, at embryonic day 19, the LP animals exhibited significantly decreased fetal to placental weight ratios compared to the control animals (Control: 5.67±0.30; LP: 4.87±0.18; $p<0.05$), indicating growth restriction[22]. Furthermore, liver weight to body weight ratios were decreased in the LP animals (Control: 0.091±0.004; LP: 0.056±0.006; $p<0.05$), indicating hepatic growth restriction at embryonic day 19. However, by 3 weeks of age, the LP male offspring caught up to the control offspring in terms of body weight (Control: 50.30±1.15g; LP: 48.00±2.17g)[22]. Similarly, there were no significant differences in the liver weight to body weight ratios between the LP animals and control animals at 3 weeks of age (Control: 0.0391±0.001; LP: 0.0360±0.001)[22], indicating recovered liver growth. At 4 months of age, these patterns continued and there were no differences in body weight (Control: 565.50±8.21g; LP: 579.00±18.74g) or liver weight to body weight ratios (Control: 0.0314±0.001; LP: 0.0306±0.001) between the control and LP offspring[22]. There were 10-14 offspring per experimental group.

2.3.2 Maternal protein restriction 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 (2g/kg), measured blood glucose levels were significantly elevated ($p<0.05$) in LP animals at the 10-, 15-, 30-, and 60-minute time points (Figure 2.2A). Blood glucose levels in the LP animals returned to the same levels as the control animals by the 120-minute 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 to the control animals (Figure 2.2B) 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 phosphorylated IRS-1 (S302 and S1101), all markers of insulin sensitivity[29,30]. Protein expression of these proteins was unchanged between control and LP animals, suggesting no difference in insulin sensitivity between the experimental groups at 4 months of age (Figure 2.3).

2.3.3 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 the *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 (Figure 2.4). Hepatic G6Pase and 11β-HSD1 mRNA were significantly increased ($p<0.05$, 1.6 fold) in the LP offspring (Figure 2.4).

2.3.4 The levels of hepatic LXRα protein are decreased, concomitant with an increase in G6Pase, 11β-HSD1 and GR 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 to the control animals (Figure 2.5). PEPCK protein expression was not different between the two groups. We also

investigated the protein expression of GR since we saw increases in the expression of 11β-HSD1. GR protein expression increased in LP offspring compared to control offspring ($p<0.05$, 1.3 fold).

2.3.5 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 to immunoprecipitate LXRα. After using *MatInspector* (Genomatix, Munich, Germany) 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 to the control animals ($p<0.05$) (Figure 2.6). The non-specific binding of immunoglobulin G (IgG) was tested and found to be minimal (Ct value > 34, data not shown).

2.3.6 Acetylation of lysine residues 9 and 14 on histone H3 is decreased surrounding the transcriptional start site 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 maternal protein restriction. 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* (Figure 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 (Figures 2.7A and 2.7B). Again, the non-specific 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 maternal protein restriction model of IUGR.

2.3.7 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. Quantitative real-time 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 was decreased in LP offspring compared to control offspring ($p<0.05$) (Figure 2.8).

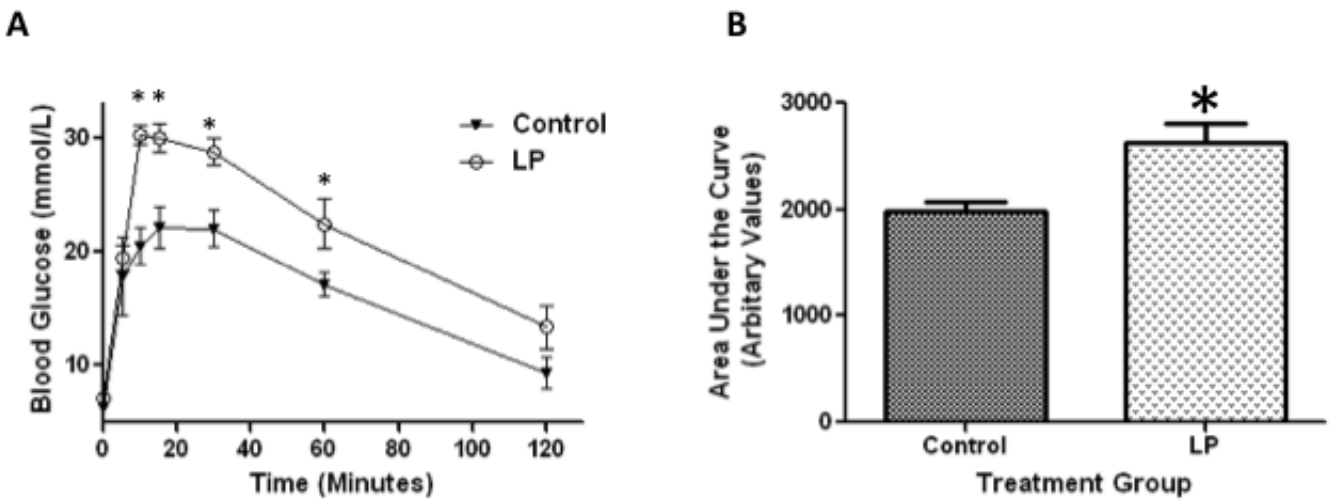

Figure 2.2: **A)** Intraperitoneal glucose tolerance tests (2g/kg) administered to fasted male offspring at 4 months of age. Control and LP animals were analyzed together at each time point (t=0, 5, 10, 15, 30, 60, 120 minutes) using the *Student's* unpaired t-test. **B)** Area under the curve of Control and LP animals. Area under the curve was calculated using *GraphPad Prism* software. (Control n=6, LP n=10). Results are expressed as the mean $\pm$ standard error (SEM). * = Statistically significant ($p<0.05$).

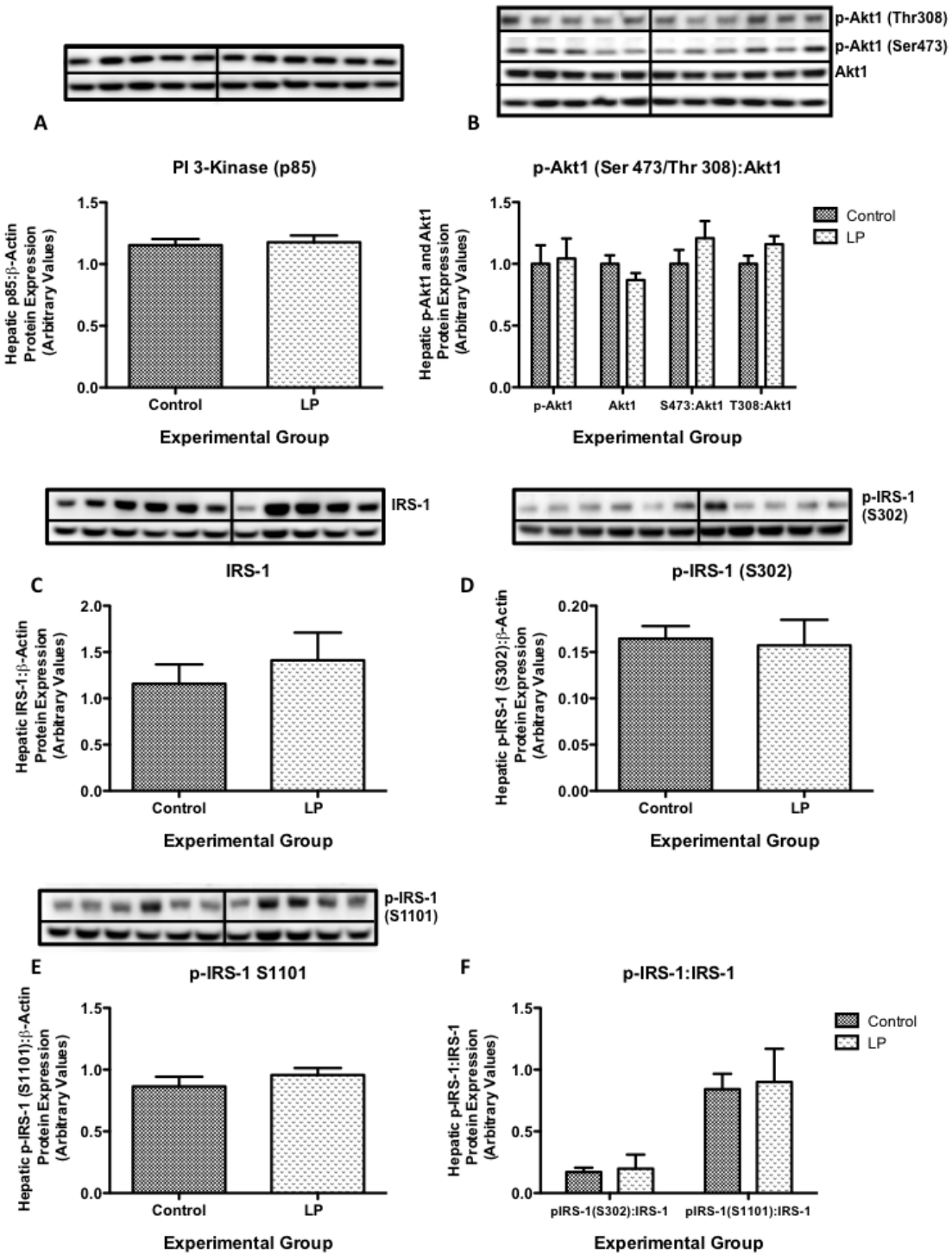

Figure 2.3: The effect of maternal low protein during gestation on the *in vivo* hepatic levels of **A)** p85 protein (85 kDa), and **B)** ratio of p-Akt1 [T308 and S473] to Akt1 (61 kDa), **C)** IRS-1 (180 kDa), **D)** phospho-IRS-1 [S302], **E)** phospho-IRS-1 [S1101] and **F)** ratio of phospho-IRS-1 [S302/S1101]:IRS-1 in 130-day-old offspring. Data were obtained from western immunoblotting experiments. Immunoblots were quantified using densitometry and normalized to β-actin (42 kDa) protein expression. Data are represented as arbitrary values. Results are expressed as the mean ± standard error (SEM). * = Statistically significant. n=5-6 per experimental group.

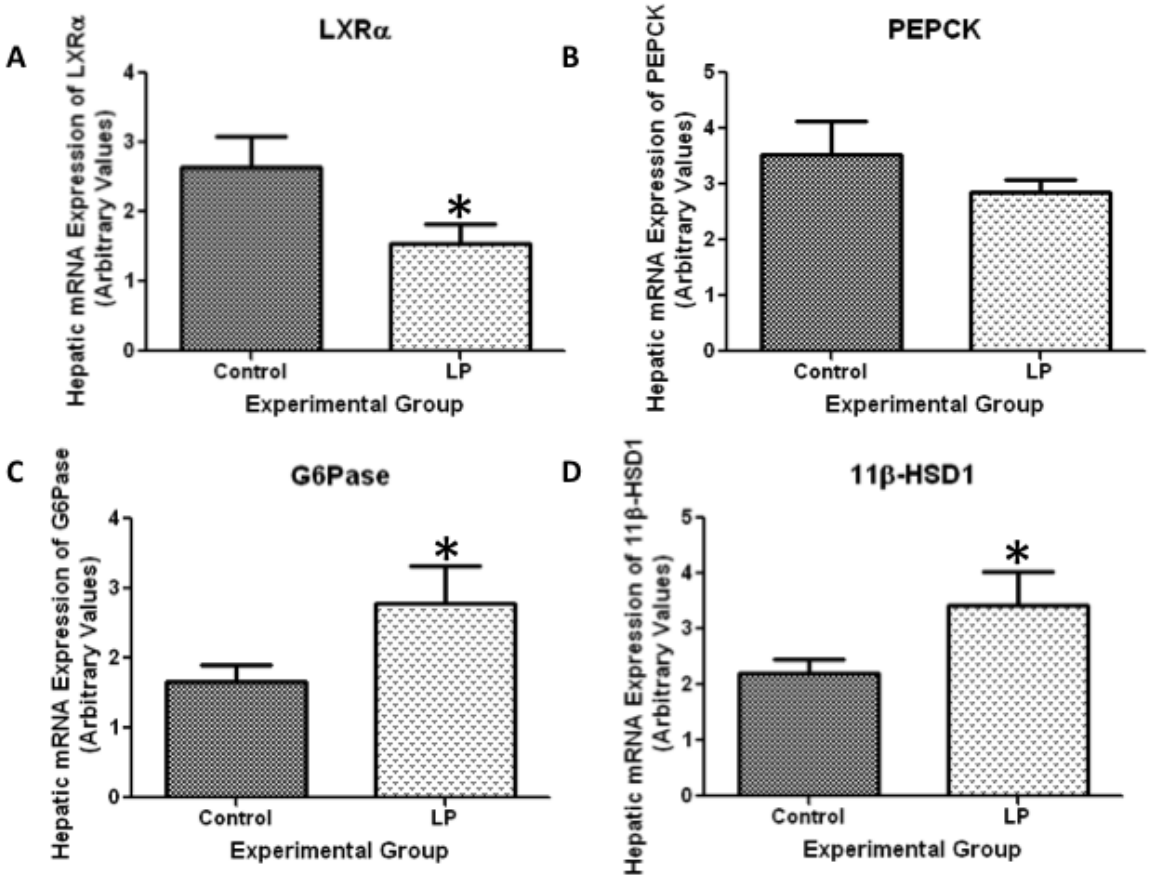

Figure 2.4: The effect of LP on *in vivo* hepatic levels of **A**) LXRα mRNA, **B**) PEPCK mRNA, **C**) G6Pase mRNA, and **D**) 11β-HSD1 mRNA in control and LP offspring at 4 months of age. Data were quantified from qRT-PCR (*Taqman*®) and the ΔΔCt method. Data are represented as arbitrary values and were analyzed using the *Student's* unpaired t-test. Results are expressed as the mean ± standard error (SEM). * = Statistically significant. n=4-6 per experimental group.

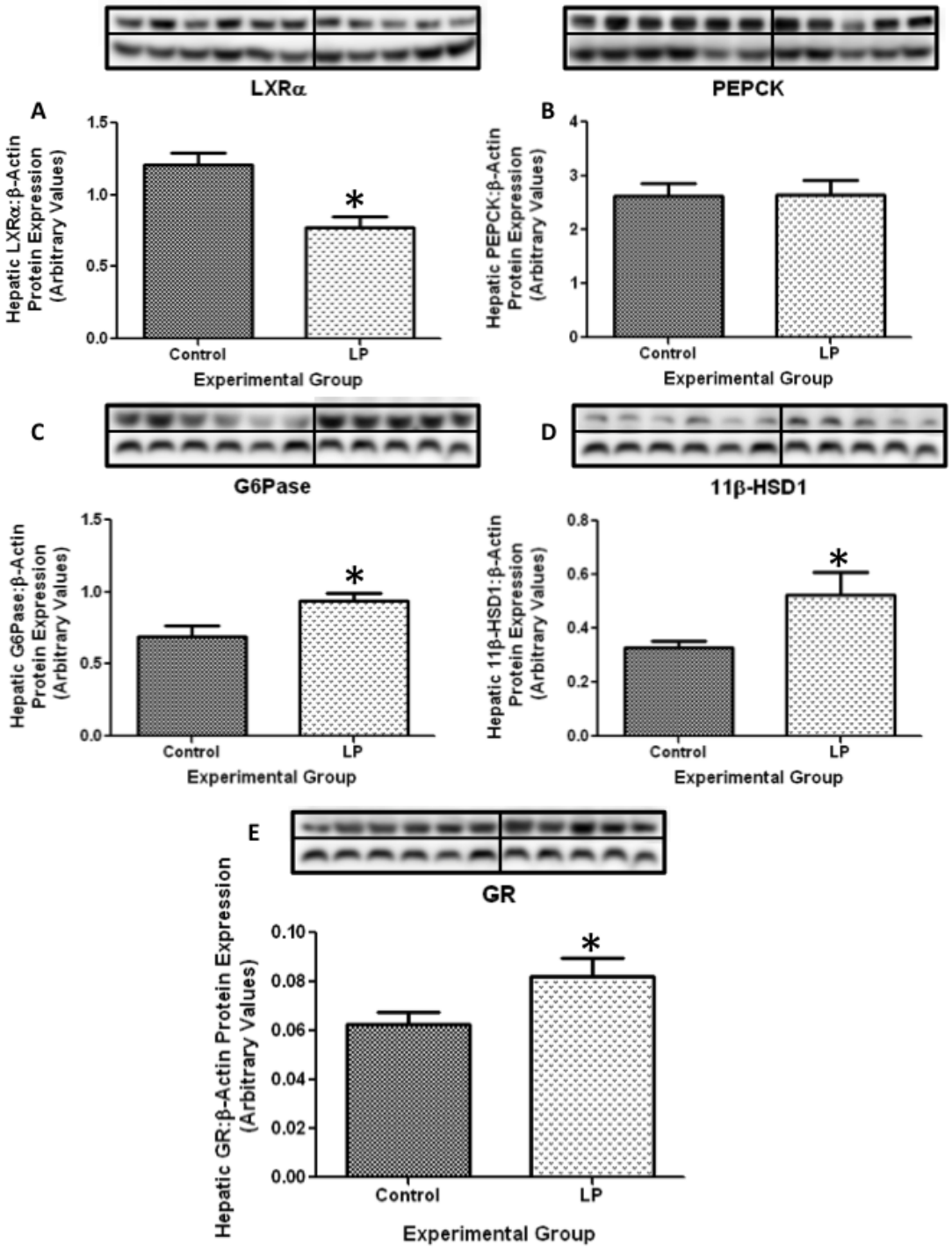

Figure 2.5: The effect of LP on the *in vivo* hepatic levels of **A**) LXRα protein (50 kDa), **B**) PEPCK protein (62 kDa), **C**) G6Pase protein (36 kDa), **D**) 11β-HSD1 protein (34 kDa) and **E)** GR protein (90-95 kDa) in control and LP offspring at 4 months of age. Data were obtained from western immunoblotting experiments. Immunoblots were quantified using densitometry and normalized to β-actin (42 kDa) protein expression. Data are represented as arbitrary values. Results are expressed as the mean $\pm$ standard error (SEM). * = Statistically significant. n=4-6 per experimental group.

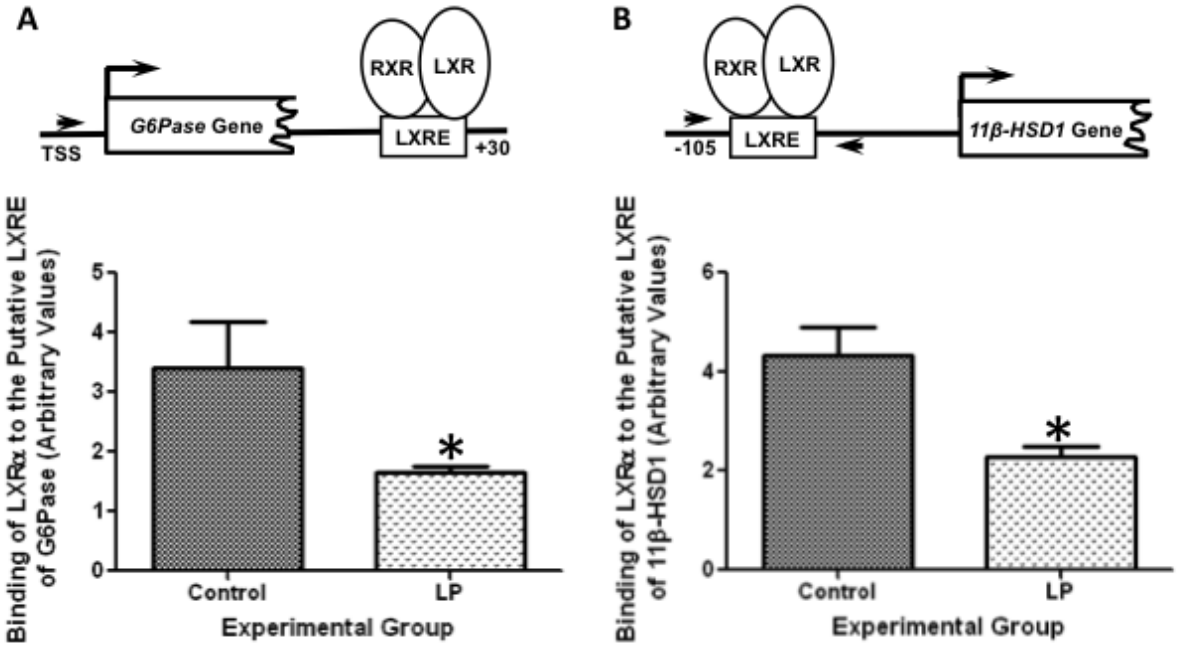

Figure 2.6: The effect of LP on the *in vivo* hepatic binding of LXRα to the promoters of **A**) *G6Pase* (+22 bp to +46 bp) and **B**) *11β-HSD1* (-114 bp to -90 bp) in control and LP offspring at 4 months of age. Putative LXRE sites were determined using the *MatInspector* software from Genomatix. Livers were immunoprecipitatied with antibodies specific to LXRα. Quantification was performed using qRT-PCR (*Sso-Fast EvaGreen)* with primers specific to the proposed LXRE sites. The relative amount of immunoprecipitated genomic DNA was normalized to total genomic DNA. Data are represented as arbitrary values using the ΔΔCt method. Results are expressed as the mean ± standard error (SEM). * = Statistically significant. n=4-6 per experimental group.

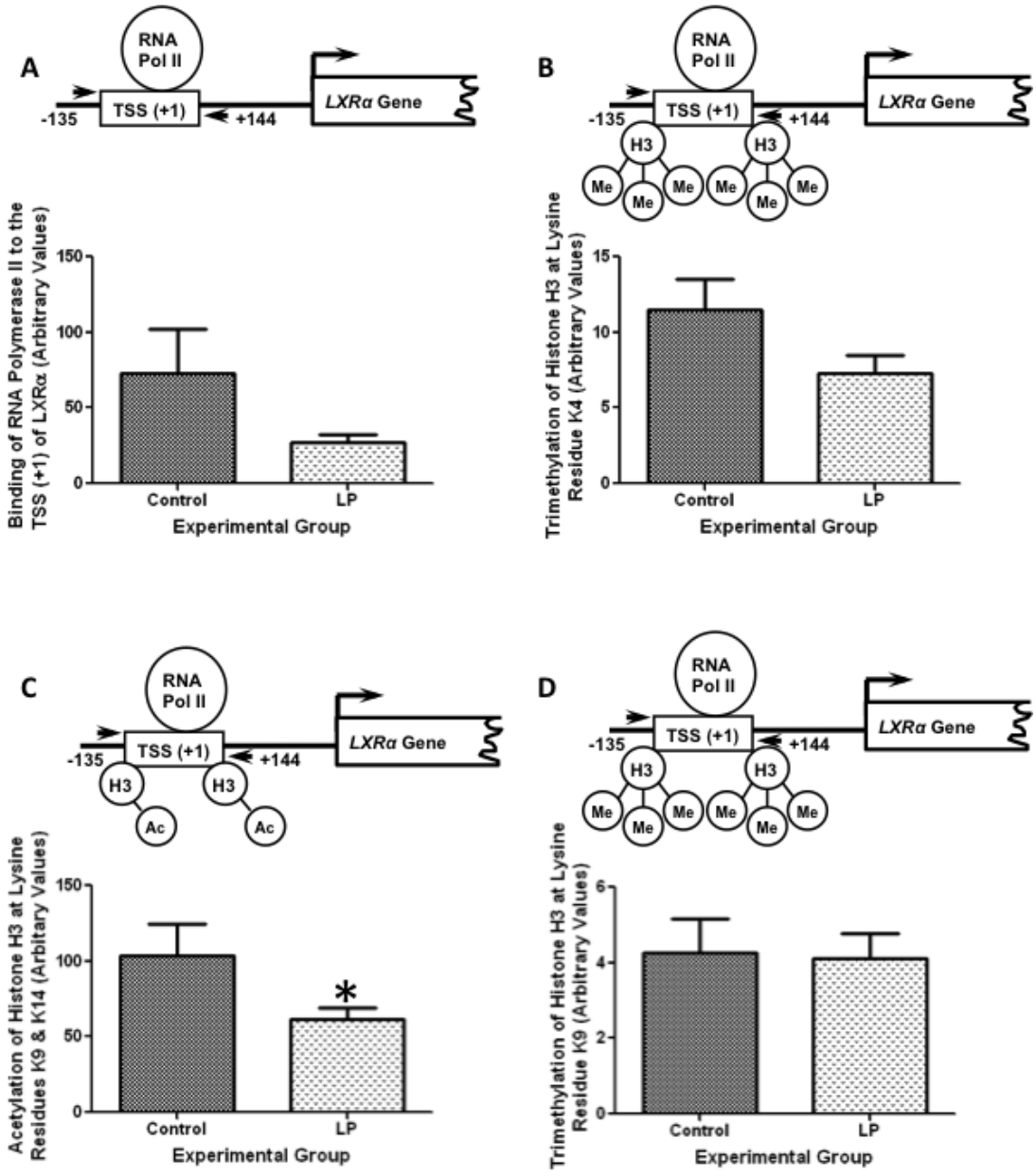

Figure 2.7: The effect of LP on the *in vivo* transcriptional and epigenetic regulation of the *LXRα* transcriptional start site (-135 bp to +144 bp) at 4 months of age. **A)** Binding of RNA Polymerase II to the *LXRα* TSS, **B)** Trimethylation of histone H3 lysine 4, **C)** Acetylation of histone H3 lysine 9 and 14 and **D)** Trimethylation of histone H3 lysine 9. Primers were designed based on sequencing from *Ensembl*. Livers were immunoprecipitated with antibodies specific to RNA polymerase II, trimethyled histone H3 [K4], acetylated histone H3 [K9, 14], and trimethylated histone H3 [K9]. Quantification was performed using qRT-PCR (*Sso-Fast EvaGreen)* with primers specific to the proposed LXR element sites. The relative amount of immunoprecipitated genomic DNA was normalized to total genomic DNA. Data are represented as arbitrary values using the ΔΔCt method. Results are expressed as the mean $\pm$ standard error (SEM). * = Statistically significant. n=4-6.

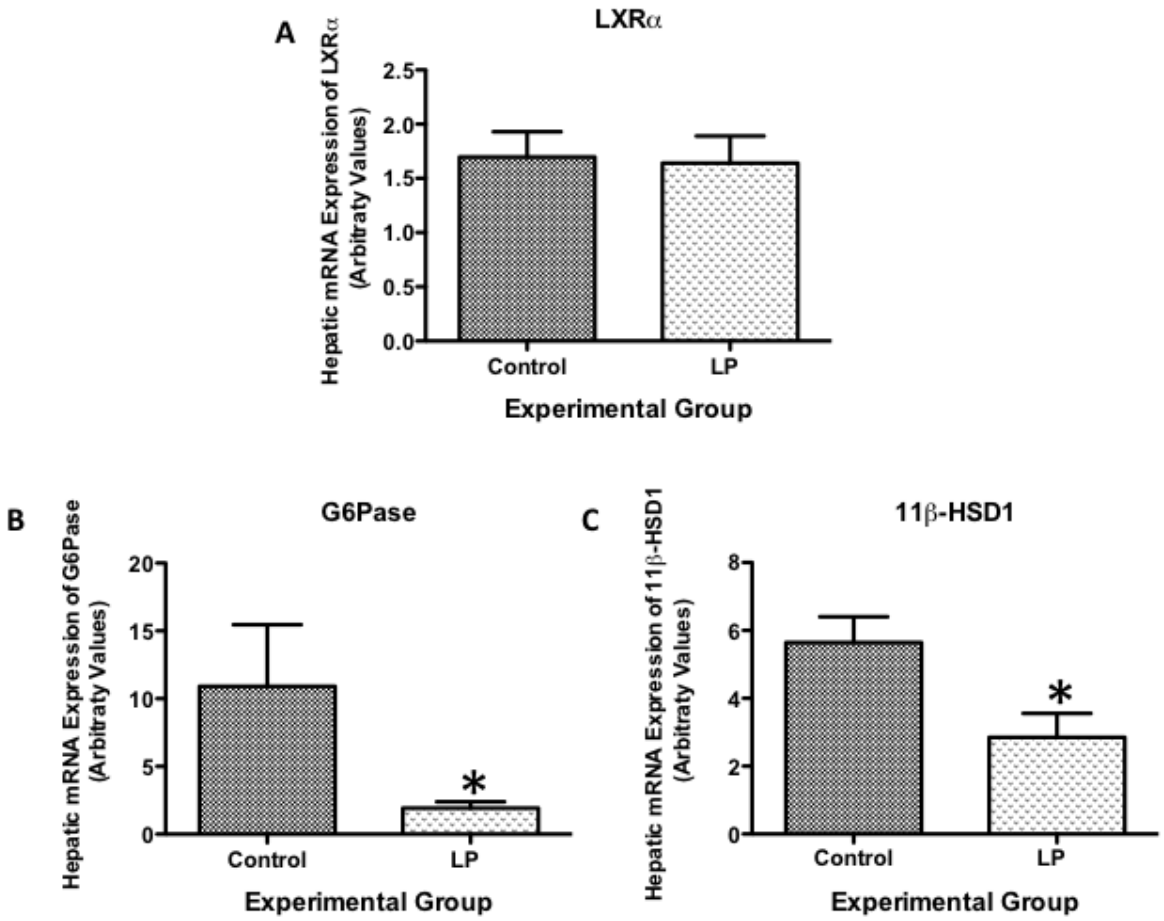

Figure 2.8: The effect of LP on *in vivo* hepatic levels of **A**) LXRα mRNA, **B**) G6Pase mRNA, **C**) 11β-HSD1 mRNA in control and LP offspring at embryonic day 19. Data were quantified from qRT-PCR (*Taqman*®) and the ΔΔCt method. Data are represented as arbitrary values and were analyzed using the *Student's* unpaired t-test. Results are expressed as the mean ± standard error (SEM). * = Statistically significant. n=4-9 per experimental group.

2.4 Discussion

Our current 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 previous studies have indicated that maternal protein restriction leads to glucose dysregulation[8-10]. 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[18,19,31,32].

Given placental insufficiency in humans can produce protein deficiency in the fetus[33], this LP model shares features in common with PI-IUGR[34]. Previous studies done in our lab 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[22]. While switching the low protein offspring to a control diet at weaning led to glucose intolerance[10], little is known about how catch-up growth due to early restoration of protein[22] 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[22]. 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[10]. Collectively, both studies further support of the main tenets of the Thrifty Phenotype hypothesis[35].

We previously found that LXRα expression and binding could be influenced by maternal diet by 3 weeks of age in the offspring[22], but the expression of LXRα at 4 months was unknown. In this study we demonstrated that in LP offspring, hepatic LXRα mRNA and protein was decreased at 4 months of age compared to control offspring. Interestingly, even though LXR expression was decreased in our LP animals, previous plasma analyses indicate no differences in the levels of circulating cholesterol and triglycerides in the same cohort of animals[22]. Given aberrant LXRα expression and activity can alter the expression of genes involved in hepatic gluconeogenesis (*e.g.* PEPCK, G6Pase, and 11β-HSD1)[18-20,32], we next examined whether the 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, but it does lead to an enhanced glucose response (*e.g.* a greater area under the curve during glucose tolerance tests), both of which we also observed[36]. To directly implicate whether alterations in LXRα expression influenced the binding of LXRα to the promoter of *G6Pase*, we then employed chromatin immunoprecipitation to examine the *in vivo* binding of LXRα to its a 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 (+22 to +46) 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 lead to non-insulin-dependent diabetes[37], 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 diet during gestation[38], and low protein offspring fed a high sucrose diet (500 g/kg) postpartum[39]. 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.

Dysregulation of glucocorticoids may also play a role in impairing glucose homeostasis in our LP model. Our study demonstrated an increase in 11β-HSD1 mRNA along with elevated 11β-HSD1 protein levels in the LP offspring. In addition, we found increases in protein expression of the glucocorticoid receptor, GR. Interestingly, while previous nutrient restriction models have demonstrated no change in 11β-HSD1 expression in the adipose tissue of adult rat offspring[40], 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. Furthermore, the increased expression of GR also indicates induction of glucocorticoid activity. Previous reports have found GR response elements on the promoter of

G6Pase[41,42], suggesting dual regulation of the *G6Pase* promoter by GR and LXR. As well, LXR induction has been demonstrated to inhibit GR expression[21]. Since glucocorticoids have stimulatory effects on the expression of gluconeogenic genes such G6Pase and PEPCK[41,43,44], the sustained overproduction of glucocorticoids would lead to an augmented glucose response, as observed in the IPGTT. 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 and GR expression.

Previous studies strongly suggest the role of epigenetics in mediating the effects of fetal programming long-term into adulthood[39,45-48]. 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 *LXR*α. Considering acetylation of histone H3 [K9,14] and methylation of histone H3 [K4] are both known to hallmarks of chromatin opening[49-51], 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. Additionally, 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[22]. 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 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 LXRα suppresses glucose production, it may be a suitable target as a therapeutic intervention to prevent glucose intolerance. Animal studies have widely demonstrated that administration of LXR agonists (i.e. GW3965, T0901317) lead to improved glucose tolerance[19,32]. Given that during the newborn period in the rat there is a high rate of replication, neogenesis and apoptosis leading to extensive liver remodeling[52], 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[53]. Therefore it is plausible that LXRα agonists, administered in neonatal life, a period of liver plasticity, may prevent glucose intolerance long-term through activation of hepatic LXRα. While LXR agonists appear promising, the negative effects of LXRα activation must also be considered given it can activate lipogenesis through increased expression of fatty acid synthase (FAS), acetyl-CoA carboxylase (ACC) and the master lipid regulator, sterol regulatory element binding protein-1c (SREBP-1c)[54].

In summary, our study demonstrates for the first time the role of LXRα in mediating the transcriptional regulation of hepatic gluconeogenic genes in our rat LP model. In these offspring, decreased expression of hepatic LXRα 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 LXRα in reducing glucose production, it serves as a possible therapeutic target of intervention due to its antidiabetic properties. Further studies will be necessary to find a suitable balance between antidiabetic and lipogenic actions of LXRα before it could be considered as an ideal candidate for preventing glucose intolerance in IUGR offspring.

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Chapter Three:

Administration of the Liver X Receptor Agonist GW3965 During the Neonatal Period Leads to Impaired Glucose Tolerance in Non-Maternal Protein Restricted Adult Male Rats

3.1 Introduction

Epidemiological studies have indicated a strong connection between impaired development and growth *in utero* and the risk of developing chronic diseases[1,2]. Low birth weight infants are often a result of intrauterine growth restriction (IUGR). The "Thrifty Phenotype Hypothesis" postulates that during gestation the fetus programs itself for short-term survival *in utero*, and that these adaptations become maladaptive in postnatal life due to a mismatch in the environments[3,4]. These maladaptive changes may have detrimental effects on the individual in later life by increasing their risk of developing cardiovascular disease, hypertension, obesity, and type 2 diabetes[5-9].

The maternal protein restriction (MPR) model of IUGR is a well-studied model for examining the developmental origins of health and disease. Typically, in models of MPR the mother is given a low protein diet (5-8%) during the gestation period (and often the weaning period). Previous studies have demonstrated that MPR leads to impaired glucose homeostasis, impaired pancreatic development and function and altered hepatic function in the offspring[10-14]. Other studies have also found detrimental effects in the kidneys and heart, in addition to changes in the longevity of MPR offspring[15-18]. Studies from our own lab have found elevated cholesterol in MPR offspring due to altered epigenetic regulation of the *Cyp7a1* gene[19]. More recently, our lab has uncovered that MPR during gestation (and not weaning) leads to suppressed Liver X Receptor (LXR) expression, facilitating the transcriptional induction of glucose-6-phosphatase (G6Pase) and 11β-hydroxysteroid dehydrogenase 1 (11β-HSD1) in male MPR offspring. These findings implicate LXR as a key factor in mediating the detrimental programming effects of IUGR[20].

LXR is a nuclear receptor that exists as two isoforms: LXRα and LXRβ. LXRα is generally found in the liver, intestines, adipose tissue and macrophages[21,22], while LXRβ is expressed ubiquitously[23]. LXR was first implicated in the regulation of cholesterol metabolism since its endogenous ligands were mainly derivatives of cholesterol[24,25]. Furthermore, LXR has been connected to lipid metabolism by stimulating the expression of many lipogenic genes including sterol regulatory element binding protein-1 (SREBP-1), fatty acid synthase (FAS), acetyl-CoA carboxylase (ACC), and stearoyl-CoA desaturase-1 (SCD-1)[26-30]. More recently, LXR has been found to be able to bind glucose and act as a glucose sensor[31]. In addition, LXR has demonstrated the ability to downregulate genes involved in the gluconeogenic pathway such as phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase[32,33]. Finally, LXR has been associated with glucocorticoid regulation through repression of 11β-HSD1, an essential enzyme involved in the conversion of inactive glucocorticoids to their active form.

Studies have been exploring the possibility of intervening during the neonatal period to reverse the programming observed in IUGR offspring. Certain organs like the liver and pancreas continue to develop even after birth and display great plasticity, especially in rodents[34,35]. Given that there is still a great deal of hepatic and pancreatic neogenesis, differentiation, replication, and apoptosis during the early neonatal period, it represents a critical time point for possible intervention[34,36]. For instance, in an elegant study done by Stoffers and colleagues (2006), administration of the glucagon-like peptide-1 (GLP-1) analogue Exendin-4™ during the neonatal period completely prevented the development of diabetes in uterine artery ligated IUGR rats[37]. They postulate that this change is mediated through restoration of the pancreatic and duodenal

homeobox 1 protein (Pdx-1), an essential transcription factor required for pancreatic development and β-cell maturation[37,38]. Further studies done by the Raab and colleagues (2009) found that administration of Exendin-4™ during the neonatal period prevented hepatic insulin resistance and reduced hepatic oxidative stress[39]. Thus, these studies demonstrate how a short-term intervention during the neonatal period can permanently alter organ function in adult life.

Given that LXR has been demonstrated to improve glucose tolerance[33,40] and our lab has previously implicated reduced LXR expression in partly mediating the effects of impaired glucose homeostasis and overexpression of gluconeogenic genes in MPR male adult rat offspring[20], we attempt in this study to prevent the development of impaired glucose homeostasis in later life (4 months of age) by administering the LXR agonist GW3965 during the early neonatal period. In this experimental paradigm, where the MPR dams receive the low protein diet during both gestation and weaning (herein termed the "LP2" experimental group), no changes in glucose homeostasis were found between control and MPR diet offspring. However, in the control diet offspring, administration of the LXR agonist during the neonatal period led to impaired glucose homeostasis. This impaired glucose homeostasis was accompanied by increased gluconeogenic gene expression (G6Pase, PEPCK) and increased lipogenic gene expression (SCD-1). Furthermore these offspring displayed signs of hyperinsulinemia and hypertriglyceridemia in addition to the observed hyperglycemia, which all encompass features of the metabolic syndrome[41].

3.2 Materials and Methods

3.2.1 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 virgin female Wistar rats at breeding age (250 g) were purchased from Charles River (La Salle, St-Constant, Quebec, Canada) and were allowed to acclimatize to their new environment for two weeks. Rats were housed at room temperature on a 12-12 hour light-dark cycle. Females were housed in separate cages and were cohabitated with a male for mating upon entering pro-estrous. Conception was confirmed under a microscope by 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. The "control" offspring and dams (C-V and C-GW) received 20% protein throughout life, while "protein restricted" dams (LP2-V and LP2-GW) received low protein chow (8%) throughout gestation and then restored on a 20% protein chow after the weaning period (21 days after birth) (Figure 3.1). All offspring were allowed to feed on the control diet ad-libitum after the weaning period.

Beginning at postnatal day 5, pups received daily intraperitoneal injections of 25mg/kg of the LXR agonist GW3965 (LP-GW and C-GW) or equal volume of the vehicle DMSO (LP2-V and C-V). The pups received daily injections of the LXR agonist or vehicle until postnatal day 15 (Figure 3.1). In our initial experiments, where the LP2-

pups received 50mg/kg of the LXR agonist, a subset of pups (4 LP2-V pups and 6 LP2-GW pups) was sacrificed at postnatal day 21 and livers from the pups were extracted. The livers were flash frozen for further molecular analysis. All further litters with greater than 10 pups were arbitrarily culled down to 9-10 pups to ensure a consistent litter size per dam. In total, there were two dams for each experimental group (8 dams total) with 9-10 pups per litter.

After intraperitoneal glucose tolerance tests at postnatal day 120-125, all offspring were sacrificed using a lethal dose (50mg/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 in other models of protein restriction, impaired glucose tolerance was not observed earlier than 4 months[42]. Following sacrifice, liver and blood were immediately extracted and flash frozen at -80^{o}C for molecular analysis. We did not examine the female offspring in this study to prevent confounding factors related to their estrous cycle and hormone profile. More importantly, the maternal low protein model has been demonstrated to exhibit early life programming effects in a sexually dimorphic manner, which was not the focus of this investigation[19,42,43]. For molecular analysis, at least one to two male pups from each of two separate dams were arbitrarily chosen. All available male pups were used for the intraperitoneal glucose tolerance tests.

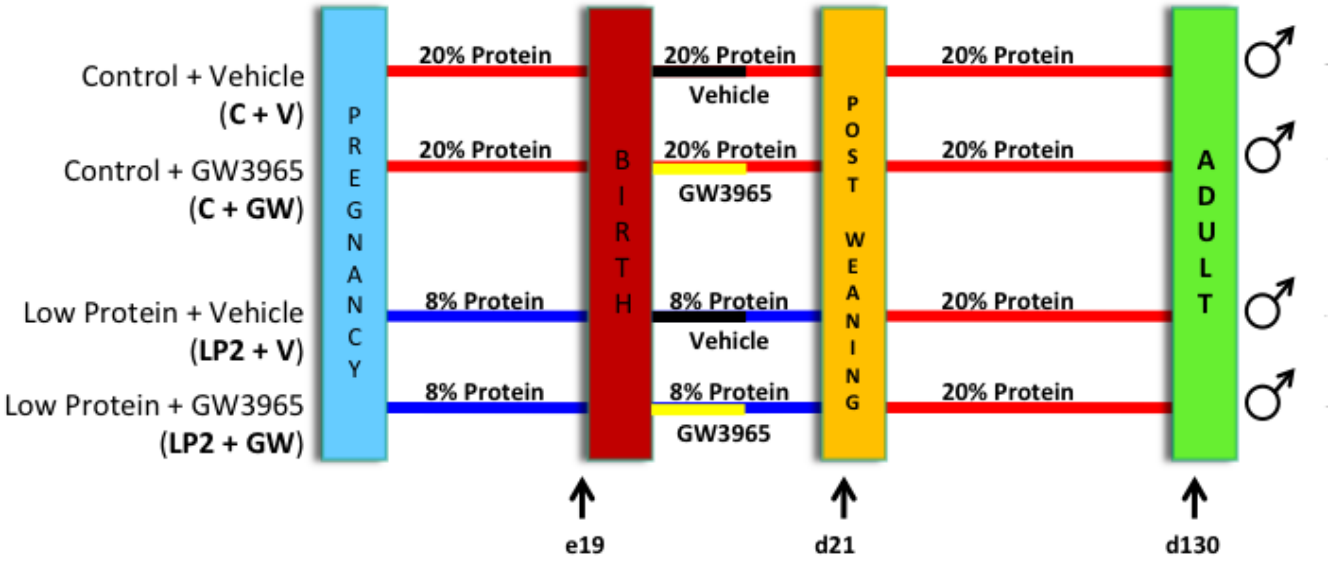

Figure 3.1: **Experimental Paradigm for Neonatal Administration of the Liver X Receptor Agonist GW3965 in Non-MPR and MPR Male Offspring**. Briefly, control dams were given a control (20% protein) diet throughout life. Control offspring also received a control diet after weaning (postnatal day 21). Low protein dams (LP2) received a low protein (8% protein) diet throughout gestation and the weaning period. Low protein offspring (LP2) received a control (20%) diet after weaning. Vehicle (V) animals received the vehicle DMSO during postnatal days 5-15, while agonist animals (GW) received GW3965 during postnatal days 5-15. Animals are sacrificed at approximately 4 months of age (postnatal day 130).

3.2.2 Glucose Tolerance Tests

At postnatal day 120-125, male offspring were subject to an intraperitoneal glucose tolerance test (IPGTT) as previously described[20]. Prior to the IPGTT, the animals were fasted overnight for 14-16 hours. Animals were awake throughout the experiment. Blood glucose measurements were obtained using a Bayer Breeze® 2 Blood Glucose Meter (Bayer, New York, USA). Fasted blood glucose levels were obtained prior to the glucose injection. Animals then received 2g/kg of glucose via injection into the intraperitoneal cavity. Blood glucose was sampled at the tail vein at t= 0, 5, 10, 15, 30, 60, and 120 minutes. Area under the curve for each animal was calculated using *GraphPad Prism™* software. IPGTTs were performed on 5 LP2-V, 7 LP2-GW, 4 C-V, and 8 C-GW male rats.

3.2.3 Plasma Assays for Fasted Resting Blood Triglyceride and Insulin Levels

At 4 months of age, blood was collected from sacrificed animals and transferred to tubes containing EDTA. The blood was then centrifuged and plasma was collected and stored at -20^{o}C until analysis. At time of analysis, samples were thawed and triglyceride levels were measured using the Cobas® Trig/GB colorimetric enzymatic kit (Roche Diagnostics, Laval, Canada). Insulin levels were measured using the ALPCO™ Insulin (Rat) ELISA kit (ALPCO Diagnostics, Salem, New Hampshire, USA). The manufacturer's instructions were followed for both kits.

3.2.4 Tissue Protein Extraction and Western Immunoblotting

Protein extraction and western immunoblotting protocols have been previously described[19,20].

Tissue protein was extracted from the medial lobe of snap frozen offspring livers using a lysis buffer solution (pH 7.4, Tris-HCl 50mM, NP-40 1%, Sodium-deoxycholate 0.25%, NaCl 150mM, EDTA 1mM, NaF 50mM, Na3VO4 1mM 1mM, β-Glycerophosphate 25mM). Prior to tissue homogenization, a mini protease inhibitor tablet was added to the lysis buffer.

Firstly, a small chunk of snap frozen liver was added to 600µl of RIPA buffer. The tissue was then homogenized with the IKA T10 Basic S1 Dispersing Tool (IKA Works Inc, Wilmington, NC) for 10-15 seconds at speed 6. After letting the homogenized tissue sit on ice for 5 minutes, the tissue was then sonicated. Following sonication, the tissue was rotated at 4^{o}C for 5 minutes and then centrifuged for 15 minutes at 300g and 4^{o}C. The supernatant was retained for further centrifugation at 20,000g for 20 minutes at 4^{o}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 12-well gradient polyacrylamide gels (Invitrogen, Carlsbad, CA, USA) and transferred onto PVDF membrane (Millipore, Etobicoke, Ontario, Canada). Amido black staining and Coomassie brilliant blue staining confirmed sufficient transfer of proteins onto the membrane.

Immunoblots were probed using LXRα (Liver X Receptor (1:1000; cat# sc-13068)), PEPCK (1:2000; cat# sc-32879), G6Pase-α (1:1000; cat# sc-25840), 11β-HSD1 (1:800; cat# sc-20175), and SREBP-1c (1:1000; cat# sc-366) all from Santa Cruz Biotechnology (Santa Cruz, California). In addition, FAS (1:1000; cat# 3180S) and ACC (1:1000; cat# 3662S) antibodies were obtained from Cell Signaling (Danvers, Massachusetts) and SCD-1 (1:1000; cat# Ab-19862) antibodies were obtained from Abcam Inc., Massachusetts. Monoclonal HRP conjugated β-Actin (1:50,000; cat#A3854, Sigma-Aldrich, Oakville, Ontario, Canada) diluted in 5% milk-TBS-Tween-20 (0.1%) buffer and HRP conjugated donkey anti-rabbit or donkey anti-mouse IgG (1:10,000, cat# 711-035-152 and 715-035-150, respectively, 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 enhanced chemiluminescence detection system (Thermo Scientific, Waltham, MA, USA).

3.2.5 Statistical Analysis

All data are represented as a mean of an arbitrary value ± Standard Error of the Mean (SEM), unless indicated as raw values. Glucose tolerance tests, areas under the curve and metabolic data were analyzed using a one-way ANOVA followed by Tukey's post-hoc test. Quantified western immunoblot bands were analyzed using the unpaired *Student's t-test*. All data with a *p*-value less than 0.05 were considered statistically significant.

3.3 Results

3.3.1 Neonatal Administration of the LXR Agonist GW3965 Leads to Altered Hepatic Expression of 11β-HSD1 and SREBP-1c at Postnatal Day 21 in MPR Male Rat Offspring

Initially, we used a small cohort of pups to determine whether neonatal administration of GW3965 would lead to changes that would persist beyond the administration period (postnatal days 5-15). Thus, we culled 4 LP2-V pups and 6 LP2-GW pups at postnatal day 21, one week after the administration period, and examined the expression of LXR-target genes via western immunoblotting. There were no changes in protein expression of LXR, G6Pase, or PEPCK at this time point in these animals (Figures 3.2 A,B&D). At postnatal day 21, even after the administration of GW3965 had halted, we found a significant reduction in the protein expression of 11β-HSD1, which is negatively regulated by LXR ($p<0.05$) (Figure 3.2C). In addition, we also found a significant increase in the protein expression of SREBP-1c, a protein that is positively regulated by LXR ($p<0.05$) (Figure 3.2E). Thus, given that there were expression changes in certain LXR target genes even after the administration of the LXR agonist GW3965 was halted, we were encouraged to continue another cohort of animals that included a "control" diet group (C-V and C-GW).

3.3.2 Neonatal Administration of the LXR Agonist GW3965 Does not Alter Whole Body Weight or Wet Liver Weights at 4 Months of Age

At postnatal day 130, the animals were sacrificed and weighed. After the whole body weights were weighed, the liver was extracted and weighed. The C-GW males were significantly heavier than LP2-V animals at 4 months of age ($p<0.05$) (Figure 3.3A). There were no differences in weight between any other groups. However, there appeared to be a trend where the control diet offspring were heavier than low protein diet offspring. For wet liver weights, LP-V liver weights were significantly less than both C-V and C-GW experimental groups ($p<0.05$) (Figure 3.3B). LP-GW liver weights were also significantly less than C-GW liver weights ($p<0.05$). Again, there appeared to be a trend where the control diet animals exhibited increased liver weights compared to low protein animals.

3.3.3 Neonatal Administration of the LXR Agonist GW3965 Leads to Impaired Glucose Homeostasis in Control Male Offspring

Intraperitoneal glucose tolerance tests were administered to the offspring at postnatal days 120-125 to assess whole body glucose handling in response to a glucose load. At the 60-minute time point the C-GW animals displayed significant hyperglycemia versus all of the other groups ($p<0.05$) (Figure 3.4A). At the 120-minute time point the C-GW animals displayed significant hyperglycemia only against the LP2-V experimental group ($p<0.05$).

The areas under the curve of each animal were determined using *GraphPad Prism™* software and then statistically analyzed as a gross assessment of glucose tolerance. The areas under the curve for C-GW offspring were significantly increased compared to all other groups ($p<0.05$) (Figure 3.4B). This suggests that the C-GW offspring were glucose intolerant compared to the rest of the experimental groups.

3.3.4 Neonatal Administration of the LXR Agonist GW3965 Leads to Fasting Hyperglycemia, Hyperinsulinemia and Hypertriglyceridemia in Non-MPR (C-GW) Male Offspring

In the control diet animals, administration of GW3965 led to significant elevation of fasting blood glucose levels (as assessed prior to the IPGTT) (Figure 3.5A). Fasted insulin levels in the C-GW experimental group were significantly elevated compared to both LP2-V and LP2-GW experimental groups ($p<0.05$) (Figure 3.5B). Only C-GW fasted triglycerides were significantly elevated compared to LP2-V and LP2-GW animals ($p<0.05$) (Figure 3.5C).

Given the results observed in our plasma analyses and glucose tolerance tests, we decided to exclusively pursue the molecular mechanisms underlying the detrimental effects of neonatal GW3965 administration in control diet (20% protein diet) animals only. There was a strong rationale to examine these animals since they displayed signs of impaired glucose tolerance (Figures 3.4A&B), fasted hyperglycemia (Figure 3.5A), hyperinsulinemia (Figure 3.5B), and phypertriglyceridemia (Figure 3.5C) – all of which encompass symptoms of the metabolic syndrome[41].

3.3.5 Neonatal Administration of the LXR Agonist GW3965 Leads to Increased Protein Expression of Gluconeogenic Genes in Non-MPR Male Offspring at 4 Months of Age

To assess whether the impaired glucose tolerance observed in C-GW animals was due to the increased expression of gluconeogenic genes, we employed western immunoblotting with antibodies specific to G6Pase and PEPCK, both LXR-target genes. We also examined the expression of 11β-HSD1 to observe whether these changes in circulating glucose were indirectly related to alterations in bioactive glucocorticoid production. At 4 months of age, neonatal administration of GW3965 in non-MPR (C-GW) rats led to significantly increased expression of the gluconeogenic genes, PEPCK and G6Pase ($p<0.05$) (Figures 3.6B&C). In addition, there was a significant increase in the expression of 11β-HSD1 ($p<0.05$), indicating the possibility of increased glucocorticoid conversion (Figure 3.6D). Interestingly, there were no changes in LXR expression between C-V and C-GW animals (Figure 3.6A).

3.3.6 Neonatal Administration of the LXR Agonist GW3965 Leads to Increased Protein Expression of the Lipogenic Gene SCD-1 in Non-MPR Male Offspring at 4 Months of Age

Given C-GW animals exhibited increased circulating triglycerides (Figure 3.5C) we next decided to examine the protein expression of genes involved in hepatic *de novo* lipogenesis. We measured the expression of SREBP-1, FAS, ACC, and SCD-1 using

western immunoblotting. At 4 months of age, we found significantly elevated protein expression of SCD-1 in C-GW animals compared to C-V animals ($p<0.05$) (Figure 3.7D). There were no significant differences in any of the other hepatic lipogenic genes (Figures 3.7 A-C).

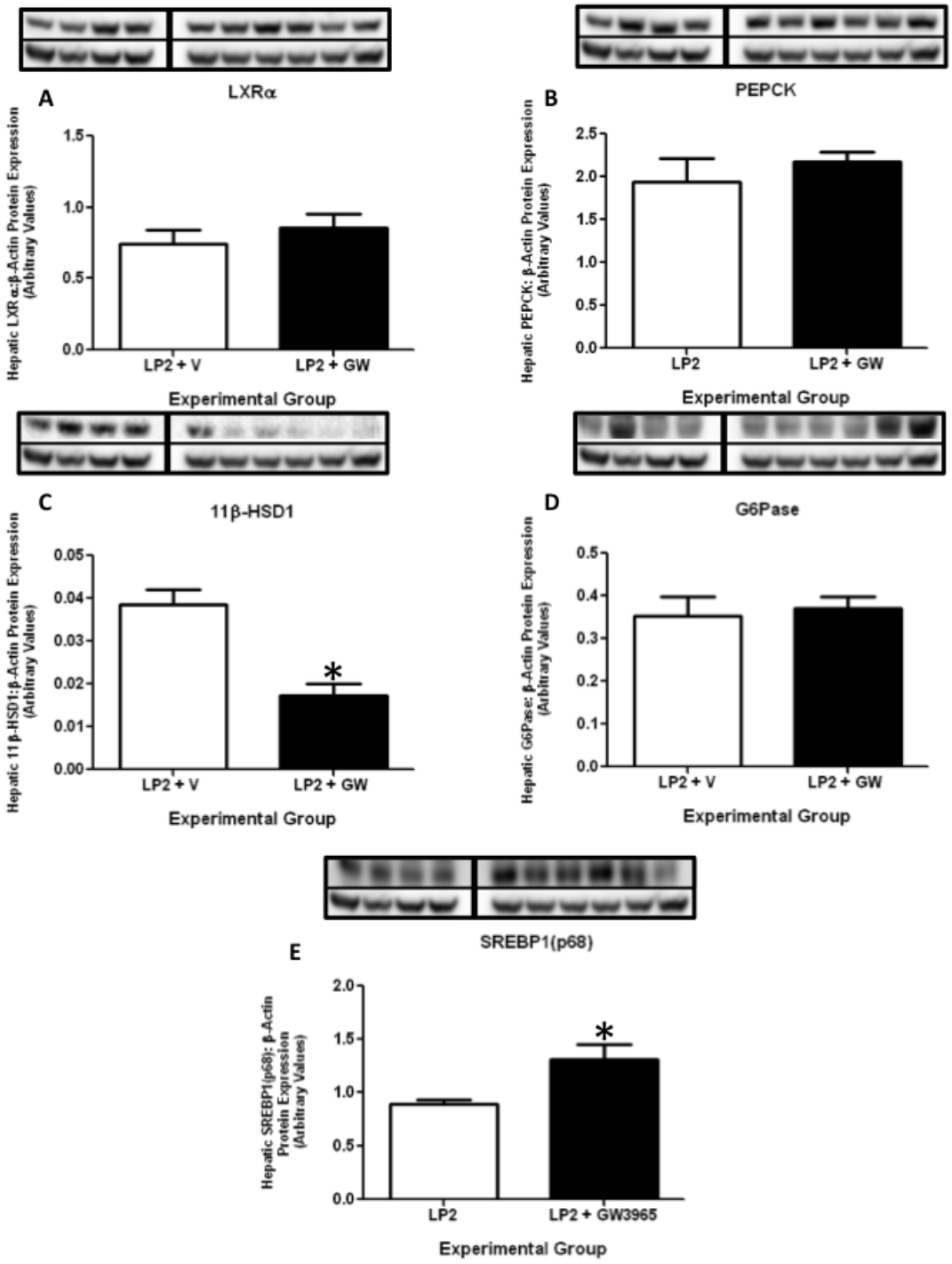

Figure 3.2: The effect of neonatal GW3965 administration (50mg/kg) on the *in vivo* hepatic levels of A) LXRα protein (50 kDa), B) PEPCK protein (62 kDa), C) G6Pase protein (36 kDa), D) 11β-HSD1 protein (34 kDa), and E) SREBP-1 protein (68 kDa) in LP2-V and LP2-GW male offspring at 21 days of age. Data were obtained from western immunoblotting experiments. Immunoblots were quantified using densitometry and normalized to β-actin (42 kDa) protein expression. Data were analyzed using the unpaired *Student's t-test*. Data are represented as arbitrary values. Results are expressed as the mean $\pm$ standard error (SEM). * = Statistically significant from LP2-V. n=4-6 per experimental group.

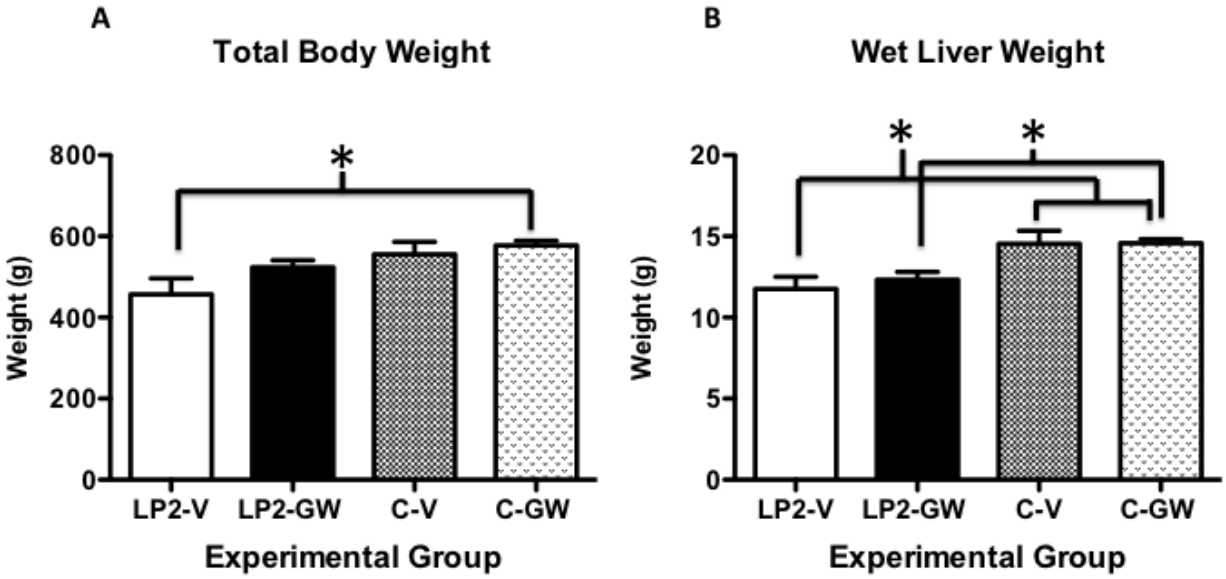

Figure 3.3: The effect of neonatal GW3965 administration (25mg/kg) on A) body weight and B) liver weight in LP2-V, LP2-GW, C-V, and C-GW male offspring at 4 months of age. Data are represented in grams (g). Data were analyzed using the one-way ANOVA followed by Tukey's post-hoc test. Results are expressed as the mean $\pm$ standard error (SEM). * = Statistically significant. n=5-9 per experimental group.

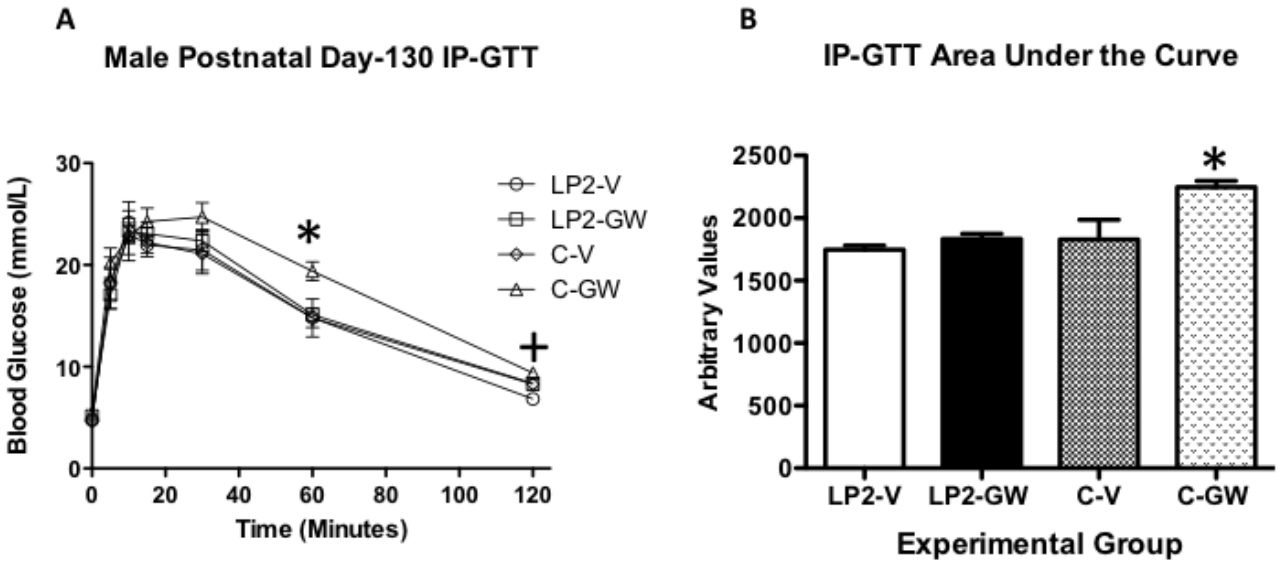

Figure 3.4: A) Intraperitoneal glucose tolerance tests (2g/kg) administered to fasted male offspring at 4 months of age. LP2-V, LP2-GW, C-V, and C-GW animals were analyzed together at each time point (t=0, 5, 10, 15, 30, 60, 120 minutes) using the one-way ANOVA followed by Tukey's post-hoc test. B) Area under the curve of LP2-V, LP2-GW, C-V, and C-GW animals. Area under the curve was calculated using *GraphPad Prism* software and analyzed using the one-way ANOVA followed by Tukey's post-hoc test. n=4-8 per experimental group. Results are expressed as the mean $\pm$ standard error (SEM). * = Statistically significant (C-GW versus all groups; $p<0.05$). + = Statistically significant (C-GW versus LP2-V; $p<0.05$).

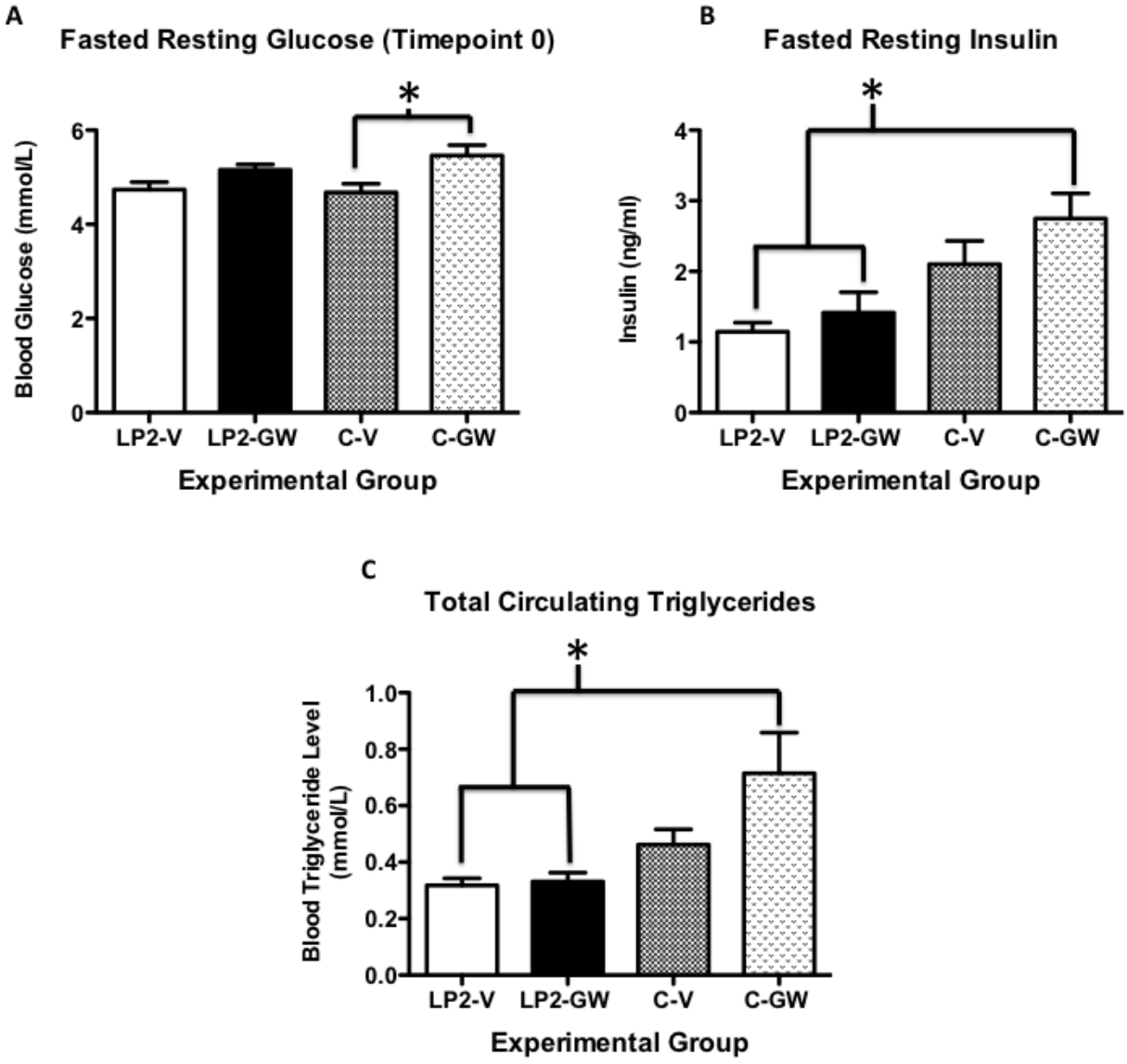

Figure 3.5: The effect of neonatal GW3965 administration (25mg/kg) on A) fasted resting glucose, B) fasted resting insulin, and C) fasted resting triglyceride levels in LP2-V, LP2-GW, C-V, and C-GW male offspring at 4 months of age. Fasted resting glucose was obtained prior to the glucose tolerance test. Fasted resting insulin and fasted resting triglyceride levels were obtained from the procedures described in the methods section. Data are represented as raw values. Data were analyzed using the one-way ANOVA followed by Tukey's post-hoc test. n=4-8 per experimental group. Results are expressed as the mean $\pm$ standard error (SEM). * = Statistically significant ($p<0.05$).

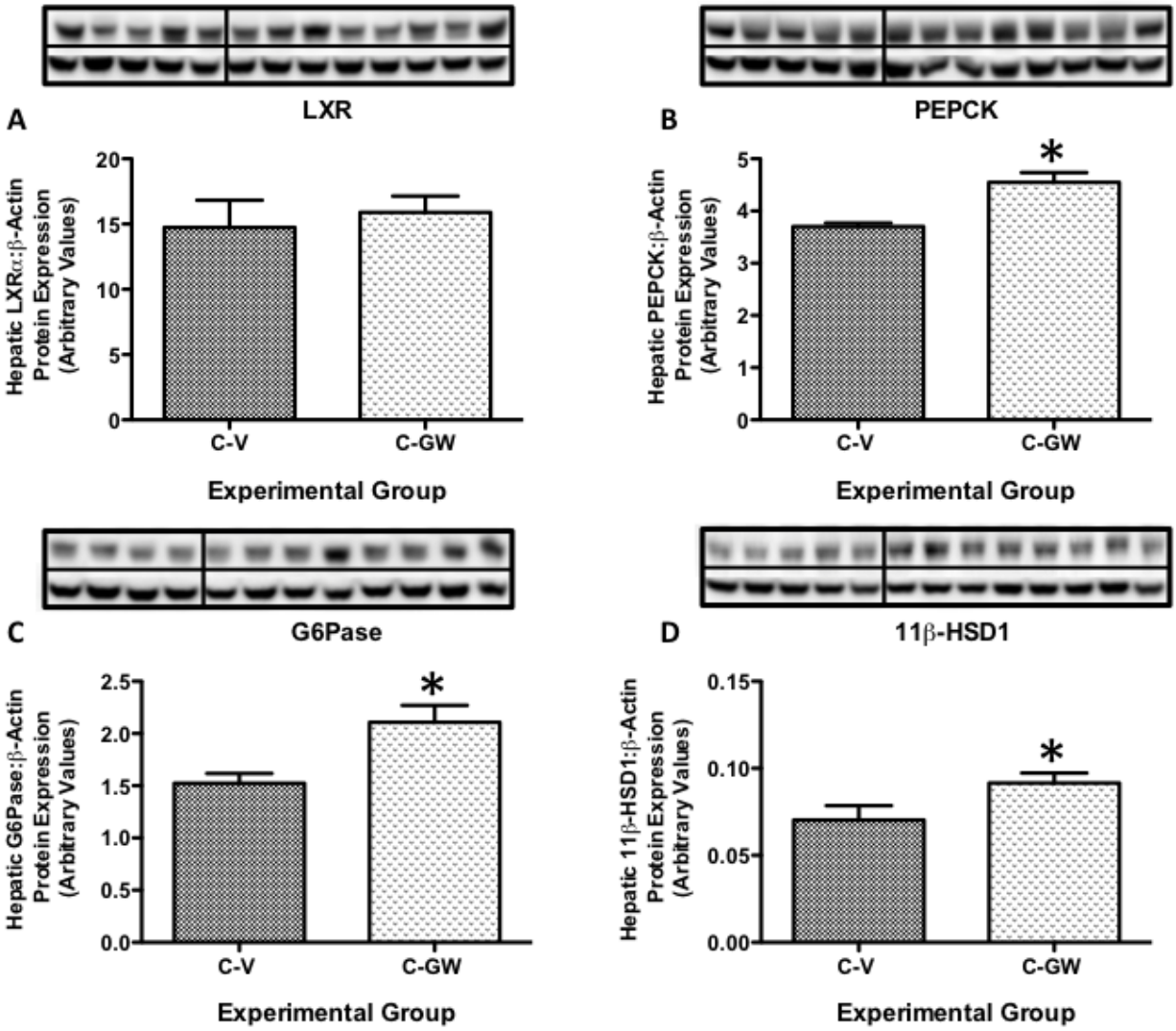

Figure 3.6: The effect of neonatal GW3965 administration (25mg/kg) on the *in vivo* hepatic levels of A) LXRα protein (50 kDa), B) PEPCK protein (62 kDa), C) G6Pase protein (36 kDa), and D) 11β-HSD1 (34 kDa) protein in C-V and C-GW male offspring at 4 months of age. Data were obtained from western immunoblotting experiments. Immunoblots were quantified using densitometry and normalized to β-actin (42 kDa) protein expression. Data are represented as arbitrary values. Data were analyzed using an unpaired *Student's t-test*. Results are expressed as the mean $\pm$ standard error (SEM). * = Statistically significant from C-V. n=4-8 per experimental group.

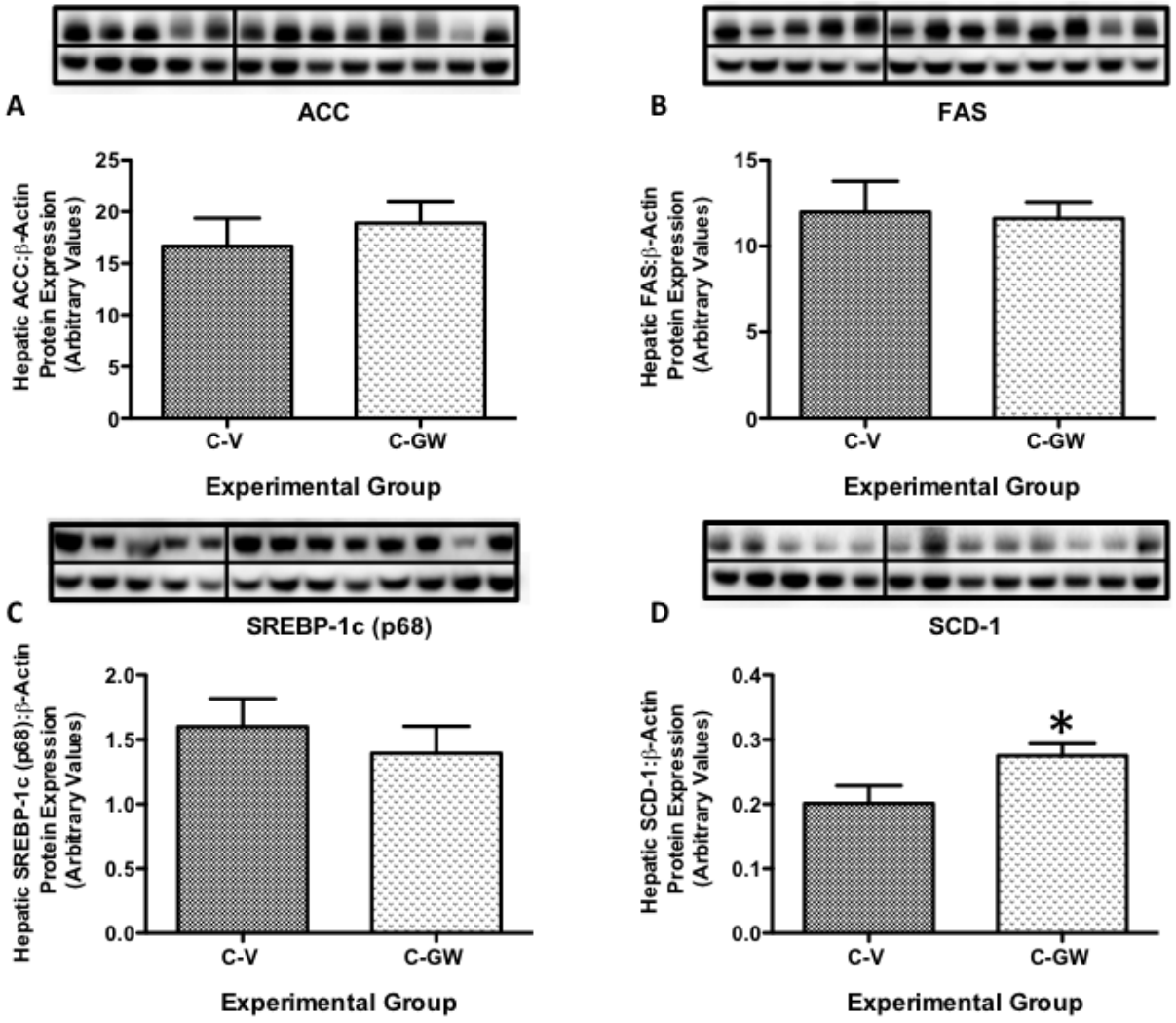

Figure 3.7: The effect of neonatal GW3965 administration (25mg/kg) on the *in vivo* hepatic levels of A) ACC protein (265 kDa), B) FAS protein (273 kDa), C) SREBP-1c protein (68 kDa), and D) SCD-1 (37 kDa) protein in C-V and C-GW male offspring at 4 months of age. Data were obtained from western immunoblotting experiments. Immunoblots were quantified using densitometry and normalized to β-actin (42 kDa) protein expression. Data are represented as arbitrary values. Data were analyzed using an unpaired *Student's t-test*. Results are expressed as the mean $\pm$ standard error (SEM). * = Statistically significant from C-V. n=4-8 per experimental group

3.4 Discussion

Our study demonstrates for the first time that administration of the Liver X Receptor agonist GW3965 during the rat neonatal period has permanent and profound effects on the expression of hepatic gluconeogenic and lipogenic genes in adult life. From our results, it appears that activation of LXR activity in neonatal life in non-MPR offspring leads to the generation of a phenotype very similar to the metabolic syndrome: impaired glucose tolerance, hyperinsulinemia, hypertriglyceridemia, increased gluconeogenic gene expression, and increased lipogenic gene expression.

Our initial hypothesis and prediction was that administration of GW3965 during the neonatal period would rescue the maternal protein restricted animals from developing impaired glucose homeostasis. However, this was not the case, as we did not observe impaired glucose tolerance in our "LP2" MPR model. Given that the male offspring of MPR dams did not exhibit impaired glucose tolerance at our time of measurement (4 months), we were not able to assess whether or not administration of GW3965 would rescue the impaired glucose tolerance phenotype. Although the literature has previously found impaired glucose tolerance in the offspring of MPR, studies have also found that male rats at 4 months of age do not yet exhibit the impaired glucose tolerance phenotype[11,42]. It is likely the time point that we chose for the study was too early and our animals had not yet developed an impaired glucose homeostasis phenotype. For instance, Hales and colleagues (1996) found that their MPR rats did not exhibit impaired glucose tolerance at 3 months, but developed worsened glucose tolerance at 15 months of age[11]. Chamson-Reig and colleagues (2009) also did not find impaired glucose tolerance in male offspring at 130 days of age but they did find signs of insulin resistance[42]. Females,

however, did develop impaired glucose tolerance at 130 days of age[42]. Recent studies in our lab did find impaired glucose tolerance at 130 days of age in MPR male rat offspring, however the offspring in that study had the normal protein diet restored earlier (immediately after birth in the aforementioned study versus after weaning in this study)[20]. Thus, a variety of factors may have played a role in why our MPR animals did not develop impaired glucose homeostasis.

Interestingly, there were permanent implications when the control diet animals received neonatal administration of GW3965. When the control diet animals received GW3965 during the neonatal period (C-GW), they developed impaired glucose tolerance, fasted hyperglycemia, as well as patterns of hyperinsulinemia and hypertriglyceridemia. In addition, these changes were reflected in the increased protein expression levels of various hepatic gluconeogenic genes (G6Pase, PEPCK) and lipogenic (SCD-1) genes. Moreover, enhanced protein expression of 11β-HSD1 was found in C-GW animals compared to C-V animals, suggesting an increase in the production of glucocorticoids, which would then indirectly lead to increased gluconeogenesis.

A permanent increase in the expression of G6Pase and PEPCK, two essential enzymes in the regulation of hepatic glucose production could very well lead to impaired glucose tolerance[44,45]. Furthermore, the liver is a principle source for insulin clearance[46-48]. Given that the gluconeogenic genes G6Pase and PEPCK are overexpressed in our C-GW animals and that they display patterns of hyperinsulinemia, hepatic insulin resistance may be a major contributor to the impaired glucose phenotype in these animals. To further support this, previous studies in liver-specific insulin receptor knockout mice have demonstrated hepatic insulin resistance to be a major contributor of impaired hepatic

function and glucose intolerance[49]. Additionally, the study by Michael and colleagues (2000) also found severe hyperinsulinemia in their liver-specific insulin receptor knockout mice[49]. Hyperinsulinemia is strongly associated with impaired glucose tolerance as well as obesity and hypertension[50]. In addition, chronic hyperinsulinemia is a great risk factor for the development of impaired glucose tolerance, insulin resistance, and ultimately diseases such as type 2 diabetes and the metabolic syndrome[51].

The increased expression of G6Pase and PEPCK may also be attributed to increased protein expression of 11β-HSD1. Considering that 11β-HSD1 is responsible for the conversion of inactive glucocorticoids to active glucocorticoids, long-term increases in this enzyme may lead to chronic elevation of glucocorticoids in the body. Glucocorticoids play a major role in the induction of G6Pase and PEPCK[52-54], thus the elevation of 11β-HSD1 protein expression may play a role in the impaired glucose homeostasis and fasted hyperglycemia seen in the C-GW experimental group.

In addition to impaired glucose homeostasis, neonatal exposure to GW3965 in non-MPR male offspring led to the increase in circulating triglycerides. Furthermore, there was a significant increase in the protein expression of SCD-1. However, no increases in other lipogenic genes were observed. While FAS and ACC are responsible for the *de novo* synthesis of long chain fatty acids, SCD-1 is vital for the production of unsaturated fatty acids and ultimately triglycerides in addition to other essential lipids[55]. Elevated SCD-1 activity has been implicated in the development of many chronic diseases including diabetes and obesity[56-59]. In contrast, studies in SCD-1 knockout mice demonstrate that these mice exhibit reduced adiposity, increased sensitivity to insulin and are resistant to weight gain[60]. These mice SCD-1 knockout mice also displayed increased

expression of genes related to lipid oxidation and a reduction in the expression of lipogenic genes[60]. Furthermore, another study was able to prevent diet-induced obesity and improve postprandial glucose and insulin levels in high-fat diet mice through administration of antisense oligonucleotide inhibitors of SCD-1[61]. Lastly, studies have also demonstrated the involvement of SCD-1 in insulin signaling and carbohydrate intake induced adiposity, implicating its possible role in glucose homeostasis[62,63]. Thus, the increase in SCD-1 expression seen in male C-GW offspring is likely to play a factor in the possible hypertriglyceridemia and impaired glucose homeostasis seen in these animals.

Of particular interest is that all of the genes that changed in our model are transcriptionally regulated by LXR. Yet, LXR expression was unchanged at all time points examined. This necessitates a mechanism that allows the temporary neonatal administration of GW3965 to continue into adulthood. An ideal candidate for such a change would be some sort of epigenetic change such as DNA methylation or a post-translational modification like histone acetylation/methylation. For instance, in addition to the study done by Stoffers and colleagues (2006), where neonatal administration of Exendin-4™ prevented the development of diabetes in IUGR rats[37], it was subsequently discovered by Pinney and colleagues (2011) that this change was effected through an epigenetic mechanism[64]. More specifically, they found that neonatal administration of Exendin-4™ permanently restored histone H3 acetylation, decreased histone H3 lysine 9 dimethylation, and restored histone H3 lysine 4 trimethylation, all signs of chromatin opening, on the *Pdx-1* promoter in adult IUGR animals[64]. Furthermore, neonatal administration of Exendin-4™ prevented direct DNA methylation around the *Pdx-1*

promoter in adult IUGR rats, another sign of permissive transcriptional status. Thus, it is highly probable that the changes mediated by our neonatal administration of GW3965 in non-MPR rats (C-GW experimental group) are a result of long lasting epigenetic changes that manifest themselves in adulthood.

Another possible mechanism that may mediate the long-term effects of neonatal GW3965 administration is through "endoplasmic reticulum (ER) stress" and the subsequent accumulation of misfolded of proteins. Previous studies have implicated the role of ER stress and impaired protein synthesis and folding in the development of chronic disease[65,66]. Additionally, ER stress has also been linked to insulin resistance and diabetes[67-69]. More recently, LXR has been linked with both ER stress and insulin resistance[70]. In the study done by Jwa *et al.* (2012), administration of piperine, an LXR antagonist, led to the amelioration of ER stress and improved insulin resistance in mice fed a high fat diet[70]. The authors postulate that the link between LXR, ER stress, and insulin resistance may involve the role of LXR in inducing the lipogenic genes and subsequent lipid accumulation. Studies from our own laboratory have also implicated insulin resistance with attenuated protein synthesis in a similar model of MPR used in this study[71]. However, the role of LXR in relation to these changes was not investigated.

These findings are significant as there are several cases in which induction of LXR may occur during pregnancy. One example is gestational diabetes, which is reported to occur in 2-25% of pregnancies in the US and is on the rise[72-74]. Considering that glucose itself has been found to be a direct agonist for LXR[31], elevated glucose exposure to the fetus during pregnancy may act to induce LXR activation. A common consequence of gestational diabetes is that the infants go on to develop impaired glucose tolerance

later on in life[75]. The Pima Indian population exemplifies this phenomenon, where approximately 45% of individuals born to gestational diabetic mothers develop type 2 diabetes[76,77]. Currently, the mechanism behind why this happens is poorly understood. One possible reason for why this occurs is due to an increased glucose load to the fetus and development of hyperinsulinemia in the fetus/neonate. As a consequence, many infants born to diabetic mothers develop hypoglycemia immediately after birth due to the elevated circulating levels of insulin during pregnancy[78]. Insulin is a potent stimulator of LXR and LXR is also an essential mediator of insulin downstream transcriptional regulation[79,80]. Thus, in addition to elevated glucose activation of LXR, prolonged hyperinsulinemia during the prenatal and neonatal period also may contribute to increased activation of LXR and a phenotype similar to the model used in this study. Moreover, gestational diabetes has been found to alter cholesterol transport in the placenta, which may affect oxysterol (endogenous LXR activators) concentrations in both the mother and the fetus[81]. Thus, our model of neonatal LXR exposure may very well mimic the molecular mechanisms underlying gestational diabetes and how it programs the development of impaired glucose tolerance in later life in the offspring.

In summary, we have produced a phenotype characteristic of the metabolic syndrome in non-maternal protein restricted male offspring through neonatal administration of the LXR agonist GW3965. Likely through an epigenetic mechanism, these changes induced during the neonatal period are permanent and result in a phenotype that includes impaired glucose tolerance, fasted hyperglycemia, fasted hyperinsulinemia, hypertriglyceridemia, and increased expression of gluconeogenic and lipogenic genes. Understanding the role of LXR induction during the neonatal period may help uncover

novel roles of LXR and other transcription factors underlying the mechanisms involved in the early life programming of chronic disease. For example, exposure to a diet high in sugar and cholesterol (natural agonists for LXR) during the neonatal period may pose considerable risk for a developing infant, regardless of birth weight and/or growth restriction due to the possible induction of LXR and subsequent programmed effects.

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Chapter Four: Discussion

Discussion

4.1 Summary

It is now widely recognized that the intrauterine environment may play a role in the development of chronic, non-communicable diseases in later adult life. Intrauterine growth restriction (IUGR) occurs in many complicated and high-risk pregnancies. The evidence strongly suggests that IUGR and the birth of a low birth weight infant increases the risk of developing obesity, heart disease, hypertension, and diabetes[1-5]. The "Barker Hypothesis" or "Thrifty Phenotype Hypothesis" postulates that the development of these chronic diseases is a result of a programming mechanism that occurs during the developmental period[6,7]. In addition, other complications in pregnancy such as gestational diabetes mellitus and maternal obesity may also lead to placental complications and increase the risk of developing chronic disease in the offspring[8-10]. Given the widespread prevalence of non-communicable chronic diseases, it is imperative to understand the mechanisms underlying how the programming of adult chronic disease occurs during the developmental period. Yet, the molecular mechanisms behind how these programming changes occur are still largely unknown. While a few mechanisms have been postulated (*e.g.* epigenetic mechanisms, nuclear receptor signaling, oxidative stress, endoplasmic reticulum stress, *etc.*), there is still much to be discovered[11].

Due to its role in glucose[12,13], lipid[13], and cholesterol homeostasis[14], the Liver X Receptor (LXR) presents itself as an attractive candidate for mediating some of the effects seen in the fetal programming of adult diseases such as type 2 diabetes, hypertension, atherosclerosis, and obesity. Overall the present studies provide strong

evidence for the involvement of the LXR in the programming of adult chronic disease. Based on the work presented in this thesis and others[15,16] we strongly believe that LXR is implicated in the programming of adult disease, and more specifically, impaired glucose tolerance, in our model of maternal protein restriction. We also strongly believe that the permanent actions of LXR are both transcriptional and epigenetic in nature.

In the first study (Chapter 2) we hypothesized that maternal protein restriction and the early restoration of protein (at birth) would lead to impaired glucose homeostasis. We further hypothesized that the impaired glucose homeostasis would at least be partly mediated through alterations in expression of LXR and its downstream target genes involved in gluconeogenesis. The study presents evidence for the epigenetic downregulation of LXR expression in a model of maternal protein restriction with early protein restoration. This repression then leads to altered transcriptional regulation of LXR downstream target genes involved directly (G6Pase) and indirectly (11β-HSD1) in the induction of gluconeogenesis. The increased hepatic production of glucose then results in the observed impaired glucose tolerance observed at postnatal day 130 (Figure 4.1).

Given MPR leads to decreases in LXR expression, accompanied by impaired glucose tolerance, we sought to investigate whether neonatal intervention with the LXR agonist GW3965 would rescue the IUGR phenotype seen in our previous studies[15,17]. We hypothesized that administration of GW3965 during the neonatal period would prevent adverse outcomes and prevent the programming of chronic disease in adulthood. The study did not support our hypothesis and the results were unanticipated. Surprisingly, neonatal administration of GW3965 led to impaired glucose tolerance and fasted hyperglycemia in the young adult males of the control diet offspring even though LXR

agonists have consistently been demonstrated to have antidiabetic effects[18,19]. Instead, the study presents evidence that neonatal overexposure to an LXR agonist may in fact be detrimental, as it appears to induce a metabolic syndrome-like phenotype in adulthood: fasted hyperglycemia, impaired glucose homeostasis, fasted hyperinsulinemia, hypertriglyceridemia, and increased expression of gluconeogenic and lipogenic genes. Many questions in this model remain unanswered. For example, is epigenetic regulation occurring at the promoter regions of *LXR*, *G6Pase*, and *11β-HSD1*, parallel to the low protein model (seen in Chapter 2)? Besides LXR, what are other nuclear receptors (*e.g.* GR, ER, PPAR) and signaling pathways (*e.g.* insulin signaling pathways, cAMP signaling pathways) are involved in the programing of the resulting phenotype? Why is LXR exhibiting the opposite of its antidiabetic effects when administered in early life versus later life (antidiabetic effects)? Investigation of these questions would provide much insight on the mechanisms involved in the early life programming of adult disease.

Together, these two studies provide strong evidence for the involvement of LXR in the programming of adult disease. In both models, MPR and neonatal overexposure to an LXR agonist, there is altered LXR expression leading to a long lasting phenotype in adulthood. In addition, both models demonstrated similar phenotypes and gene expression profiles, suggesting a conserved mechanism with regards to LXR expression and activity.

4.2 Limitations and Improvements

No study is without its limitations and the present study is no exception. Firstly, we decided only to examine males in both of the studies presented. This was to prevent

confounding variables associated with either sex, namely differences attributed to female estrous cycling, and differing hormone profiles in either sex. Previous studies have also demonstrated exacerbated effects of developmental programming in males[20]. Although it was our decision was to investigate only the male-specific effects of our models, by neglecting investigation of the females we leave an entire half of the study open to question. Other studies have investigated the differences between the sexes and have found different mechanisms of programming between males and females[20-22]. For instance, Chamson-Reig and colleagues (2009) found that both males and females display altered glucose metabolism, however males were insulin resistant, while females were insulin deficient[21]. Thus, investigation of females would have provided much insight into the mechanisms behind fetal programming and how each sex responds to each insult differently.

Secondly, our evaluation of hepatic gluconeogenesis was limited by the fact that we only explored the expression profiles of the genes involved in gluconeogenesis (*e.g.* G6Pase, PEPCK). While this provides a good measure of gluconeogenesis, it does not necessarily imply increased activity of these enzymes. We did attempt to do an *in vivo* measure of hepatic gluconeogenic activity through a pyruvate challenge test as described by Yao *et al.* (2006)[23] and Meyer zu Schwabedissen *et al.* (2011)[24]. However, due to limited experience with the technique and the animals not responding well to the pyruvate challenge and other complications, we were not confident in the results obtained from the experiment. Another option to measure activity would have been to do hepatic microsomal extractions and colorimetric measurements of the enzyme products over time as previously described[25-28]. In addition, we speculate that an increase in glucocorticoid

synthesis may have contributed to the observed increase in gluconeogenic genes since there were increases in 11β-HSD1 and GR expression. However, we did not measure the levels of hepatic or circulating glucocorticoids. Unfortunately, this was due to the lack of plasma samples at the time of experimentation. Again, measurement of 11β-HSD1 enzyme activity would also help solidify the present findings. These considerations will be taken into account for future cohorts and experiments.

Our assessment of insulin resistance may also have been improved. Although we did assess protein expression levels for markers of insulin sensitivity in the liver to give us a specific evaluation of hepatic insulin resistance, a few other experiments may have been done to fully assess insulin resistance. Firstly, a whole body insulin tolerance test (or even better, a hyperinsulinemic euglycemic clamp experiment) could be employed, although this would not give a direct measure of hepatic insulin utilization. Secondly, other markers of insulin resistance could be measured, such as interactions between insulin signaling molecules and receptors through protein complex immunoprecipitation experiments (Co-IP)[29].

In the second study (Chapter 3), the main issue was choosing the inappropriate maternal protein restriction model with respect to glucose tolerance. We decided on restoring the protein in our MPR rats after weaning instead of immediately after birth for two reasons. The first reason was based on results obtained previously in our lab by Sohi and colleagues[15] whereby MPR offspring restored on a control protein diet after the weaning period displayed elevated circulating and hepatic cholesterol and altered epigenetic regulation of the *Cyp7a1* gene in adulthood, implicating that this model was consistent in inducing a chronic disease programmed phenotype. Studies by Chamson-

Reig *et al.* (2009) have also demonstrated long-term effects in the offspring employing a similar model of MPR[21]. The second reason was based on promising results from our initial pilot study, whereby we used the post-weaning protein restoration model and found that there was a significant difference between the LP2-V and LP2-GW animals at postnatal day 21 in the protein expression of 11β-HSD1 and SREBP-1 (Figure 3.2). Although no changes were found in the protein expression of LXR, G6Pase, or PEPCK, this still provided encouragement to continue the study because there were significant changes in the expression of some LXR-target genes even one week after administration of the LXR agonist discontinued. Thus, we continued the study with a second cohort of animals taken to postnatal day 130 as presented in Chapter 3. At postnatal day 130, we did not observe an impaired glucose tolerance phenotype in the MPR animals. Thus, there was no "rescue" or "prevention" of the phenotype. In hindsight, we would have chosen the MPR model more similar to the one in Chapter 2, in which the offspring were restored on a regular protein diet immediately after birth and displayed consistent impaired glucose homeostasis at postnatal day 130[17].

Finally, considerations must be taken into account with respect to the animals themselves. Caution must be taken when interpreting the current data in the context of what happens in the human on a physiological and pathophysiological basis. In terms of development, it appears that both the rat and human experience similar patterns of postnatal development for the liver and pancreas, although the rat does go through a higher degree of liver remodeling and maturation during the first 28 days after birth[30]. However, the human liver does not reach full maturation until approximately 5 years after birth, suggesting a similar pattern of postnatal development. Another consideration

pertinent to the presented studies is that human and rat hepatocytes have been demonstrated to respond to the LXR agonist GW3965 quite differently through gene expression profile experiments[31]. For instance, LXR agonists repressed the expression of GLUT2, glucokinase (GCK), and pyruvate kinase (PKLR), in human hepatocytes but not rat hepatocytes[31]. Moreover, LXR-mediated transcriptional activation of Cyp7a1 appears to only occur in rats but not humans or other mammals[32]. Thus, it is possible that the effects seen in our experiments in neonatal rats may not necessarily mimic the effects proposed in humans. Lastly, all experiments were performed in the fasted state. There is a significant difference in metabolic gene expression profiles in the fasted and non-fasted state[33]. Considering how important nutritional status is in the regulation of hepatic gluconeogenesis and lipogenesis[34,35] it is important to take into account the nutritional status of the animal in the assessment of its metabolic status. Although the fasted state allows us to examine the metabolic profiles of the animals without confounding variables (*e.g.* variable food and water intake), we are curious to see what differences may occur in the non-fasted state.

4.3 A New Hypothesis

Although we found strong evidence to support our hypothesis in the first study (Chapter 2), the hypothesis in the second study (Chapter 3) must be reconsidered. Given that neonatal administration did not lead to the rescue of an impaired glucose tolerance phenotype, a new hypothesis must be proposed to address the current findings and/or a new experiment must be conducted to re-examine the hypothesis. In the case of the latter, we would redesign the study such that we use a maternal protein restriction model that

consistently produces a glucose intolerance phenotype. If we were to redesign the study to examine the possibility of a phenotype rescue, it would likely be the MPR model used in our Chapter 2 study whereby the MPR offspring were restored protein in their diet at an earlier time point (immediately after birth). However, we can examine a modified hypothesis because we did find an altered phenotype in the control diet animals given the LXR agonist. This suggested hypothesis, which aims to cover both Chapters 2 and 3, would be as follows: ***We hypothesize that alterations in LXR expression during the neonatal period through various intrauterine insults leads to the long-term programming of impaired glucose homeostasis and ultimately the development of diseases such as type 2 diabetes and the metabolic syndrome*.**

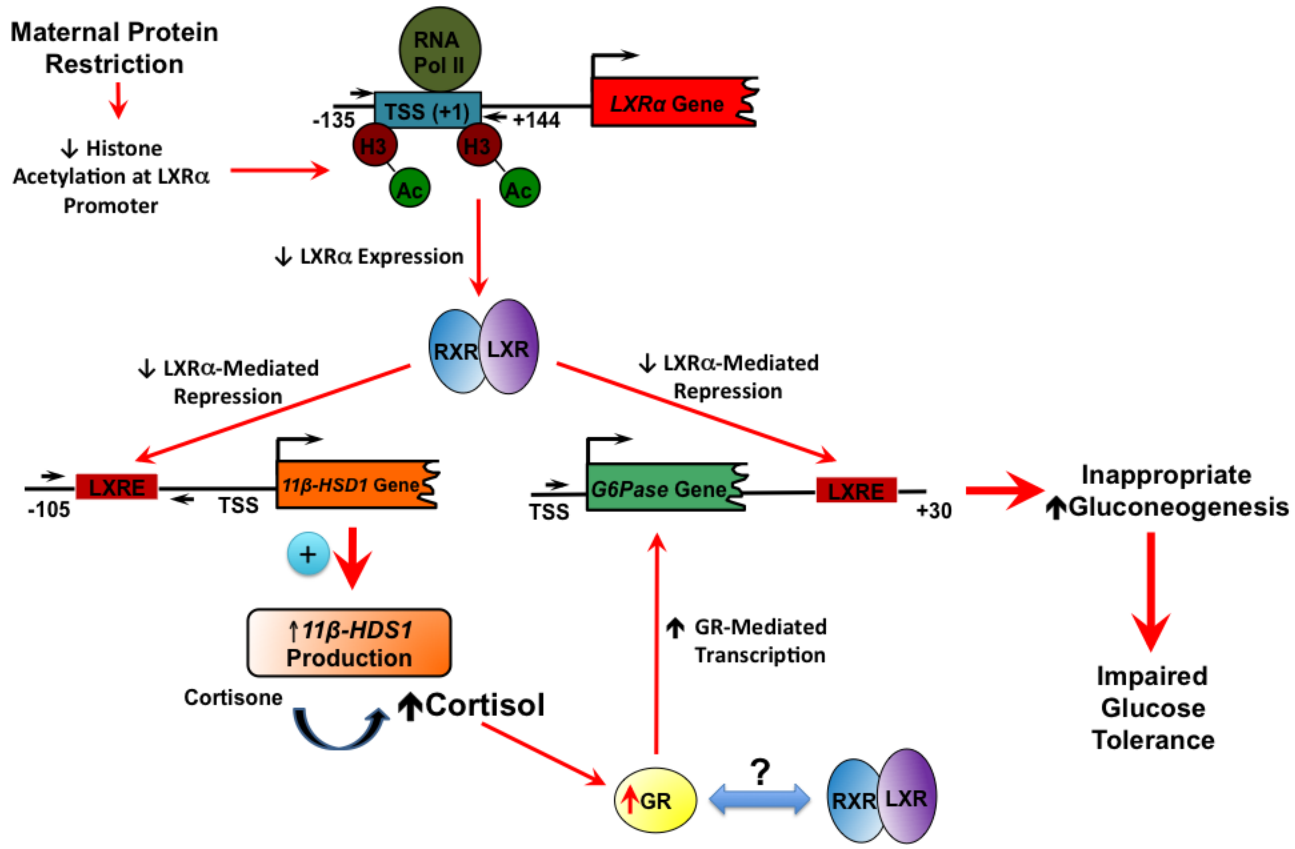

Figure 4.1: A Working Hypothesis for the Role of LXR in Mediating Impaired Glucose Tolerance in a Model of Maternal Protein Restriction.

4.4 Future Directions

Though we have begun to characterize the role of LXR in mediating the programming effects of adult disease, the issue remains quite complex and there is still much to be investigated. As previously mentioned, our model neglects the question of what happens in the female offspring of MPR animals and females administered GW3965 during the neonatal period. Although we do have preliminary evidence that the females do experience impaired glucose tolerance, it is to a much lesser degree than the males. This is supported by other studies, whereby males are more susceptible to the effects of fetal programming and display exacerbated phenotypes compared to females[20]. The mechanisms underlying these sexually dimorphic observations are still in question. Furthermore, it would be worthwhile to investigate more time points in the study for several reasons. Firstly, investigation of early time points may reveal critical time points at which the programming is occurring. A longitudinal study similar to the one employed in the Chamson-Reig *et al.* study (2009) would reveal when specific changes in metabolism occur. For instance by examining both the postnatal day 85 time point and the postnatal day 130 time point, Chamson-Reig and colleagues (2009) were able to find that impaired glucose tolerance did not occur until at least early adulthood (postnatal day 130)[21].

In contrast, it would be of great interest to investigate the current models at later time points. Our current time point for sacrifice occurs at postnatal day 130, which is still a young age for the rat. The development of many chronic diseases occurs later on in life, once the effects of aging are compounded with early life insults. For instance, in the Hales *et al.* study (1996), the effects of MPR in the offspring were not observed at 3

months of age, however they occurred at 15 months of age[36]. It is quite possible that the animals in our model have not had the chance to develop other pathologies such as obesity, hypertension, atherosclerosis, and heart disease. Future studies may include the 6, 12, and 15-month time point to observe the progression of the metabolic syndrome and cardiovascular disease. A longitudinal study might also be possible, given the proper funding and resources. Of interest is also the fact that MPR animals have demonstrated decreased longevity, especially in models of accelerated catch-up growth[36-38]. Although some mechanisms have been postulated (*e.g.* oxidative stress, altered insulin signaling, impaired mitochondrial function)[39-42], it is still unclear whether the decreased lifespan is a result of the development of chronic disease or if the decreased lifespan is itself programmed in early life.

Another area of relevance is the possibility of examining a "double hit" model. In this model, an insult during pregnancy is utilized to induce IUGR (insults may include maternal protein restriction, total maternal nutrient restriction, uterine artery ligation and hypoxia) and then a second insult is compounded to the intrauterine insult in postnatal life. A common postnatal insult is through the feeding of a high fat or "western diet" that usually generates an obese phenotype[43]. Double hit models are of great interest because they are especially relevant in today's society given the increasing prevalence of the consumption of high sugar and high fat "western diets". This is especially important as the population begins to develop chronic diseases at a younger age due to poor lifestyle choices in diet and lack of exercise[44,45].

The second study (Chapter 3) is still largely incomplete, and additional experiments are required to expand on the current findings. Firstly, quantitative real-time

PCR experiments are required to investigate whether steady-state levels of mRNA are increased in our genes of interest (LXR, G6Pase, PEPCK, 11β-HSD1, SCD-1, ACC, FAS, SREBP-1). Although the long-term expression of LXR was not altered by neonatal GW3965 treatment, it is still conceivable that LXR activity itself is enhanced long-term. Thus, chromatin immunoprecipitation should be employed to investigate this possibility by measuring the binding of LXR to its target promoters. In addition, post-translational and epigenetic mechanisms need to be further explored to help explain the long lasting effects of neonatal LXR agonist treatment. For example, administration of the LXR agonist T0901317 in chick embryo hepatocytes has been demonstrated to increase activity of the LXR/RXR heterodimer in addition to increasing the acetylation of histone H3, lysine 4 around the LXRE of the *ACC* gene promoter (a downstream target of LXR activation)[46]. Hence, the agonists themselves may be responsible for mediating epigenetic changes. Histone modifications are likely key mechanisms that may be involved in the altered expression of our genes of interest. Investigation of acetylated histone H3 lysine 9, trimethylated histone H3 lysine 27, trimethylated histone H3 lysine 4, and binding of RNA polymerase at the promoters of our genes of interest may provide a better understanding of the transcriptional regulation occurring at these gene promoters. Furthermore, the examination of direct DNA methylation through bisulfite sequencing experiments may provide further clues on epigenetic regulation.

4.5 Conclusion

It is clear from the results of the present study that the Liver X Receptor is emerging as a key factor in mediating the early life programming of adult chronic

diseases. In both studies, we found that alterations in LXR expression or activation either short-term during the neonatal period or long-term in adulthood led to detrimental effects in adulthood. Whether these changes are due to LXR directly or due to cross talk with other nuclear receptors and transcription factors is presently unknown. However, it is clear that LXR is involved and it is directly influencing the transcription of various genes involved in hepatic gluconeogenesis. From both studies, these genes appear to be critical points of regulation, whereby aberration in expression can lead to phenotype changes: G6Pase, PEPCK, 11β-HSD1, SCD-1 and possibly GR.

Studies from other groups as well as from colleagues in our lab and have also demonstrated the involvement of LXR in the programming of adult disease. Sohi *et al.* (2011)[15] have previously demonstrated elevated LXR expression concomitant with increased LXR binding to the *Cyp7a1* gene in young MPR rats, while Ma *et al.* (2013, unpublished data) has demonstrated elevated LXR expression in adult male offspring of pregnant rats exposed to moderate doses of nicotine (as seen in moderate smokers). Moreover, van Straten and colleagues (2012) have demonstrated altered levels of LXR in embryonic day 19.5 MPR offspring as well[47]. These studies further cement the role of LXR in the programming of adult disease.

While we provide evidence for the involvement of LXR in the programming of chronic disease in IUGR animals, there are many other transcription factors and mechanisms that may contribute to the programming of adult disease. These transcription factors include other nuclear receptors such as the peroxisome proliferator-activated receptors (PPAR), glucocorticoid receptor (GR), and estrogen receptor (ER)[17]. In addition, other mechanisms of action may include elevated oxidative stress and

endoplasmic reticulum stress and altered epigenetic profiles[17]. It should be noted that it is likely a combination of all of these factors and mechanisms that contribute to the programming of adult disease. Similarly, there is a high likelihood of cross talk between LXR and the other nuclear receptors, transcription factors, and co-activators/co-repressors. For instance, studies have demonstrated possible cross talk between LXR and GR based on the existence of putative GR binding sites on the *LXR* promoter[48]. Furthermore, GR may require LXR to induce its effects and LXR induction has been demonstrated to suppress GR-mediated actions[49,50]. Thus, the mediators of the developmental programming of adult disease are extremely complex and multifactorial in nature.

Finally, and most importantly, once we discover these mechanisms and collect the data, what becomes the next task? The pivotal task ahead is learning how to translate these data and prevent the development of these chronic diseases, which are now devastating health care systems around the world. Intervention at early and critical time points in development to prevent adverse outcomes is key. As research continues, more interventional approaches are being employed in animal models. A few examples include: administration of key transcription factor and nuclear receptor agonists (such as Exendin-4™)[51], administration of antioxidants such as tempol, resveratrol, vitamin C (ascorbic acid)[52-54], and folic acid supplementation[55,56]. Clearly, there are many avenues to take for the intervention of programmed adult diseases, further adding to the complexity of how these mechanisms work and how they can be reversed[57]. It is imperative that we come to understand not only the molecular mechanisms behind the

fetal programming of chronic adult diseases but also how we can use this understanding to prevent further development of these diseases.

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Appendix II: Curriculum Vitae

(Peter) Thin Xuan Vo

Department of Physiology and Pharmacology
The University of Western Ontario
London, Ontario, Canada
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Education

- MSc. **University of Western Ontario**. Master of Science Candidate. Department of Physiology and Pharmacology; Department of Obstetrics and Gynaecology. 2011-Present.

- B.MSc. **University of Western Ontario**. Bachelor of Medical Sciences. Honors Specialization in Physiology. *Western Scholar*. 2006-2010.

Employment Experience

Teaching Assistant - Physiology **3130Y at the University of Western Ontario** — September 2011 - Current

Awards and Recognition

Received **first prize** for best oral presentation at the 3rd Annual Diabetes Research Day in **London, Ontario** — November 2012

Received **first prize** for poster competition at the Developmental Origins of the Metabolic Syndrome Symposium in **Ann Arbor, Michigan** — October 2012

Received the **Obstetrics and Gynaecology Graduate Scholarship** — September 2012

Received the Society for **Gynecologic Investigation President's Presenter Award** at the Society of Gynecologic Investigation's (SGI) 59th Annual Meeting in **San Diego, California** — March 2012

Received the **Children's Health Research Institute (CHRI) Travel Grant** to present research abroad — January 2012

Received the **Western Graduate Research Scholarship** — September 2011 & September 2012

Publications

Vo T, Revesz A, Sohi G, Ma N, Hardy DB. Maternal protein restriction leads to enhanced hepatic gluconeogenic gene — April 2013

expression in adult male rat offspring due to impaired expression of the liver X receptor. J Endocrinol. 2013 Jun;218(1):85-97.

Vo T, Hardy DB. Molecular mechanisms underlying the fetal programming of adult disease. J Cell Commun Signal. 2012 Aug;6(3):139-53. Epub 2012 May 24 — May 2012

Presented and/or Published Abstracts

Vo TX, Revesz A, Sohi G, Ma N, Hardy DB. Maternal Protein Restriction Decreases Histone Acetylation Surrounding the Promoter of the *Liver X Receptor* Leading to Impaired Expression of Hepatic Gluconeogenic Genes and Ultimately Impaired Glucose Homeostasis in Adult Male Rat Offspring. **Poster presentation** at the Paul Harding Awards Research Day (OB/GYN Department) in **London, ON.** — May 2013

Vo TX, Sohi G, Revesz A, Hardy DB. Maternal Protein Restriction Results in Altered Transcriptional Regulation of Hepatic Liver X Receptor (LXRα) Target Genes Leading to Impaired Glucose Homeostasis in Adult Rat Offspring. **Oral presentation** at 3rd Annual Diabetes Research Day in **London, ON.** — November 2012

Revesz A, Sohi G, **Vo T**, Ma NL, Hardy DB. Elevated Hepatic miR-29 Expression in Male Growth Restricted Rats is Inversely Correlated with its Target Insulin-like Growth Factor 1 (IGF-1) Longterm. **Submitted abstract** to the 60th Society for Gynecologic Investigation Annual Scientific Meeting in **Orlando, FL.** — October 2012

Vo TX, Sohi G, Revesz A, Hardy DB. Maternal Protein Restriction Results in Altered Transcriptional Regulation of Hepatic Liver X Receptor (LXRα) Target Genes Leading to Impaired Glucose Homeostasis in Adult Rat Offspring. **Poster presentation** at the Developmental Origins of the Metabolic Syndrome Symposium in **Ann Arbor, MI.** — October 2012

Vo TX, Sohi G, Revesz A, Hardy DB. The Role of The Lixer X Receptor in the Impairment of Glucose Homeostasis in Maternal Protein Restricted Rat Offspring. **Poster presentation** at the Paul Harding Awards Research Day (OB/GYN Department) in **London, ON.** — May 2012

Vo TX, Sohi G, Revesz A, Hardy DB. Maternal Protein — March 2012

Restriction Results in Altered Transcriptional and Epigenetic Regulation of Hepatic Liver X Receptor (LXRα) Target Genes Leading to Impaired Glucose Homeostasis in Adult Rat Offspring. **Oral presentation** at the 59th Society for Gynecologic Investigation Annual Scientific Meeting in **San Diego, CA.**

Vo TX, Sohi G, Revesz A, Hardy DB. Maternal Protein Restriction Results in Altered Transcriptional and Epigenetic Regulation of Hepatic Liver X Receptor (LXRα) Target Genes Leading to Impaired Glucose Homeostasis in Adult Rat Offspring. **Poster presentation** at London Health Research Day in **London, ON.** — March 2012

Vo TX, Sohi G, Revesz A, Hardy DB. Maternal Protein Restriction Results in Altered Transcriptional and Epigenetic Regulation of Hepatic Liver X Receptor (LXRα) Target Genes Leading to Impaired Glucose Homeostasis in Adult Rat Offspring. **Poster presentation** at the Annual Eastern Canadian Perinatal Investigator's Meeting in **Kingston, ON.** — November 2011

Extracurricular Activities

- Volunteered in Kilema, Tanzania for three weeks with the Canada Africa Community Health Alliance (CACHA) — July – August 2012
- Summited Mt. Kilimanjaro (5895m) via the Lemosho Route — August 2012
- Volunteering with the Children's Aid Society as a "Big Brother" — October 2011 - Current